MODIFIED GUIDE RNAS

SEQUENCE LISTING

[0000] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on December 7, 2017, is named 01155-0004-00PCT_SeqList.txt and is 118,877 bytes in size.

[0001] This application claims the benefit of priority to United States Provisional Application No. 62/431,756, which was filed on December 8, 2016, and which is incorporated by reference in its entirety.

[0002] This disclosure relates to the field of gene editing using CRISPR/Cas systems, a part of the prokaryotic immune system that recognizes and cuts exogenous genetic elements. The CRISPR/Cas system relies on a single nuclease, termed CRISPR-associated protein 9 (Cas9), which induces site-specific breaks in DNA. Cas9 is guided to specific DNA sequences by small RNA molecules termed guide RNA (gRNA). Guide RNA comprises trRNA (also known as tracrRNA) and crisprRNA (crRNA). The trRNA and crRNA may be contained within a single guide RNA (sgRNA) or in two separate RNA molecules of a dual guide RNA (dgRNA). Cas9 in combination with trRNA and crRNA or an sgRNA is termed the Cas9 ribonucleoprotein complex (RNP).

[0003] Oligonucleotides, and in particular RNA, are sometimes degraded in cells and in serum by endonuclease or exonuclease cleavage. Improved methods and compositions for preventing such degradation, improving stability of gRNAs and enhancing gene editing efficiency is desired, especially for therapeutic applications.

SUMMARY

[0004] In some embodiments, therapeutic genome editing tools are provided comprising modified guide RNAs. The modified guide RNAs described herein may improve the stability of the guide RNA and the guide RNA/Cas9 complex and improve the activity of Cas9 (e.g., SpyCas9 and equivalents) to cleave target DNA. In some embodiments, the guide RNA is an sgRNA. In some embodiments, the guide RNA is a dgRNA. In some embodiments, the guide RNA is a tracrRNA. In some embodiments, the guide RNA is a crRNA.

[0005] The guide RNAs described herein comprise at least one modified nucleotide. Modifications may include 2'-O-methyl (2'-O-Me), 2'-O-(2-methoxyethyl) (2'-O-moe), 2'-fluoro (2'-F), phosphorothioate (PS) bond between nucleotides, G-C substitutions, and

inverted abasic linkages between nucleotides and equivalents thereof. Embodiments of the invention include:

[0006] In some embodiments, a single guide RNA (sgRNA) is encompassed comprising a 5' end modification and one or more modification in one or more of: the upper stem region; the hairpin 1 region; and the hairpin 2 region, wherein the 5' end modification comprises at least two phosphorothioate linkages within the first seven nucleotides at the 5' end of the 5' terminus. In some instances, the modification is a 2'-O-methyl (2'-O-Me) modified nucleotide. In some embodiments, the modification is a 2'-fluoro (2'-F) modified nucleotide.

[0007] In some embodiments, the sgRNA comprises modifications at US1 to US12 and/or a modification at H1-1 and/or a modification in H2-1. In some embodiments, the sgRNA comprises modifications at H1-1 to H1-12 and/or H2-1 to H2-15. In some embodiments, the sgRNA comprises one or more modifications in each of the upper stem region, the hairpin 1 region, and the hairpin 2 region. In some embodiments, the sgRNA comprises a modified nucleotide between hairpin 1 and hairpin 2 regions. In some embodiments, the sgRNA comprises a modification in the lower stem region.

[0008] In some embodiments, the sgRNA comprises a modification at the 5' terminus and/or the 3' terminus. In some embodiments, the sgRNA comprises a 3' end modification in the 3' terminus. In some embodiments, the sgRNA comprises modifications on at least two of the last four nucleotides at the 3' end of the 3' terminus. In some embodiments, the sgRNA comprises a 5' end modification in the 5' terminus. In some embodiments, the sgRNA comprises modifications on at least two of the first four nucleotides at the 5' end of the 5' terminus. In some embodiments, the sgRNA comprises a 3' end modification in the 3' terminus and a 5' end modification in the 5' terminus. In some embodiments, the sgRNA comprises modifications on at least two of the last four nucleotides at the 3' end of the 3' terminus and on at least two of the first four nucleotides at the 5' end of the 5' terminus. In some instances, these modifications are 2'-O-Me, 2'-F, 2'-O-moe, or phosphorothioate (PS) bonds linking the nucleotides. In some embodiments, the sgRNA comprises PS bonds between at least two of the last four nucleotides at the 3' end of the 3' terminus and/or at least two of the first four nucleotides at the 5' end of the 5' terminus. In some instances, the sgRNA comprises 5' terminus and 3' terminus with more than one modification as described herein, such as, with PS bonds and 2'-O-Me modifications.

[0009] In some embodiments, the sgRNA comprises a modification in the bulge region. In some embodiments, 50% of the nucleotides in the bulge region are modified, wherein the modification is 2'-O-Me or 2'-F.

[0010] In some embodiments, the sgRNA comprises a modification in the nexus region. In some embodiments, the sgRNA comprises modifications at N15, N16, N17, and/or N18 in the nexus region, wherein the modification is 2'-O-Me or 2'-F. In some instances, N16, N17, and N18 are linked with PS bonds.

[0011] In some embodiments, the sgRNA comprises at least the first three nucleotides at the 5' end of the 5' terminus, and the last three nucleotides at the 3' end of the 3' terminus are modified.

[0012] In some embodiments, the sgRNA comprises modifications at the 3' terminus and/or 5' terminus. In some instances, the first four nucleotides at the 5' end of the 5' terminus, and the last four nucleotides at the 3' end of the 3' terminus are linked with phosphorothioate (PS) bonds. In some embodiments, the 5' and 3' modification comprises 2'-O-Me or 2'-O-moe. In some embodiments, the 5' and 3' modification comprises 2'-F. In some embodiments, the 5' and/or 3' modification comprises PS bonds linking nucleotides. In some embodiments, the 5' and/or 3' modification comprises one or more of 2'-O-Me, 2'-O-moe, 2'-F, and PS bonds linking nucleotides.

[0013] In some embodiments, the sgRNA comprises modifications at the first four nucleotides at the 5' end of the 5' terminus and the last four nucleotides at the 3' end of the 3'terminus. In some instances, these modifications are linking PS bond (i.e., PS bonds that link the first four and last four nucleotides). In some embodiments, the sgRNA further comprises 2'-O-Me modifications at the first three nucleotides at the 5' end of the 5' terminus and the last three nucleotides at the 3' end of the 3' terminus.

[0014] In some embodiments, the sgRNA comprises modifications at the first four nucleotides at the 5' end of the 5' terminus and the last four nucleotides at the 3' end of the 3' terminus, wherein the modifications are at least PS bonds linking the four nucleotides, and further wherein the first three nucleotides at the 5' end of the 5' terminus and the last three nucleotides at the 3' end of the 3' terminus comprise 2'-O-Me, 2'-O-moe, or 2'-F modifications.

[0015] In some embodiments, the sgRNA comprises modifications LS1, LS6, LS7, LS8, LS11, and LS12, wherein the modification is 2'-O-Me or 2'-F.

[0016] In some embodiments, the sgRNA comprises modifications at each of the nucleotides in the bulge region, wherein the modification is 2'-O-Me or 2'-F.

[0017] In some embodiments, the sgRNA comprises modifications at each of the nucleotides in the upper stem region, wherein the modification is 2'-O-Me or 2'-F.

[0018] In some embodiments, the sgRNA comprises modifications at each of the nucleotides in the hairpin 1 region, wherein the modification is 2'-O-Me or 2'-F.

[0019] In some embodiments, the sgRNA comprises modifications at each of the nucleotides in the hairpin 2 region, wherein the modification is 2'-O-Me or 2'-F.

[0020] In some embodiments, an sgRNA is encompassed comprising 2'-O-Me modified nucleotides at the following positions:

a. the first three nucleotides at the 5' end of the 5' terminus;
b. LS1, LS6, LS7, LS8, LS11, and/or LS12 in the lower stem region;
c. B1 and/or B2 in the bulge region;
d. each nucleotide in the upper stem region;
e. N16, N17, and/or N18 in the nexus region;
f. each nucleotide in the hairpin 1 region;
g. each nucleotide in the hairpin 2 region; and
h. the last four nucleotides at the 3' end of the 3' terminus.

In some embodiments, B3-B6 are modified with 2'-O-Me. In some instances, the sgRNA further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' end of the 5' terminus and three PS bonds linking the last four nucleotides at the 3' end of the 3' terminus. In some embodiments, the sgRNA comprises 2'-F modifications at LS9 and LS10. In some embodiments, the sgRNA comprises 2'F modifications at N15, N16, N17, and N18. In some embodiments, the sgRNA comprises 2'F modifications at H2-9, H2-10, H2-11, H2-12, H2-13, H2-14, and H2-15. In some embodiments, the sgRNA comprises 2'F modifications at the second to last, third to last, and fourth to last nucleotides at the 3' end of the 3' terminus.

[0021] In some embodiments, an sgRNA is encompassed comprising 2'-F modified nucleotides at the following positions:

a. LS9 and LS10 in the lower stem region;
b. N15, N16, N17, and N18 in the nexus region; and
c. H2-9, H2-10, H2-11, H2-12, H2-13, H2-14, and H2-15 in the hairpin 2 region.

In some embodiments, the sgRNA comprises 2'-F modified nucleotides at the second to last, third to last, and fourth to last nucleotides at the 3' terminus. In some embodiments, the sgRNA comprises three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' end of the 5' terminus and three PS bonds linking the last four nucleotides at the 3' end of

the 3' terminus. In some embodiments, the sgRNA comprises 2'-O-Me or 2'-F modified nucleotides at the first three nucleotides at the 5' end of the 5' terminus, and 2'-O-Me or 2'-F modified nucleotides at three of the last four nucleotides at the 3' end of the 3' terminus.

[0022] In some embodiments, an sgRNA is encompassed comprising

a. 2'-O-Me modified nucleotides at the first three nucleotides at the 5' end of the 5' terminus;
b. Optional 2'-O-Me modified nucleotides at LS1 and/or LS6;
c. 2'-O-Me modified nucleotides at US1-US12;
d. 2'-O-Me modified nucleotides at H1-1 – H1-12;
e. Optional 2'-O-Me modified nucleotide between Hairpin 1 and Hairpin 2;
f. 2'-O-Me modified nucleotides at H2-1 – H2-15; and
g. 2'-O-Me modified nucleotides at the last four nucleotides at the 3' end of the 3' terminus; and optionally

further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' end of the 5' terminus and three PS bonds linking the last four nucleotides at the 3' end of the 3' terminus.

[0023] In some embodiments, an sgRNA is encompassed comprising:

a. 2'-O-Me modified nucleotides at the first three nucleotides at the 5' end of the 5' terminus;
b. 2'-F modified nucleotides at LS1-LS6;
c. 2'-O-Me modified nucleotides at US1-US12;
d. 2'-O-Me modified nucleotides at H1-1 – H1-12;
e. a 2'-O-Me modified nucleotide between Hairpin 1 and Hairpin 2;
f. 2'-O-Me modified nucleotides at H2-1 – H2-15; and
g. 2'-O-Me modified nucleotides at the last four nucleotides at the 3' end of the 3' terminus; and optionally

further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' end of the 5' terminus and three PS bonds linking the last four nucleotides at the 3' end of the 3' terminus.

[0024] In some embodiments, an sgRNA is encompassed comprising:

a. 2'-O-Me modified nucleotides at the first three nucleotides at the 5' terminus;
b. 2'-F modified nucleotides at LS2-LS5;
c. 2'-O-Me modified nucleotides at LS1 and LS6;
d. 2'-O-Me modified nucleotides at US1-US12;

e. 2'-O-Me modified nucleotides at H1-1 – H1-12;
f. a 2'-O-Me modified nucleotide between Hairpin 1 and Hairpin 2;
g. 2'-O-Me modified nucleotides at H2-1 – H2-15; and
h. 2'-O-Me modified nucleotides at the last four nucleotides at the 3' terminus and optionally

further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' end of the 5' terminus and three PS bonds linking the last four nucleotides at the 3' end of the 3' terminus.

[0025] In some embodiments, a sgRNA is encompassed comprising:

a. 2'-O-Me modified nucleotides at the first three nucleotides at the 5' terminus;
b. 2'-O-Me modified nucleotides at US1-US12;
c. 2'-O-Me modified nucleotides at LS7, LS8, LS11, and LS12;
d. 2'-O-Me modified nucleotides at H1-1 – H1-12;
e. a 2'-O-Me modified nucleotide between Hairpin 1 and Hairpin 2;
f. 2'-O-Me modified nucleotides at H2-1 – H2-15; and
g. 2'-O-Me modified nucleotides at the last four nucleotides at the 3' terminus,

and optionally further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' end of the 5' terminus and three PS bonds linking the last four nucleotides at the 3' end of the 3' terminus.

[0026] In some embodiments, a sgRNA is encompassed comprising:

a. 2'-O-Me modified nucleotides at the first three nucleotides at the 5' terminus;
b. 2'-O-Me modified nucleotides at US1-US12;
c. 2'-O-Me modified nucleotides at LS7, LS8, LS11, and LS12;
d. 2'-F modified nucleotides at LS9 and LS10;
e. 2'-O-Me modified nucleotides at H1-1 – H1-12;
f. a 2'-O-Me modified nucleotide between Hairpin 1 and Hairpin 2;
g. 2'-O-Me modified nucleotides at H2-1 – H2-15; and
h. 2'-O-Me modified nucleotides at the last four nucleotides at the 3' terminus,

and optionally further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' end of the 5' terminus and three PS bonds linking the last four nucleotides at the 3' end of the 3' terminus.

[0027] In some embodiments, an sgRNA is encompassed comprising:

a. 2'-O-Me modified nucleotides at the first three nucleotides at the 5' terminus;
b. 2'-O-Me modified nucleotides at US1-US12;

c. 2'-O-Me modified nucleotides at LS8, LS10, and LS12;
d. 2'-O-F modified nucleotides at LS7, LS9, and LS11;
e. 2'-O-Me modified nucleotides at H1-1 – H1-12;
f. a 2'-O-Me modified nucleotide between Hairpin 1 and Hairpin 2;
g. 2'-O-Me modified nucleotides at H2-1 – H2-15; and
h. 2'-O-Me modified nucleotides at the last four nucleotides at the 3' terminus, and optionally

further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' end of the 5' terminus and three PS bonds linking the last four nucleotides at the 3' end of the 3' terminus.

[0028] In some embodiments, a sgRNA is encompassed comprising:

a. 2'-O-Me modified nucleotides at the first three nucleotides at the 5' terminus;
b. 2'-O-Me modified nucleotides at LS1, LS6, LS7, LS8, LS11, and LS12
c. 2'-O-Me modified nucleotides at US1-US12;
d. 2'-O-Me modified nucleotides at H1-1 – H1-12;
e. a 2'-O-Me modified nucleotide between Hairpin 1 and Hairpin 2;
f. 2'-O-Me modified nucleotides at H2-1 – H2-15; and
g. 2'-O-Me modified nucleotides at the last four nucleotides at the 3' terminus, and optionally

further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' end of the 5' terminus and three PS bonds linking the last four nucleotides at the 3' end of the 3' terminus

[0029] In some embodiments, a sgRNA is encompassed comprising:

a. 2'-O-Me modified nucleotides at the first three nucleotides at the 5' terminus;
b. 2'-O-Me modified nucleotides at LS1, LS6, LS7, LS8, LS11, and LS12;
c. 2'-F modified nucleotides at LS9 and LS10;
d. 2'-O-Me modified nucleotides at US1-US12;
e. 2'-O-Me modified nucleotides at H1-1 – H1-12;
f. a 2'-O-Me modified nucleotide between Hairpin 1 and Hairpin 2;
g. 2'-O-Me modified nucleotides at H2-1 – H2-15; and
h. 2'-O-Me modified nucleotides at the last four nucleotides at the 3' terminus, and optionally

further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' end of the 5' terminus and three PS bonds linking the last four nucleotides at the 3' end of the 3' terminus.

[0030] In some embodiments, a sgRNA is encompassed comprising:

a. 2'-O-Me modified nucleotides at the first three nucleotides at the 5' end of the 5' terminus;
b. 2'-O-Me modified nucleotides at US1-US12;
c. 2'-O-Me modified nucleotides at H1-1 – H1-12;
d. a 2'-O-Me modified nucleotide between Hairpin 1 and Hairpin 2;
e. 2'-O-Me modified nucleotides at H2-1 – H2-8;
f. 2'-F modified nucleotides at H2-9 – H2-15;
g. 2'-F modified nucleotides at the second from last, third from last, and fourth from last nucleotide at the 3' terminus; and
h. a 2'-O-Me modified nucleotide at the last nucleotide at the 3' terminus, and optionally

further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' end of the 5' terminus and three PS bonds linking the last four nucleotides at the 3' end of the 3' terminus.

[0031] In some embodiments, a sgRNA is encompassed comprising:

a. 2'-O-Me modified nucleotides at the first three nucleotides at the 5' end of the 5' terminus;
b. 2'-O-Me modified nucleotides at US1-US12;
c. 2'-O-Me modified nucleotides at H1-2, H1-4, H1-6, H1-8, H1-10, and H1-12;
d. 2'-F modified nucleotides at H1-1, H1-3, H1-5, H1-7, H1-9, and H1-11;
e. a 2'-F modified nucleotide between Hairpin 1 and Hairpin 2;
f. 2'-F modified nucleotides at H2-2, H2-4, H2-6, H2-8, H2-10, H2-12; and H2-14;
g. 2'-O-Me modified nucleotides at H2-1, H2-3, H2-5, H2-7, H2-9, H2-11; H2-13, and H2-15;
h. 2'-F modified nucleotides at the second from last, and fourth from last nucleotide at the 3' terminus; and
i. 2'-O-Me modified nucleotide at the third from last and last nucleotide at the 3' end of the 3' terminus,

and optionally further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' end of the 5' terminus and three PS bonds linking the last four nucleotides at the 3' end of the 3' terminus.

[0032] In some embodiments, a sgRNA is encompassed comprising:

a. 2'-O-Me modified nucleotides LS8, LS10, LS12, H1-2, H1-4, H1-6, H1-8, H1-10, H1-12, H2-1, H2-3, H2-5, H2-7, H2-9, H2-11, H2-13, and H2-15; and

b. 2'-F modified nucleotides at LS7, LS9, LS11; H1-1, H1-3, H1-5, H1-7, H1-9, H1-11, H1-13, H2-2, H2-4, H2-6, H2-8, H2-10, H2-12, and H2-14, and optionally further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' end of the 5' terminus and three PS bonds linking the last four nucleotides at the 3' end of the 3' terminus; and optionally further comprising:

c. 2'-O-Me modified nucleotides at the last and third to last nucleotide at the 3' end of the 3' terminus; and/or

d. 2'-F modified nucleotides at the second to last, fourth to last, and/or last nucleotide at the 3' end of the 3' terminus.

[0033] In some embodiments, a sgRNA is encompassed comprising the nucleic acids of any of SEQ ID Nos: 228-353, including the modifications of Table 4. In some embodiments, a sgRNA is encompassed comprising any of SEQ ID Nos: 228-332, including the modifications of Table 4. In some embodiments, an sgRNA is encompassed comprising any of SEQ ID Nos: 235-240, 265-285, and 309-329, including the modifications of Table 4. In some embodiments, an sgRNA is encompassed comprising SEQ ID No: 240. In some embodiments, a sgRNA is encompassed comprising SEQ ID No. 240, including the modifications of Table 4. In some embodiments, a sgRNA is encompassed comprising SEQ ID No: 242. In some embodiments, a sgRNA is encompassed comprising SEQ ID No: 358. In additional embodiments, a sgRNA comprising nucleic acids having at least 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 85, 80, 75, or 70% identity to the nucleic acids of any one of SEQ ID Nos: 235-240, 265-285, and 309-329, wherein the modification at each nucleotide of the sgRNA that corresponds to a nucleotide of the reference sequence identifier in Table 4, is identical to or equivalent to the modification shown in the reference sequence identifier in Table 4, optionally further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' end of the 5' terminus and three PS bonds linking the last four nucleotides at the 3' end of the 3' terminus. In some embodiments, the sgRNA further comprises at least three PS bonds linking the nucleotides in the hairpin 1 region. In some embodiments, the sgRNA further comprises at least three PS bonds linking the nucleotides in

the hairpin 2 region. In some embodiments, the sgRNA further comprises at least three PS bonds linking the nucleotides in the upper stem region. In some embodiments, the sgRNA forms a ribonucleoprotein complex with *S. pyogenes* Cas9.

FIGURE LEGENDS

[0034] FIG 1 shows percent editing as measured by next-generation sequence (NGS) of mouse transthyretin (TTR) gene following transfection of Neuro2A cells with modified crRNAs together with Cas9 mRNA and unmodified trRNA (TR000002).

[0035] FIG 2 shows percent editing as measured by NGS of mouse TTR gene following transfection of Neuro2A cells with modified trRNAs together with unmodified crRNA (CR000686) and Cas9 mRNA.

[0036] FIG 3 shows percent editing as measured by NGS of mouse TTR gene following transfection of Neuro2A cells with Cas9 mRNA and crRNAs and trRNAs having G-C pairings not found in parental sequences.

[0037] FIG 4 shows percent editing as measured by NGS of mouse TTR gene following transfection of Neuro2A cells with modified crRNAs and trRNAs together with Cas9 mRNA. Standard deviations follow the value.

[0038] FIG 5 shows percent editing as measured by NGS of mouse TTR gene following transfection of Neuro2A cells with modified sgRNAs together with Cas9 mRNA.

[0039] FIG 6 shows percent editing as measured by NGS of mouse TTR gene following transfection of Neuro2A cells with modified crRNAs and unmodified trRNA (TR000002) together with Cas9 mRNA. The asterisk denotes a dual guide that for technical reasons did not show activity in this experiment. This dual guide was tested again in the experiment represented in Figure 9, in which it showed editing activity.

[0040] FIG 7 shows percent editing as measured by NGS of mouse TTR gene following transfection of Neuro2A cells with unmodified crRNA (CR000686) and modified trRNAs together with Cas9 mRNA.

[0041] FIG 8 shows percent editing as measured by NGS of mouse TTR gene following transfection of Neuro2A cells with Cas9 mRNA and crRNA and trRNA pairings with G-C pairings or G-U mismatches not found in the parental sequences.

[0042] FIG 9 shows percent editing as measured by NGS of mouse TTR gene following transfection of Neuro2A cells with modified crRNAs and modified trRNAs together with Cas9 mRNA. Standard deviations follow the value.

[0043] FIG 10 shows percent editing as measured by NGS of mouse TTR gene following transfection of Neuro2A cells with modified sgRNAs together with Cas9 mRNA.

[0044] FIG 11 shows percent editing as measured by NGS of mouse Factor VII (FVII) gene following transfection of Neuro2A cells with modified sgRNAs together with Cas9 mRNA.

[0045] FIGs 12A and 12B show percent editing as measured by NGS of mouse TTR (FIG 12A) or FVII (FIG 12B) following transfection of Neuro2A cells with modified crRNAs and unmodified trRNA together with Cas9 mRNA.

[0046] FIGs 13A and 13B shows percent editing as measured by NGS of mouse TTR (FIG 13A) or FVII (FIG 13B) following transfection of Neuro2A cells with modified trRNAs and unmodified crRNA together with Cas9 mRNA.

[0047] FIGs 14A, 14B, 14C, and 14D show interferon alpha (IFN-alpha, 14A), interleukin 6 (IL-6, 14B), monocyte chemotactic protein 1 (MCP-1, 14C), and tumor necrosis factor alpha (TNF-alpha, 14D) levels in serum after in vivo administration of LNPs comprising Cas9 mRNA and sgRNAs.

[0048] FIGs 15A, 15B, and 15C show in vivo results following administration of LNPs comprising Cas9 mRNA and sgRNAs. FIG 15A shows percentage of total editing in liver. FIG 15B shows serum TTR levels. FIG 15C shows the mean and standard deviation for the results of FIG 15A. FIG 15D summarizes modifications to the G000209 sgRNA (SEQ ID NO: 228). FIG 15E summarizes modifications to the G000267 sgRNA (SEQ ID NO: 234). In FIG 15D and 15E, the nucleotides in bold are 2'-O-Me modified.

[0049] FIGs 16A, 16B, 16C, and 16D show interferon alpha (IFN-alpha, 16A), tumor necrosis factor alpha (TNF-alpha, 16B), interleukin 6 (IL-6, 16C), and monocyte chemotactic protein 1 (MCP-1, 16D) levels in serum after in vivo administration of LNPs comprising Cas9 mRNA and sgRNAs.

[0050] FIGs 17A, 17B, 17C, and 17D show in vivo results following administration of LNPs comprising Cas9 mRNA and sgRNAs. FIG 17A shows percentage of total editing in liver. FIG 17B shows the mean and standard deviation for the results of FIG 17A. FIG 17C shows serum TTR levels. FIG 17D shows the mean and standard deviation for the results of FIG 17B.

[0051] FIGs 18A, 18B, and 18C show in vivo results following administration of LNPs comprising Cas9 mRNA and sgRNAs. FIG 18A shows percentage of total editing in liver. FIG 18B summarizes liver editing data. FIG 18C shows serum TTR levels. MPK = milligrams per kilogram; BLOD = below level of detection.

[0052] FIGs 19A, 19B, 19C, and 19D show interferon alpha (IFN-alpha, 19A), monocyte chemotactic protein 1 (MCP-1, 19B), interleukin 6 (IL-6, 19C), and tumor necrosis factor alpha (TNF-alpha, 19D) levels in serum after in vivo administration of LNPs comprising Cas9 mRNA and sgRNAs.

[0053] FIGs 20A and 20B show editing in liver of FVII locus (FIG 20A) and TTR locus (FIG 20B) following in vivo administration of LNPs comprising Cas9 mRNA and sgRNAs.

[0054] FIGs 21A, 21B, and 21C show schematics of an annotated sgRNA (SEQ ID NO: 341) (FIG 21A), non-annotated dgRNA CR000686 (SEQ ID NO: 1) and TR000002 (SEQ ID NO: 188) (FIG 21B), and annotated dgRNA CR000686 (SEQ ID NO: 1) and TR000002 (SEQ ID NO: 188) (FIG 21C).

[0055] FIG 22A, 22B, and 22C show in vivo results following administration of LNPs comprising Cas9 mRNA and sgRNAs. FIG 22A shows percentage of total editing of TTR locus in liver. FIG 22B summarizes liver editing data. FIG 22C shows serum TTR levels.

[0056] FIG 23A, 23B, and 23C show in vivo results following administration of LNPs comprising Cas9 mRNA and sgRNAs. FIG 23A shows percentage of total editing of TTR locus in liver. FIG 23B summarizes liver editing data. FIG 23C shows serum TTR levels.

[0057] FIGs 24A, 24B, and 24C show editing in primary mouse hepatocytes following administration of LNPs comprising Cas9 mRNA and sgRNAs. FIG24A shows editing percentage of total editing of TTR locus. FIG24B shows normalized transforms of editing percentage as a function of mRNA dose used to calculate EC50. FIG 24C shows EC50 values for the LNPs tested.

DETAILED DESCRIPTION

[0058] Provided herein are modified guide RNAs, including dual and single guide RNAs for use in gene editing methods. The modified guides are more stable and show improved in vitro and in vivo efficacy as compared to their non-modified counterparts. Sequences of engineered and tested guide RNAs are shown in Table 4.

Table 4:

SEQ ID NO	Name	Alias	Description	Sequence
	crRNA			
1	CR000686		unmodified	CCAGUCCAGCGAGGCAAAGGGUUUUAGAGCUAUGCUGUUUUG
2	CR003393	CR686-1	upper	CCAGUCCAGCGAGGCAAAGGGUUUUAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
3	CR003394	CR686-2	partial upper	CCAGUCCAGCGAGGCAAAGGGUUUUAGAGCUAmUmGmCmUmGmUmUmUmUmG
4	CR003395	CR686-3	partial upper	CCAGUCCAGCGAGGCAAAGGGUUUUAGAGCUAUGCmUmGmUmUmUmUmG
5	CR003396	CR686-4	partial upper	CCAGUCCAGCGAGGCAAAGGGUUUUAGAGCUAUGCUGUmUmUmUmG
6	CR003397	CR686-5	lower	CCAGUCCAGCGAGGCAAAGGmGmUmUmUmUmAGAGCUAUGCUGUUUUG
7	CR003398	CR686-6	lower walk	CCAGUCCAGCGAGGCAAAGGmGUUUUAGAGCUAUGCUGUUUUG
8	CR003399	CR686-7	lower walk	CCAGUCCAGCGAGGCAAAGGGmUUUUAGAGCUAUGCUGUUUUG
9	CR003400	CR686-8	lower walk	CCAGUCCAGCGAGGCAAAGGGUmUUUAGAGCUAUGCUGUUUUG
10	CR003401	CR686-9	lower walk	CCAGUCCAGCGAGGCAAAGGGUUmUUAGAGCUAUGCUGUUUUG
11	CR003402	CR686-10	lower walk	CCAGUCCAGCGAGGCAAAGGGUUUmUAGAGCUAUGCUGUUUUG
12	CR003403	CR686-11	lower walk	CCAGUCCAGCGAGGCAAAGGGUUUUmAGAGCUAUGCUGUUUUG
13	CR003404	CR686-12	partial lower	CCAGUCCAGCGAGGCAAAGGGmUmUmUmUAGAGCUAUGCUGUUUUG
14	CR003405	CR686-13	partial lower	CCAGUCCAGCGAGGCAAAGGGUmUmUUAGAGCUAUGCUGUUUUG
15	CR003406	CR686-GC1	Lower GC	CCAGUCCAGCGAGGCAAAGGGGCGCAGAGCUAUGCUGUUUUG
16	CR003407	CR686-GC3	Upper GC	CCAGUCCAGCGAGGCAAAGGGUUUUAGAGCUAUGCUGGCGCG
17	CR003408	CR686-GC5	Lower Upper GC	CCAGUCCAGCGAGGCAAAGGGGCGCAGAGCUAUGCUGGCGCG
18	CR003409	CR686 all OMe		mCmCmAmGmUmCmCmAmGmCmGmAmGmGmCmAmAmAmGmGmGmUmUmUmUmAmGmAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
19	CR003393 - mod only		upper	GUUUUAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
20	CR003394 - mod only		partial upper	GUUUUAGAGCUAmUmGmCmUmGmUmUmUmUmG

SEQ ID NO	Name	Alias	Description	Sequence
21	CR003395 - mod only		partial upper	GUUUUAGAGCUAUGCmUmGmUmUmUmUmG
22	CR003396 - mod only		partial upper	GUUUUAGAGCUAUGCUGUmUmUmUmG
23	CR003397 - mod only		lower	mGmUmUmUmUmAGAGCUAUGCUGUUUUG
24	CR003398 - mod only		lower walk	mGUUUUAGAGCUAUGCUGUUUUG
25	CR003399 - mod only		lower walk	GmUUUUAGAGCUAUGCUGUUUUG
26	CR003400 - mod only		lower walk	GUmUUUAGAGCUAUGCUGUUUUG
27	CR003401 - mod only		lower walk	GUUmUUAGAGCUAUGCUGUUUUG
28	CR003402 - mod only		lower walk	GUUUmUAGAGCUAUGCUGUUUUG
29	CR003403 - mod only		lower walk	GUUUUmAGAGCUAUGCUGUUUUG
30	CR003404 - mod only		partial lower	GmUmUmUmUAGAGCUAUGCUGUUUUG
31	CR003405 - mod only		partial lower	GUmUmUUAGAGCUAUGCUGUUUUG
32	CR003721	CR686-14	upper and lower	CCAGUCCAGCGAGGCAAAGGmGUUUmUmAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
33	CR003722	CR686-15	lower combo	CCAGUCCAGCGAGGCAAAGGmGUUUmUmAGAGCUAUGCUGUUUUG
34	CR003723	CR686-16	upper, lower combo	CCAGUCCAGCGAGGCAAAGGmGUUUUmAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
35	CR003724	CR686-17	lower combo	CCAGUCCAGCGAGGCAAAGGmGUUUUmAGAGCUAUGCUGUUUUG
36	CR003725	CR686-18	upper, lower combo	CCAGUCCAGCGAGGCAAAGGmGUUUUAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
37	CR003726	CR686-19	nexus walk	CCAGUCCAGCGAGGCAAAGGGUUUUAmGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
38	CR003727	CR686-20	nexus walk	CCAGUCCAGCGAGGCAAAGGGUUUUAGmAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
39	CR003728	CR686-21	nexus walk	CCAGUCCAGCGAGGCAAAGGGUUUUAfGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
40	CR003729	CR686-22	nexus walk	CCAGUCCAGCGAGGCAAAGGGUUUUAGfAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
41	CR003730	CR686-23	2'F lower walk	CCAGUCCAGCGAGGCAAAGGGfUUUUAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG

SEQ ID NO	Name	Alias	Description	Sequence
42	CR003731	CR686-24	2'F lower walk	CCAGUCCAGCGAGGCAAAGGGUfUUUAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
43	CR003732	CR686-25	2'F lower walk	CCAGUCCAGCGAGGCAAAGGGUUfUUAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
44	CR003733	CR686-26	2'F lower walk	CCAGUCCAGCGAGGCAAAGGGUUUfUAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
45	CR003734	CR686-27	2'F lower combo	CCAGUCCAGCGAGGCAAAGGfGfUfUfUfUfAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
46	CR003735	CR686-28	lower alt	CCAGUCCAGCGAGGCAAAGGfGmUfUmUfUmAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
47	CR003736	CR686-29	lower alt	CCAGUCCAGCGAGGCAAAGGmGfUmUfUmUfAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
48	CR003737	CR686-GC6	Lower GC	CCAGUCCAGCGAGGCAAAGGGUCUCAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
49	CR003738	CR686-GC7	Lower C walk	CCAGUCCAGCGAGGCAAAGGGCUUUAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
50	CR003739	CR686-GC8	Lower C walk	CCAGUCCAGCGAGGCAAAGGGUCUUAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
51	CR003740	CR686-GC9	Lower C walk	CCAGUCCAGCGAGGCAAAGGGUUCUAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
52	CR003741	CR686-GC10	Lower C walk	CCAGUCCAGCGAGGCAAAGGGUUUCAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
53	CR003721-mod only	CR686-14-mod only	upper and lower	mGUUUmUmAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
54	CR003722-mod only	CR686-15-mod only	lower combo	mGUUUmUmAGAGCUAUGCUGUUUUG
55	CR003723-mod only	CR686-16-mod only	upper, lower combo	mGUUUUmAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
56	CR003724-mod only	CR686-17-mod only	lower combo	mGUUUUmAGAGCUAUGCUGUUUUG
57	CR003725-mod only	CR686-18-mod only	upper, lower combo	mGUUUUAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG

SEQ ID NO	Name	Alias	Description	Sequence
58	CR003726-mod only	CR686-19-mod only	nexus walk	GUUUUAmGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
59	CR003727-mod only	CR686-20-mod only	nexus walk	GUUUUAGmAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
60	CR003728-mod only	CR686-21-mod only	nexus walk	GUUUUAfGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
61	CR003729-mod only	CR686-22-mod only	nexus walk	GUUUUAGfAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
62	CR003730-mod only	CR686-23-mod only	2'F lower walk	GfUUUUAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
63	CR003731-mod only	CR686-24-mod only	2'F lower walk	GUfUUUAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
64	CR003732-mod only	CR686-25-mod only	2'F lower walk	GUUfUUAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
65	CR003733-mod only	CR686-26-mod only	2'F lower walk	GUUUfUAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
66	CR003734-mod only	CR686-27-mod only	2'F lower combo	fGfUfUfUfUfAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
67	CR003735-mod only	CR686-28-mod only	lower alt	fGmUfUmUfUmAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
68	CR003736-mod only	CR686-29-mod only	lower alt	mGfUmUfUmUfAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
69	CR003737-mod only	CR686-GC6-mod only	Lower GC	GUCUCAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
70	CR003738-mod only	CR686-GC7-mod only	Lower C walk	GCUUUAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
71	CR003739-mod only	CR686-GC8-mod only	Lower C walk	GUCUUAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
72	CR003740-mod only	CR686-GC9-mod only	Lower C walk	GUUCUAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
73	CR003741-mod only	CR686-GC10-mod only	Lower C walk	GUUUCAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
74	CR000705		unmodified	UUACAGCCACGUCUACAGCAGUUUUAGAGCUAUGCUGUUUUG
75	CR004188	CR705-1	upper	UUACAGCCACGUCUACAGCAGUUUUAGAmGmCmUmAmUmGmCmUmGmUmUmUmG
76	CR004189	CR705-2	partial upper	UUACAGCCACGUCUACAGCAGUUUUAGAGCUAmUmGmCmUmGmUmUmUmUmG
77	CR004190	CR705-3	partial upper	UUACAGCCACGUCUACAGCAGUUUUAGAGCUAUGCmUmGmUmUmUmUmG
78	CR004191	CR705-4	partial upper	UUACAGCCACGUCUACAGCAGUUUUAGAGCUAUGCUGUmUmUmUmG
79	CR004192	CR705-5	lower	UUACAGCCACGUCUACAGCAmGmUmUmUmUmAGAGCUAUGCUGUUUUG
80	CR004193	CR705-6	lower walk	UUACAGCCACGUCUACAGCAmGUUUUAGAGCUAUGCUGUUUUG
81	CR004194	CR705-7	lower walk	UUACAGCCACGUCUACAGCAGmUUUUAGAGCUAUGCUGUUUUG
82	CR004195	CR705-8	lower walk	UUACAGCCACGUCUACAGCAGUmUUUAGAGCUAUGCUGUUUUG

SEQ ID NO	Name	Alias	Description	Sequence
83	CR004196	CR705-9	lower walk	UUACAGCCACGUCUACAGCAGUUmUUAGAGCUAUGCUGUUUUG
84	CR004197	CR705-10	lower walk	UUACAGCCACGUCUACAGCAGUUUmUAGAGCUAUGCUGUUUUG
85	CR004198	CR705-11	lower walk	UUACAGCCACGUCUACAGCAGUUUUmAGAGCUAUGCUGUUUUG
86	CR004199	CR705-14	upper and lower	UUACAGCCACGUCUACAGCAmGUUUmUmAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
87	CR004200	CR705-15	lower combo	UUACAGCCACGUCUACAGCAmGUUUmUmAGAGCUAUGCUGUUUUG
88	CR004201	CR705-16	upper, lower combo	UUACAGCCACGUCUACAGCAmGUUUUmAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
89	CR004202	CR705-17	lower combo	UUACAGCCACGUCUACAGCAmGUUUUmAGAGCUAUGCUGUUUUG
90	CR004203	CR705-18	upper, lower combo	UUACAGCCACGUCUACAGCAmGUUUUAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
91	CR004204	CR705-19	nexus walk	UUACAGCCACGUCUACAGCAGUUUUAmGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
92	CR004205	CR705-20	nexus walk	UUACAGCCACGUCUACAGCAGUUUUAGmAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
93	CR004206	CR705-21	nexus walk	UUACAGCCACGUCUACAGCAGUUUUAfGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
94	CR004207	CR705-22	nexus walk	UUACAGCCACGUCUACAGCAGUUUUAGfAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
95	CR004208	CR705-23	2'F lower walk	UUACAGCCACGUCUACAGCAGfUUUUAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
96	CR004209	CR705-24	2'F lower walk	UUACAGCCACGUCUACAGCAGUfUUUAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
97	CR004210	CR705-25	2'F lower walk	UUACAGCCACGUCUACAGCAGUUfUUAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
98	CR004211	CR705-26	2'F lower walk	UUACAGCCACGUCUACAGCAGUUUfUAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
99	CR004212	CR705-27	2'F lower combo	UUACAGCCACGUCUACAGCAfGfUfUfUfUfAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG

SEQ ID NO	Name	Alias	Description	Sequence
100	CR004213	CR705-28	lower alt	UUACAGCCACGUCUACAGCAfGmUfUmUfUmAGAmGmCmUmAmUmG mCmUmGmUmUmUmUmG
101	CR004214	CR705-29	lower alt	UUACAGCCACGUCUACAGCAmGfUmUfUmUfAGAmGmCmUmAmUmG mCmUmGmUmUmUmUmG
102	CR004215	CR705-GC1	Lower GC	UUACAGCCACGUCUACAGCAGGCGCAGAGCUAUGCUGUUUUG
103	CR004216	CR705-GC3	Upper GC	UUACAGCCACGUCUACAGCAGUUUUAGAGCUAUGCUGGCGCG
104	CR004188-mod only	CR705-1-mod only	upper	GUUUUAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
105	CR004189-mod only	CR705-2-mod only	partial upper	GUUUUAGAGCUAmUmGmCmUmGmUmUmUmUmG
106	CR004190-mod only	CR705-3-mod only	partial upper	GUUUUAGAGCUAUGCmUmGmUmUmUmUmG
107	CR004191-mod only	CR705-4-mod only	partial upper	GUUUUAGAGCUAUGCUGUmUmUmUmG
108	CR004192-mod only	CR705-5-mod only	lower	mGmUmUmUmUmAGAGCUAUGCUGUUUUG
109	CR004193-mod only	CR705-6-mod only	lower walk	mGUUUUAGAGCUAUGCUGUUUUG
110	CR004194-mod only	CR705-7-mod only	lower walk	GmUUUUAGAGCUAUGCUGUUUUG
111	CR004195-mod only-mod only	CR705-8-mod only	lower walk	GUmUUUAGAGCUAUGCUGUUUUG
112	CR004196-mod only	CR705-9-mod only	lower walk	GUUmUUAGAGCUAUGCUGUUUUG
113	CR004197-mod only	CR705-10-mod only	lower walk	GUUUmUAGAGCUAUGCUGUUUUG
114	CR004198-mod only	CR705-11-mod only	lower walk	GUUUUmAGAGCUAUGCUGUUUUG
115	CR004199-mod only	CR705-14-mod only	upper and lower	mGUUUmUmAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
116	CR004200-mod only	CR705-15-mod only	lower combo	mGUUUmUmAGAGCUAUGCUGUUUUG
117	CR004201-mod only	CR705-16-mod only	upper, lower combo	mGUUUUmAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
118	CR004202-mod only	CR705-17-mod only	lower combo	mGUUUUmAGAGCUAUGCUGUUUUG
119	CR004203-mod only	CR705-18-mod only	upper, lower combo	mGUUUUAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
120	CR004204-mod only	CR705-19-mod only	nexus walk	GUUUUAmGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
121	CR004205-mod only	CR705-20-mod only	nexus walk	GUUUUAGmAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
122	CR004206-mod only	CR705-21-mod only	nexus walk	GAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
123	CR004207-mod only	CR705-22-mod only	nexus walk	GfAmGmCmUmAmUmGmCmUmGmUmUmUmUmG

SEQ ID NO	Name	Alias	Description	Sequence
124	CR004208-mod only	CR705-23-mod only	2'F lower walk	GfUUUUAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
125	CR004209-mod only	CR705-24-mod only	2'F lower walk	GUfUUUAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
126	CR004210-mod only	CR705-25-mod only	2'F lower walk	GUUfUUAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
127	CR004211-mod only	CR705-26-mod only	2'F lower walk	GUUUfUAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
128	CR004212-mod only	CR705-27-mod only	2'F lower combo	fGfUfUfUfUfAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
129	CR004213-mod only	CR705-28-mod only	lower alt	fGmUfUmUfUmAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
130	CR004214-mod only	CR705-29-mod only	lower alt	mGfUmUfUmUfAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
131	CR000657		unmodified	CAGGGCUCUUGAAGAUCUCCGUUUUAGAGCUAUGCUGUUUUG
132	CR004218	CR657-1	upper	CAGGGCUCUUGAAGAUCUCCGUUUUAGAmGmCmUmAmUmGmCmUmGmUmUmUmG
133	CR004219	CR657-2	partial upper	CAGGGCUCUUGAAGAUCUCCGUUUUAGAGCUAmUmGmCmUmGmUmUmUmUmG
134	CR004220	CR657-3	partial upper	CAGGGCUCUUGAAGAUCUCCGUUUUAGAGCUAUGCmUmGmUmUmUmUmG
135	CR004221	CR657-4	partial upper	CAGGGCUCUUGAAGAUCUCCGUUUUAGAGCUAUGCUGUmUmUmUmG
136	CR004222	CR657-5	lower	CAGGGCUCUUGAAGAUCUCCmGmUmUmUmUmAGAGCUAUGCUGUUUUG
137	CR004223	CR657-6	lower walk	CAGGGCUCUUGAAGAUCUCCmGUUUUAGAGCUAUGCUGUUUUG
138	CR004224	CR657-7	lower walk	CAGGGCUCUUGAAGAUCUCCGmUUUUAGAGCUAUGCUGUUUUG
139	CR004225	CR657-8	lower walk	CAGGGCUCUUGAAGAUCUCCGUmUUUAGAGCUAUGCUGUUUUG
140	CR004226	CR657-9	lower walk	CAGGGCUCUUGAAGAUCUCCGUUmUUAGAGCUAUGCUGUUUUG
141	CR004227	CR657-10	lower walk	CAGGGCUCUUGAAGAUCUCCGUUUmUAGAGCUAUGCUGUUUUG
142	CR004228	CR657-11	lower walk	CAGGGCUCUUGAAGAUCUCCGUUUUmAGAGCUAUGCUGUUUUG
143	CR004229	CR657-14	upper and lower	CAGGGCUCUUGAAGAUCUCCmGUUUmUmAGAmGmCmUmAmUmGmCmUmGmUmUmUmG
144	CR004230	CR657-15	lower combo	CAGGGCUCUUGAAGAUCUCCmGUUUmUmAGAGCUAUGCUGUUUUG
145	CR004231	CR657-16	upper, lower combo	CAGGGCUCUUGAAGAUCUCCmGUUUUmAGAmGmCmUmAmUmGmCmUmGmUmUmUmG
146	CR004232	CR657-17	lower combo	CAGGGCUCUUGAAGAUCUCCmGUUUUmAGAGCUAUGCUGUUUUG

SEQ ID NO	Name	Alias	Description	Sequence
147	CR004233	CR657-18	upper, lower combo	CAGGGCUCUUGAAGAUCUCCmGUUUUAGAmGmCmUmAmUmGmCmUmGmUmUmUmG
148	CR004234	CR657-19	nexus walk	CAGGGCUCUUGAAGAUCUCCGUUUUAmGAmGmCmUmAmUmGmCmUmGmUmUmUmG
149	CR004235	CR657-20	nexus walk	CAGGGCUCUUGAAGAUCUCCGUUUUAGmAmGmCmUmAmUmGmCmUmGmUmUmUmG
150	CR004236	CR657-21	nexus walk	CAGGGCUCUUGAAGAUCUCCGUUUUAfGAmGmCmUmAmUmGmCmUmGmUmUmUmG
151	CR004237	CR657-22	nexus walk	CAGGGCUCUUGAAGAUCUCCGUUUUAGfAmGmCmUmAmUmGmCmUmGmUmUmUmG
152	CR004238	CR657-23	2'F lower walk	CAGGGCUCUUGAAGAUCUCCGfUUUUAGAmGmCmUmAmUmGmCmUmGmUmUmUmG
153	CR004239	CR657-24	2'F lower walk	CAGGGCUCUUGAAGAUCUCCGUfUUUAGAmGmCmUmAmUmGmCmUmGmUmUmUmG
154	CR004240	CR657-25	2'F lower walk	CAGGGCUCUUGAAGAUCUCCGUUfUUAGAmGmCmUmAmUmGmCmUmGmUmUmUmG
155	CR004241	CR657-26	2'F lower walk	CAGGGCUCUUGAAGAUCUCCGUUUfUAGAmGmCmUmAmUmGmCmUmGmUmUmUmG
156	CR004242	CR657-27	2'F lower combo	CAGGGCUCUUGAAGAUCUCCfGfUfUfUfUfAGAmGmCmUmAmUmGmCmUmGmUmUmUmG
157	CR004243	CR657-28	lower alt	CAGGGCUCUUGAAGAUCUCCfGmUfUmUfUmAGAmGmCmUmAmUmGmCmUmGmUmUmUmG
158	CR004244	CR657-29	lower alt	CAGGGCUCUUGAAGAUCUCCmGfUmUfUmUfAGAmGmCmUmAmUmGmCmUmGmUmUmUmG
159	CR004245	CR657-GC1	Lower GC	CAGGGCUCUUGAAGAUCUCCGGCGCAGAGCUAUGCUGUUUUG
160	CR004246	CR657-GC3	Upper GC	CAGGGCUCUUGAAGAUCUCCGUUUUAGAGCUAUGCUGGCGCG
161	CR004218-mod only	CR657-1-mod only	upper	GUUUUAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
162	CR004219-mod only	CR657-2-mod only	partial upper	GUUUUAGAGCUAmUmGmCmUmGmUmUmUmUmG
163	CR004220-mod only	CR657-3-mod only	partial upper	GUUUUAGAGCUAUGCmUmGmUmUmUmUmG

SEQ ID NO	Name	Alias	Description	Sequence
164	CR004221-mod only	CR657-4-mod only	partial upper	GUUUUAGAGCUAUGCUGUmUmUmUmG
165	CR004222-mod only	CR657-5-mod only	lower	mGmUmUmUmUmAGAGCUAUGCUGUUUUG
166	CR004223-mod only	CR657-6-mod only	lower walk	mGUUUUAGAGCUAUGCUGUUUUG
167	CR004224-mod only	CR657-7-mod only	lower walk	GmUUUUAGAGCUAUGCUGUUUUG
168	CR004225-mod only	CR657-8-mod only	lower walk	GUmUUUAGAGCUAUGCUGUUUUG
169	CR004226-mod only	CR657-9-mod only	lower walk	GUUmUUAGAGCUAUGCUGUUUUG
170	CR004227-mod only	CR657-10-mod only	lower walk	GUUUmUAGAGCUAUGCUGUUUUG
171	CR004228-mod only	CR657-11-mod only	lower walk	GUUUUmAGAGCUAUGCUGUUUUG
172	CR004229-mod only	CR657-14-mod only	upper and lower	mGUUUmUmAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
173	CR004230-mod only	CR657-15-mod only	lower combo	mGUUUmUmAGAGCUAUGCUGUUUUG
174	CR004231-mod only	CR657-16-mod only	upper, lower combo	mGUUUUmAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
175	CR004232-mod only	CR657-17-mod only	lower combo	mGUUUUmAGAGCUAUGCUGUUUUG
176	CR004233-mod only	CR657-18-mod only	upper, lower combo	mGUUUUAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
177	CR004234-mod only	CR657-19-mod only	nexus walk	GUUUUAmGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
178	CR004235-mod only	CR657-20-mod only	nexus walk	GUUUUAGmAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
179	CR004236-mod only	CR657-21-mod only	nexus walk	GUUUUAfGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
180	CR004237-mod only	CR657-22-mod only	nexus walk	GUUUUAGfAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
181	CR004238-mod only	CR657-23-mod only	2'F lower walk	GfUUUUAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
182	CR004239-mod only	CR657-24-mod only	2'F lower walk	GUfUUUAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
183	CR004240-mod only	CR657-25-mod only	2'F lower walk	GUUfUUAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
184	CR004241-mod only	CR657-26-mod only	2'F lower walk	GUUUfUAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
185	CR004242-mod only	CR657-27-mod only	2'F lower combo	fGfUfUfUfUfAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
186	CR004243-mod only	CR657-28-mod only	lower alt	fGmUfUmUfUmAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
187	CR004244-mod only	CR657-29-mod only	lower alt	mGfUmUfUmUfAGAmGmCmUmAmUmGmCmUmGmUmUmUmUmG
	trRNA			
188	TR000002		unmodified	AACAGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAG UGGCACCGAGUCGGUGCUUUUUUU

SEQ ID NO	Name	Alias	Description	Sequence
189	TR000110	TR2-v2-1	shortened tail	AACAGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
190	TR000111	TR2-v2-2	Upper, hairpins	mAmAmCmAmGmCmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmUmUmUmU
191	TR000112	TR2-v2-3	upper only	mAmAmCmAmGmCmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
192	TR000113	TR2-v2-4	hairpin 1	AACAGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGGCACCGAGUCGGUGCUUUU
193	TR000114	TR2-v2-5	hairpin 2	AACAGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmUmUmUmU
194	TR000115	TR2-v2-6	upper, hairpin 2	mAmAmCmAmGmCmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmUmUmUmU
195	TR000116	TR2-v2-7	both hairpins	AACAGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmUmUmUmU
196	TR000117	TR2-v2-8	lower walk	AACAGCAUAGCAAGUmUmAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
197	TR000118	TR2-v2-9	lower walk	AACAGCAUAGCAAGUUAmAmAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
198	TR000119	TR2-v2-10	lower walk	AACAGCAUAGCAAGUUAAAmAmUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
199	TR000120	TR2-v2-11	partial nexus	AACAGCAUAGCAAGUUAAAAUAAGGCUAGUCCGmUmUmAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
200	TR000121	TR2-v2-12	partial nexus	AACAGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAmUmCmAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
201	TR000122	TR2-GC1	Lower GC	AACAGCAUAGCAAGUUGCGCUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU

SEQ ID NO	Name	Alias	Description	Sequence
202	TR000123	TR2-GC3	upper GC	GCCAGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAG UGGCACCGAGUCGGUGCUUUU
203	TR000124	TR2-GC5	Lower Upper GC	GCCAGCAUAGCAAGUUGCGCUAAGGCUAGUCCGUUAUCAACUUGAAAAA GUGGCACCGAGUCGGUGCUUUU
204	TR000125	TR2 all OMe		mAmAmCmAmGmCmAmUmAmGmCmAmAmGmUmUmAmAmAmAmU mAmAmGmGmCmUmAmGmUmCmCmGmUmUmAmUmCmAmAmCmU mUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmG mGmUmGmCmUmUmUmUmUmUmU
205	TR000126	TR2-v2-13	lower	mAmAmCmAmGmCmAmUmAmGmCAAGUmUmAmAmAmAmUAAGGCU AGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmA mCmCmGmAmGmUmCmGmGmUmGmCmUmUmUmU
206	TR000127	TR2-v2-14	lower	mAmAmCmAmGmCmAmUmAmGmCAAGUmUmAAAmAmUAAGGCUAG UCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmC mCmGmAmGmUmCmGmGmUmGmCmUmUmUmU
207	TR000128	TR2-v2-15	lower	mAmAmCmAmGmCmAmUmAmGmCAAGUmUmAfAfAmAmUAAGGCUA GUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAm CmCmGmAmGmUmCmGmGmUmGmCmUmUmUmU
208	TR000129	TR2-v2-16	lower alt	mAmAmCmAmGmCmAmUmAmGmCAAGUmUfAmAfAmAfUAAGGCUAG UCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmC mCmGmAmGmUmCmGmGmUmGmCmUmUmUmU
209	TR000130	TR2-v2-17	lower alt	mAmAmCmAmGmCmAmUmAmGmCAAGUfUmAfAmAfAmUAAGGCUAG UCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmC mCmGmAmGmUmCmGmGmUmGmCmUmUmUmU
210	TR000131	TR2-v2-18	nexus walk	mAmAmCmAmGmCmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUU AUCmAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmG mAmGmUmCmGmGmUmGmCmUmUmUmU
211	TR000132	TR2-v2-19	nexus walk	mAmAmCmAmGmCmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUU AUmCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmG mAmGmUmCmGmGmUmGmCmUmUmUmU

SEQ ID NO	Name	Alias	Description	Sequence
212	TR000133	TR2-v2-20	nexus walk	mAmAmCmAmGmCmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAmUCAmAmCmUmUmGmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmUmUmUmU
213	TR000134	TR2-v2-21	nexus walk	mAmAmCmAmGmCmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUmAUCAmAmCmUmUmGmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmUmUmUmU
214	TR000135	TR2-v2-22	nexus walk	mAmAmCmAmGmCmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUmUAUCAmAmCmUmUmGmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmUmUmUmU
215	TR000136	TR2-v2-23	nexus walk	mAmAmCmAmGmCmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGmUUAUCAmAmCmUmUmGmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmUmUmUmU
216	TR000137	TR2-v2-24	nexus walk	mAmAmCmAmGmCmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUfCfAmAmCmUmUmGmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmUmUmUmU
217	TR000138	TR2-v2-25	nexus walk	mAmAmCmAmGmCmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUfAfUCAmAmCmUmUmGmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmUmUmUmU
218	TR000139	TR2-v2-26	nexus walk	mAmAmCmAmGmCmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGfUfUAUCAmAmCmUmUmGmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmUmUmUmU
219	TR000140	TR2-v2-27	nexus walk	mAmAmCmAmGmCmAmUmAmGmCAAGUUAAAAUAAmGmGmCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmUmUmUmU
220	TR000141	TR2-v2-28	nexus walk	mAmAmCmAmGmCmAmUmAmGmCAAGUUAAAAUAAGGCUAGUmCmCmGUUAUCAmAmCmUmUmGmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmUmUmUmU

SEQ ID NO	Name	Alias	Description	Sequence
221	TR000142	TR2-v2-29	bulge walk	mAmAmCmAmGmCmAmUmAmGmCmAmAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmUmUmUmU
222	TR000143	TR2-v2-30	bulge walk	mAmAmCmAmGmCmAmUmAmGmCAAmGmUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmUmUmUmU
223	TR000144	TR2-GC6	Lower GC	AACAGCAUAGCAAGUUGAGAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
224	TR000145	TR2-GC7	Lower GC walk	AACAGCAUAGCAAGUUAAAGUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
225	TR000146	TR2-GC8	Lower GC walk	AACAGCAUAGCAAGUUAAGAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
226	TR000147	TR2-GC9	Lower GC walk	AACAGCAUAGCAAGUUAGAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
227	TR000148	TR2-GC10	Lower GC walk	AACAGCAUAGCAAGUUGAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
	sgRNA			
228	G000209			mC*mC*mA*GUCCAGCGAGGCAAAGGGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCmU*mU*mU*U
229	G000262	G209-1	hairpin 2	mC*mC*mA*GUCCAGCGAGGCAAAGGGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
230	G000263	G209-2	hairpins	mC*mC*mA*GUCCAGCGAGGCAAAGGGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU

SEQ ID NO	Name	Alias	Description	Sequence
231	G000264	G209-3	tetraloop	mC*mC*mA*GUCCAGCGAGGCAAAGGGUUUUAGAGCUAmGmAmAmAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCmU*mU*mU*U
232	G000265	G209-4	upper	mC*mC*mA*GUCCAGCGAGGCAAAGGGUUUUAGAmGmCmUmAGAAAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCmU*mU*mU*U
233	G000266	G209-5	upper and loop	mC*mC*mA*GUCCAGCGAGGCAAAGGGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCmU*mU*mU*U
234	G000267	G209-6	upper, loop, hairpins	mC*mC*mA*GUCCAGCGAGGCAAAGGGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
235	G000262-mod only	G209-1-mod only	hairpin 2	GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
236	G000263-mod only	G209-2-mod only	hairpins	GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
237	G000264-mod only	G209-3-mod only	tetraloop	GUUUUAGAGCUAmGmAmAmAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCmU*mU*mU*U
238	G000265-mod only	G209-4-mod only	upper	GUUUUAGAmGmCmUmAGAAAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCmU*mU*mU*U
239	G000266-mod only	G209-5-mod only	upper and loop	GUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCmU*mU*mU*U
240	G000267-mod only	G209-6-mod only	upper, loop, hairpins	GUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU

SEQ ID NO	Name	Alias	Description	Sequence
241	G000211		end mod	mU*mU*mA*CAGCCACGUCUACAGCAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCmU*mU*mU*U
242	G000282		mod6	mU*mU*mA*CAGCCACGUCUACAGCAGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
243	G000201		unmod	UUACAGCCACGUCUACAGCAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
244	G000331	G211-7	lower cr	mU*mU*mA*CAGCCACGUCUACAGCAmGUUUUmAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
245	G000332	G211-8	lower cr	mU*mU*mA*CAGCCACGUCUACAGCAfGfUfUfUfUfAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
246	G000333	G211-9	lower cr	mU*mU*mA*CAGCCACGUCUACAGCAmGfUfUfUfUmAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
247	G000334	G211-10	lower tr	mU*mU*mA*CAGCCACGUCUACAGCAGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUmUmAAAmAmUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
248	G000335	G211-11	lower tr	mU*mU*mA*CAGCCACGUCUACAGCAGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUmUmAfAfAmAmUAAGGCUAGUCCGUUAUCAmA

SEQ ID NO	Name	Alias	Description	Sequence
				mCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmU mCmGmGmUmGmCmU*mU*mU*mU
249	G000336	G211-12	lower tr	mU*mU*mA*CAGCCACGUCUACAGCAGUUUUAGAmGmCmUmAmGmAm AmAmUmAmGmCAAGUfUmAfAmAfAmUAAGGCUAGUCCGUUAUCAmA mCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmU mCmGmGmUmGmCmU*mU*mU*mU
250	G000337	G211-13	lower all	mU*mU*mA*CAGCCACGUCUACAGCAmGUUUUmAGAmGmCmUmAmG mAmAmAmUmAmGmCAAGUmUmAAAmAmUAAGGCUAGUCCGUUAUCA mAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmG mUmCmGmGmUmGmCmU*mU*mU*mU
251	G000338	G211-14	lower all	mU*mU*mA*CAGCCACGUCUACAGCAmGUUUUmAGAmGmCmUmAmG mAmAmAmUmAmGmCAAGUmUmAfAfAmAmUAAGGCUAGUCCGUUAUC AmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAm GmUmCmGmGmUmGmCmU*mU*mU*mU
252	G000339	G211-15	lower all	mU*mU*mA*CAGCCACGUCUACAGCAmGUUUUmAGAmGmCmUmAmG mAmAmAmUmAmGmCAAGUfUmAfAmAfAmUAAGGCUAGUCCGUUAUC AmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAm GmUmCmGmGmUmGmCmU*mU*mU*mU
253	G000340	G211-16	lower all	mU*mU*mA*CAGCCACGUCUACAGCAfGfUfUfUfUfAGAmGmCmUmAmG mAmAmAmUmAmGmCAAGUmUmAAAmAmUAAGGCUAGUCCGUUAUCA mAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmG mUmCmGmGmUmGmCmU*mU*mU*mU
254	G000341	G211-17	lower all	mU*mU*mA*CAGCCACGUCUACAGCAfGfUfUfUfUfAGAmGmCmUmAmG mAmAmAmUmAmGmCAAGUmUmAfAfAmAmUAAGGCUAGUCCGUUAUC AmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAm GmUmCmGmGmUmGmCmU*mU*mU*mU
255	G000342	G211-18	lower all	mU*mU*mA*CAGCCACGUCUACAGCAfGfUfUfUfUfAGAmGmCmUmAmG mAmAmAmUmAmGmCAAGUfUmAfAmAfAmUAAGGCUAGUCCGUUAUC

SEQ ID NO	Name	Alias	Description	Sequence
				AmAmCmUmUmGmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
256	G000343	G211-19	Bulge cr	mU*mU*mA*CAGCCACGUCUACAGCAGUUUUAmGmAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
257	G000344	G211-20	Bulge tr	mU*mU*mA*CAGCCACGUCUACAGCAGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCmAmAmGmUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
258	G000345	G211-21	nexus	mU*mU*mA*CAGCCACGUCUACAGCAGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUfAfUfCfAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
259	G000346	G211-22	nexus	mU*mU*mA*CAGCCACGUCUACAGCAGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAmUmCmAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
260	G000347	G211-23	lower all	mU*mU*mA*CAGCCACGUCUACAGCAfGfUfUfUfUfAmGmAmGmCmUmAmGmAmAmAmUmAmGmCmAmAmGmUmUmAfAfAmAmUAAGGCUAGUCCGUUAmUmCmAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
261	G000348	G211-24	no PS	mUmUmACAGCCACGUCUACAGCAGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmUmUmUmU
262	G000349	G211-25	2 OMe PS	mU*mU*ACAGCCACGUCUACAGCAGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmU

SEQ ID NO	Name	Alias	Description	Sequence
				mGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmUmU*mU*mU
263	G000350	G211-26	2'F hairpin	mU*mU*mA*CAGCCACGUCUACAGCAGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGfUfCfGfGfUfGfCfU*fU*fU*mU
264	G000351	G211-27	Alt hairpin	mU*mU*mA*CAGCCACGUCUACAGCAGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAfAmCfUmUfGmAfAmAfAmAfGmUfGmGfCmAfCmCfGmAfGmUfCmGfGmUfGmCfU*mU*fU*mU
265	G000331-mod only	G211-7-mod only	lower cr	mGUUUUmAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
266	G000332-mod only	G211-8-mod only	lower cr	fGfUfUfUfUfAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
267	G000333-mod only	G211-9-mod only	lower cr	mGfUfUfUfUmAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
268	G000334-mod only	G211-10-mod only	lower tr	GUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUmUmAAAmAmUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
269	G000335-mod only	G211-11-mod only	lower tr	GUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUmUmAfAfAmAmUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
270	G000336-mod only	G211-12-mod only	lower tr	GUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUfUmAfAmAfAmUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU

SEQ ID NO	Name	Alias	Description	Sequence
271	G000337-mod only	G211-13-mod only	lower all	mGUUUUmAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUmUmAAAmAmUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
272	G000338-mod only	G211-14-mod only	lower all	mGUUUUmAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUmUmAfAfAmAmUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
273	G000339-mod only	G211-15-mod only	lower all	mGUUUUmAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUfUmAfAmAfAmUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
274	G000340-mod only	G211-16-mod only	lower all	fGfUfUfUfUfAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUmUmAAAmAmUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
275	G000341-mod only	G211-17-mod only	lower all	fGfUfUfUfUfAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUmUmAfAfAmAmUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
276	G000342-mod only	G211-18-mod only	lower all	fGfUfUfUfUfAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUfUmAfAmAfAmUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
277	G000343-mod only	G211-19-mod only	Bulge cr	GUUUUAmGmAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU

SEQ ID NO	Name	Alias	Description	Sequence
278	G000344-mod only	G211-20-mod only	Bulge tr	GUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCmAmAmGmUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
279	G000345-mod only	G211-21-mod only	nexus	GUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUfAfUfCfAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
280	G000346-mod only	G211-22-mod only	nexus	GUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAmUmCmAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
281	G000347-mod only	G211-23-mod only	lower all	fGfUfUfUfUfAmGmAmGmCmUmAmGmAmAmAmUmAmGmCmAmAmGmUmUmAfAfAmAmUAAGGCUAGUCCGUUAmUmCmAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
282	G000348-mod only	G211-24-mod only	no PS	GUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmUmUmUmU
283	G000349-mod only	G211-25-mod only	2 OMe PS	GUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU
284	G000350-mod only	G211-26-mod only	2'F hairpin	GUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGfUfCfGfGfUfGfCfU*fU*fU*mU
285	G000351-mod only	G211-27-mod only	Alt hairpin	GUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAfAmCfUmUfGmAfAmAfAmAfGmUfGmGfCmAfCmCfGmAfGmUfCmGfGmUfGmCfU*mU*fU*mU
286	G000208		end mod	mC*mA*mG*GGCUCUUGAAGAUCUCCGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCmU*mU*mU*U

SEQ ID NO	Name	Alias	Description	Sequence
287	G000373		mod6	mC*mA*mG*GGCUCUUGAAGAUCUCCGUUUUAGAmGmCmUmAmGmA mAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmU mUmGmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmG mGmUmGmCmU*mU*mU*mU
288	G000352	G208-7	lower cr	mC*mA*mG*GGCUCUUGAAGAUCUCCmGUUUUmAGAmGmCmUmAmG mAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmC mUmUmGmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmC mGmGmUmGmCmU*mU*mU*mU
289	G000353	G208-8	lower cr	mC*mA*mG*GGCUCUUGAAGAUCUCCfGfUfUfUfUfAGAmGmCmUmAmG mAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmC mUmUmGmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmC mGmGmUmGmCmU*mU*mU*mU
290	G000354	G208-9	lower cr	mC*mA*mG*GGCUCUUGAAGAUCUCCmGfUfUfUfUmAGAmGmCmUmA mGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmA mCmUmUmGmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmU mCmGmGmUmGmCmU*mU*mU*mU
291	G000355	G208-10	lower tr	mC*mA*mG*GGCUCUUGAAGAUCUCCGUUUUAGAmGmCmUmAmGmA mAmAmUmAmGmCAAGUmUmAAAmAmUAAGGCUAGUCCGUUAUCAmA mCmUmUmGmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmU mCmGmGmUmGmCmU*mU*mU*mU
292	G000356	G208-11	lower tr	mC*mA*mG*GGCUCUUGAAGAUCUCCGUUUUAGAmGmCmUmAmGmA mAmAmUmAmGmCAAGUmUmAfAfAmAmUAAGGCUAGUCCGUUAUCAm AmCmUmUmGmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGm UmCmGmGmUmGmCmU*mU*mU*mU
293	G000357	G208-12	lower tr	mC*mA*mG*GGCUCUUGAAGAUCUCCGUUUUAGAmGmCmUmAmGmA mAmAmUmAmGmCAAGUfUmAfAmAfAmUAAGGCUAGUCCGUUAUCAm AmCmUmUmGmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGm UmCmGmGmUmGmCmU*mU*mU*mU

SEQ ID NO	Name	Alias	Description	Sequence
294	G000358	G208-13	lower all	mC*mA*mG*GGCUCUUGAAGAUCUCCmGUUUUmAGAmGmCmUmAmG mAmAmAmUmAmGmCAAGUmUmAAAmAmUAAGGCUAGUCCGUUAUCA mAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmG mUmCmGmGmUmGmCmU*mU*mU*mU
295	G000359	G208-14	lower all	mC*mA*mG*GGCUCUUGAAGAUCUCCmGUUUUmAGAmGmCmUmAmG mAmAmAmUmAmGmCAAGUmUmAfAfAmAmUAAGGCUAGUCCGUUAUC AmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAm GmUmCmGmGmUmGmCmU*mU*mU*mU
296	G000360	G208-15	lower all	mC*mA*mG*GGCUCUUGAAGAUCUCCmGUUUUmAGAmGmCmUmAmG mAmAmAmUmAmGmCAAGUfUmAfAmAfAmUAAGGCUAGUCCGUUAUC AmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAm GmUmCmGmGmUmGmCmU*mU*mU*mU
297	G000361	G208-16	lower all	mC*mA*mG*GGCUCUUGAAGAUCUCCfGfUfUfUfUfAGAmGmCmUmAmG mAmAmAmUmAmGmCAAGUmUmAAAmAmUAAGGCUAGUCCGUUAUCA mAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmG mUmCmGmGmUmGmCmU*mU*mU*mU
298	G000362	G208-17	lower all	mC*mA*mG*GGCUCUUGAAGAUCUCCfGfUfUfUfUfAGAmGmCmUmAmG mAmAmAmUmAmGmCAAGUmUmAfAfAmAmUAAGGCUAGUCCGUUAUC AmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAm GmUmCmGmGmUmGmCmU*mU*mU*mU
299	G000363	G208-18	lower all	mC*mA*mG*GGCUCUUGAAGAUCUCCfGfUfUfUfUfAGAmGmCmUmAmG mAmAmAmUmAmGmCAAGUfUmAfAmAfAmUAAGGCUAGUCCGUUAUC AmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAm GmUmCmGmGmUmGmCmU*mU*mU*mU
300	G000364	G208-19	Bulge cr	mC*mA*mG*GGCUCUUGAAGAUCUCCGUUUUAmGmAmGmCmUmAmG mAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmC mUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmC mGmGmUmGmCmU*mU*mU*mU

SEQ ID NO	Name	Alias	Description	Sequence
301	G000365	G208-20	Bulge tr	mC*mA*mG*GGCUCUUGAAGAUCUCCGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCmAmAmGmUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
302	G000366	G208-21	nexus	mC*mA*mG*GGCUCUUGAAGAUCUCCGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCmAAGUUAAAAUAAGGCUAGUCCGUUfAfUfCfAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
303	G000367	G208-22	nexus	mC*mA*mG*GGCUCUUGAAGAUCUCCGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCmAAGUUAAAAUAAGGCUAGUCCGUUAmUmCmAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
304	G000368	G208-23	lower all	mC*mA*mG*GGCUCUUGAAGAUCUCCfGfUfUfUfUfAmGmAmGmCmUmAmGmAmAmAmUmAmGmCmAmAmGmUmUmAfAfAmAmUAAGGCUAGUCCGUUAmUmCmAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
305	G000369	G208-24	no PS	mCmAmGGGCUCUUGAAGAUCUCCGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCmAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmUmUmUmU
306	G000370	G208-25	2 OMe PS	mC*mAG*GGCUCUUGAAGAUCUCCGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCmAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmUmU*mU*mU
307	G000371	G208-26	2'F hairpin	mC*mA*mG*GGCUCUUGAAGAUCUCCGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCmAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGfUfCfGfGfUfGfCfU*fU*fU*mU

SEQ ID NO	Name	Alias	Description	Sequence
308	G000372	G208-27	Alt hairpin	mC*mA*mG*GGCUCUUGAAGAUCUCCGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAfAmCfUmUfGmAfAmAfAmAfGmUfGmGfCmAfCmCfGmAfGmUfCmGfGmUfGmCfU*mU*fU*mU
309	G000352-mod only	G208-7-mod only	lower cr	mGUUUUmAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
310	G000353-mod only	G208-8-mod only	lower cr	fGfUfUfUfUfAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
311	G000354-mod only	G208-9-mod only	lower cr	mGfUfUfUfUmAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
312	G000355-mod only	G208-10-mod only	lower tr	GUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUmUmAAAmAmUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
313	G000356-mod only	G208-11-mod only	lower tr	GUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUmUmAfAfAmAmUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
314	G000357-mod only	G208-12-mod only	lower tr	GUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUfUmAfAmAfAmUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
315	G000358-mod only	G208-13-mod only	lower all	mGUUUUmAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUmUmAAAmAmUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
316	G000359-mod only	G208-14-mod only	lower all	mGUUUUmAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUmUmAfAfAmAmUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmG

SEQ ID NO	Name	Alias	Description	Sequence
				mUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
317	G000360-mod only	G208-15-mod only	lower all	mGUUUUmAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUfUmAfAmAfAmUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
318	G000361-mod only	G208-16-mod only	lower all	fGfUfUfUfUfAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUmUmAAAmAmUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
319	G000362-mod only	G208-17-mod only	lower all	fGfUfUfUfUfAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUmUmAfAfAmAmUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
320	G000363-mod only	G208-18-mod only	lower all	fGfUfUfUfUfAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUfUmAfAmAfAmUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
321	G000364-mod only	G208-19-mod only	Bulge cr	GUUUUAmGmAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
322	G000365-mod only	G208-20-mod only	Bulge tr	GUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCmAmAmGmUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
323	G000366-mod only	G208-21-mod only	nexus	GUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUfAfUfCfAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU

SEQ ID NO	Name	Alias	Description	Sequence
324	G000367-mod only	G208-22-mod only	nexus	GUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAmUmCmAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
325	G000368-mod only	G208-23-mod only	lower all	fGfUfUfUfUfAmGmAmGmCmUmAmGmAmAmAmUmAmGmCmAmAmGmUmUmAfAfAmAmUAAGGCUAGUCCGUUAmUmCmAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
326	G000369-mod only	G208-24-mod only	no PS	GUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmUmUmUmU
327	G000370-mod only	G208-25-mod only	2 OMe PS	GUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmUmU*mU*mU
328	G000371-mod only	G208-26-mod only	2'F hairpin	GUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGfUfCfGfGfUfGfCfU*fU*fU*mU
329	G000372-mod only	G208-27-mod only	Alt hairpin	GUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAfAmCfUmUfGmAfAmAfAmAfGmUfGmGfCmAfCmCfGmAfGmUfCmGfGmUfGmCfU*mU*fU*mU
330	G000269		end mod	mC*mC*mC*AUACUCCUACAGCACCAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCmU*mU*mU*U
331	G000283		mod6	mC*mC*mC*AUACUCCUACAGCACCAGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
332	G000285		unmod	CCCAUACUCCUACAGCACCAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU

SEQ ID NO	Name	Alias	Description	Sequence
342	G000537	G211-33	5'end 3xOMePS	mU*mU*mA*CAGCCACGUCUACAGCAGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmUmUmUmU
343	G000538	G211-34	3'end 3xOMePS	mUmUmACAGCCACGUCUACAGCAGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
344	G000539	G211-35	5xOMePS	mU*mU*mA*mC*mA*GCCACGUCUACAGCAGUUUUAGAmGmCmUmAmGmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmG*mC*mU*mU*mU*mU
345	G000541	G211-37	3xOMePS+2PS	mU*mU*mA*C*A*GCCACGUCUACAGCAGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmG*mC*mU*mU*mU*mU
346	G000542	G211-38	3xOMePS+7PS	mU*mU*mA*C*A*G*C*C*A*C*GUCUACAGCAGUUUUAGAmGmCmUmAmGmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmU*mC*mG*mG*mU*mG*mC*mU*mU*mU*mU
347	G000543	G211-39	invd abasic	(invd)UUACAGCCACGUCUACAGCAGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmUmUmUmU(invd)
348	G000544	G211-40	invd abasic + 3xOMePS	(invd)mU*mU*mA*CAGCCACGUCUACAGCAGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU(invd)

SEQ ID NO	Name	Alias	Description	Sequence
349	G000564	G211-42	3xMOE-PS	moeU*moeU*moeA*CAGCCACGUCUACAGCAGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmoeU*moeU*moeU*mU
350	G000545	G211-43	US loop PS	mU*mU*mA*CAGCCACGUCUACAGCAGUUUUAGAmGmCmUmA*mG*mA*mA*mA*mUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
351	G000546	G211-44	H1 loop PS	mU*mU*mA*CAGCCACGUCUACAGCAGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmU*mG*mA*mA*mA*mAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
352	G000547	G211-45	H2 loop PS	mU*mU*mA*CAGCCACGUCUACAGCAGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmGmUmGmGmCmAmCmCmG*mA*mG*mU*mCmGmGmUmGmCmU*mU*mU*mU
353	G000548	G211-46	all loops PS	mU*mU*mA*CAGCCACGUCUACAGCAGUUUUAGAmGmCmUmA*mG*mA*mA*mA*mUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmU*mG*mA*mA*mA*mAmAmGmUmGmGmCmAmCmCmG*mA*mG*mU*mCmGmGmUmGmCmU*mU*mU*mU
354			Mod6 (with modifications not shown in sequence listing)	mN*mN*mN*NNNNNNNNNNNNNNNNGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU N = any nucleotide
355			Invariable region only	GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU

SEQ ID NO	Name	Alias	Description	Sequence
356			Mod6 pattern; invariable region only	GUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
357			Variable and invariable region	NNNNNNNNNNNNNNNNNNNNGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
358			Mod6 with modifications shown in sequence listing	mN*mN*mN*NNNNNNNNNNNNNNNNNNGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU N = any nucleotide

[0059] "Guide RNA" and "gRNA" are used herein interchangeably to refer collectively to either an sgRNA, a trRNA (also known as tracrRNA), or a crRNA (also known as a CRISPR RNA). The crRNA and trRNA may be associated on one RNA molecule (single guide RNA [sgRNA]) or in two separate RNA molecules (dual guide RNA [dgRNA]). "Guide RNA" or "gRNA" refers to each type.

[0060] The trRNA sequences may be naturally-occurring, or the trRNA sequence may include modifications or variations compared to naturally-occurring sequences.

[0061] "Editing efficiency" or "editing percentage" or "percent editing" as used herein is the total number of sequence reads with insertions or deletions of nucleotides into the target region of interest over the total number of sequence reads following cleavage by a Cas RNP.

[0062] "Hairpin" as used herein describes a loop of nucleic acids that is created when a nucleic acid strand folds and forms base pairs with another section of the same strand. A hairpin may form a structure that comprises a loop or a U-shape. In some embodiments, a hairpin may be comprised of a RNA loop. Hairpins can be formed with two complementary sequences in a single nucleic acid molecule bind together, with a folding or wrinkling of the molecule. In some embodiments, hairpins comprise stem or stem loop structures.

[0063] "Regions" as used herein describes conserved groups of nucleic acids. Regions may also be referred to as "modules" or "domains." Regions of a gRNA may perform particular functions, e.g., in directing endonuclease activity of the RNP, for example as described in *Briner AE et al., Molecular Cell 56:333–339 (2014)*. Regions of a gRNA are described in Tables 1-3.

[0064] "Ribonucleoprotein" (RNP) or "RNP complex" as used herein describes a gRNA, for example, together with a nuclease, such as a Cas protein. In some embodiments, the RNP comprises Cas9 and gRNA.

[0065] "Stem loop" as used herein describes a secondary structure of nucleotides that form a base-paired "stem" that ends in a loop of unpaired nucleic acids. A stem may be formed when two regions of the same nucleic acid strand are at least partially complementary in sequence when read in opposite directions. "Loop" as used herein describes a region of nucleotides that do not base pair (i.e., are not complementary) that may cap a stem. A "tetraloop" describes a loop of 4 nucleotides. As used herein, the upper stem of a sgRNA may comprise a tetraloop.

[0066] In certain embodiments involving dgRNA, a "stem" region as used herein describes a secondary structure of nucleotides that forms a base-paired region between certain

regions of a crRNA and trRNA (e.g., the lower and upper stem regions of each RNA). The "stem" region of a dgRNA may also be referred to in the art as a "flagpole" region.

[0067] "Treatment" as used herein covers any administration or application of a therapeutic for disease in a subject, and includes inhibiting the disease, arresting its development, relieving one or more symptoms of the disease, curing the disease, or preventing reoccurrence of one or more symptoms of the disease.

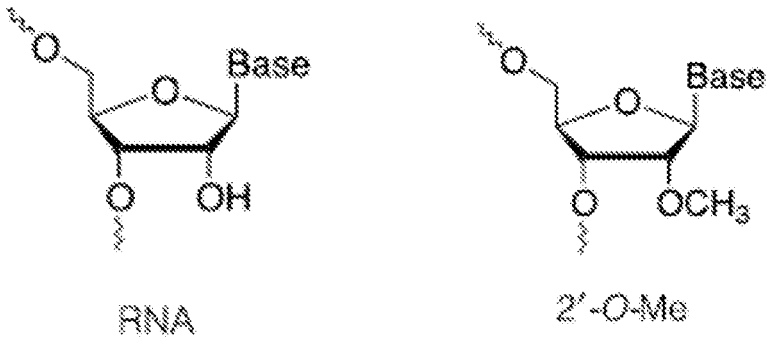
1. Types of Modifications

A. 2'-O-methyl modifications

[0068] Modified sugars are believed to control the puckering of nucleotide sugar rings, a physical property that influences oligonucleotide binding affinity for complementary strands, duplex formation, and interaction with nucleases. Substitutions on sugar rings can therefore alter the confirmation and puckering of these sugars. For example, 2'-O-methyl (2'-O-Me) modifications can increase binding affinity and nuclease stability of oligonucleotides, though as shown in the Examples, the effect of any modification at a given position in an oligonucleotide needs to be empirically determined.

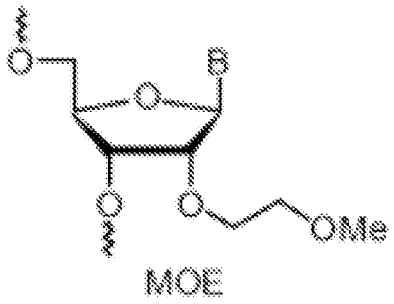
[0069] The terms "mA," "mC," "mU," or "mG" may be used to denote a nucleotide that has been modified with 2'-O-Me.

[0070] Modification of a ribonucleotide as 2'-O-methyl ribonucleotide can be depicted as follows:



B. 2'-O-(2-methoxyethyl) modifications

[0071] In some embodiments, the modification may be 2'-O-(2-methoxyethyl) (2'-O-moe). Modification of a ribonucleotide as a 2'-O-moe ribonucleotide can be depicted as follows:



MOE

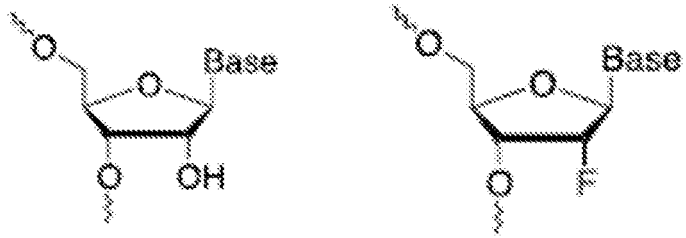
[0072] The terms "moeA," "moeC," "moeU," or "moeG" may be used to denote a nucleotide that has been modified with 2'-O-moe.

C. 2'-fluoro modifications

[0073] Another chemical modification that has been shown to influence nucleotide sugar rings is halogen substitution. For example, 2'-fluoro (2'-F) substitution on nucleotide sugar rings can increase oligonucleotide binding affinity and nuclease stability.

[0074] In this application, the terms "fA," "fC," "fU," or "fG" may be used to denote a nucleotide that has been substituted with 2'-F.

[0075] Substitution of 2'-F can be depicted as follows:



RNA 2'F-RNA

Natural composition of RNA 2'F substitution

D. Phosphorothioate modifications

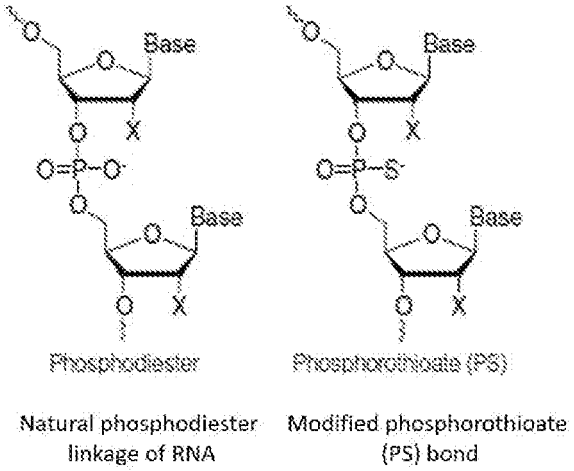
[0076] Phosphorothioate (PS) linkage or bond refers to a bond where a sulfur is substituted for one nonbridging phosphate oxygen in a phosphodiester linkage, for example

in the bonds between nucleotides bases. When phosphorothioates are used to generate oligonucleotides, the modified oligonucleotides may also be referred to as S-oligos.

[0077] A "*" may be used to depict a PS modification. In this application, the terms A*, C*, U*, or G* may be used to denote a nucleotide that is linked to the next (e.g., 3') nucleotide with a PS bond.

[0078] In this application, the terms "mA*," "mC*," "mU*," or "mG*" may be used to denote a nucleotide that has been substituted with 2'-O-Me and that is linked to the next (e.g., 3') nucleotide with a PS bond. Similarly, the terms "fA*," "fC*," "fU*," or "fG*" may be used to denote a nucleotide that has been substituted with 2'-F and that is linked to the next (e.g., 3') nucleotide with a PS bond. Equivalents of a PS linkage or bond are encompassed by embodiments described herein.

[0079] The diagram below shows the substitution of S- into a nonbridging phosphate oxygen, generating a PS bond in lieu of a phosphodiester bond:

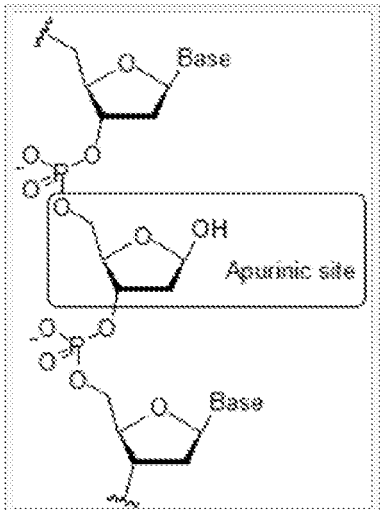


E. G-C substitutions

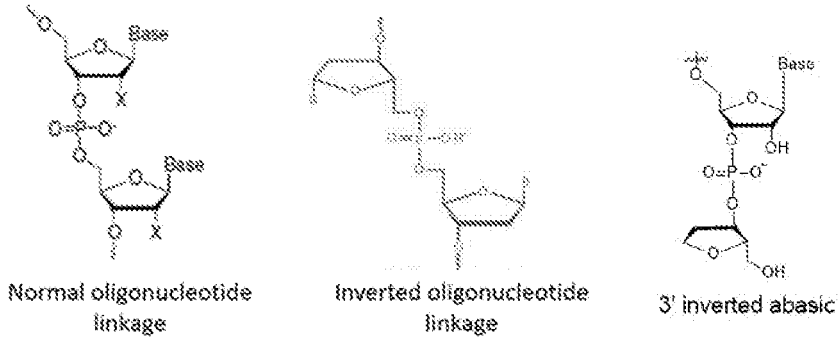
[0080] In some embodiments, gRNAs are modified with sequence substitutions that do not comprise chemical modifications. In some embodiments, modified gRNAs are engineered with G-C pairings (e.g., in lower and/or upper stem regions) that are not found in the parental gRNA sequence. In some embodiments, modified gRNAs are engineered with G-U mismatches ("GU wobbles" or mismatch pairings) that are not found in the parental gRNA sequence.

F. Inverted abasic modifications

[0081] Abasic nucleotides refer to those which lack nitrogenous bases. The figure below depicts an oligonucleotide with an abasic (also known as apurinic) site that lacks a base:



[0082] Inverted bases refer to those with linkages that are inverted from the normal 5' to 3' linkage (i.e., either a 5' to 5' linkage or a 3' to 3' linkage). For example:



[0083] An abasic nucleotide can be attached with an inverted linkage. For example, an abasic nucleotide may be attached to the terminal 5' nucleotide via a 5' to 5' linkage, or an abasic nucleotide may be attached to the terminal 3' nucleotide via a 3' to 3' linkage. An inverted abasic nucleotide at either the terminal 5' or 3' nucleotide may also be called an inverted abasic end cap. In this application, the terms "invd" indicates an inverted abasic nucleotide linkage.

[0084] The above modifications and their equivalents are included within the scope of the embodiments described herein.

2. Guide RNA Compositions

[0085] Compositions comprising guide RNA are encompassed. In some embodiments, the guide RNA comprises a trRNA. In some embodiments, the guide RNA comprises a crRNA. In some embodiments, the guide RNA comprises a crRNA and trRNA. In some embodiments, the guide RNA comprises a crRNA and trRNA on one RNA molecule as a sgRNA. In some embodiments, the guide RNA comprises a crRNA and trRNA on two RNA molecules as a dgRNA. In a dgRNA, the two RNA molecules may associate via base pairing.

[0086] In some embodiments, the guide RNA comprises a 5' terminus region. In some embodiments, the guide RNA does not comprise a 5' terminus region. In some embodiments, the 5' terminus region comprises a "spacer" region as described in *Briner AE et al., Molecular Cell 56:333–339 (2014)* for sgRNA (but applicable herein to all guide RNAs). In some embodiments, the 5' terminus region comprises a 5' end modification. A 5' terminus region with or without a spacer region may be associated with a crRNA, trRNA, sgRNA and/or dgRNA. The spacer region is also sometimes referred to herein, and by others, as a "guide region," "guide domain" or "targeting domain." A "target sequence" as used herein refers to a sequence of nucleic acid to which the guide region/domain directs a nuclease for cleavage. In some embodiments, a spyCas9 protein may be directed by a guide region/domain to a target sequence of a target nucleic acid molecule by the nucleotides present in the spacer region. In some embodiments, the guide RNA does not comprise a spacer region.

[0087] In some embodiments, the guide RNAs described herein comprise or consist of any of the sequences shown in Table 4. Note, however, that where a sequence shows a guide/spacer region, it should be recognized that the composition may comprise this region or not. Further, guide RNAs are encompassed that comprise the modifications of any of the sequences shown in Table 4, and identified therein by SEQ ID No. That is, the nucleotides may be the same or different, but the modification pattern shown may be the same or similar to a modification pattern of a guide sequence of Table 4. A modification pattern includes the relative position and identity of modifications of the gRNA or a region of the gRNA (e.g. 5' terminus region, lower stem region, bulge region, upper stem region, nexus region, hairpin 1 region, hairpin 2 region, 3' terminus region). In some embodiments, the modification pattern contains at least 50%, 55%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, and 99% of the modifications of any one of the sequences shown in the sequence column of Table

4, or over one or more regions of the sequence. In some embodiments, the modification pattern is at least 50%, 55%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, and 99% identical to the modification pattern of any one of the sequences shown in the sequence column of Table 4. In some embodiments, the modification pattern is at least 50%, 55%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, and 99% identical over one or more regions of the sequence shown in Table 4, e.g., a 5' terminus region, lower stem region, bulge region, upper stem region, nexus region, hairpin 1 region, hairpin 2 region, and/or 3' terminus region. For example, in some embodiments, a guide RNA is encompassed wherein the modification pattern is least 50%, 55%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, and 99% identical to the modification pattern of a sequence over the 5' terminus region. In some embodiments, a guide RNA is encompassed wherein the modification pattern is least 50%, 55%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, and 99% identical over the lower stem. In some embodiments, a guide RNA is encompassed wherein the modification pattern is least 50%, 55%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, and 99% identical over the bulge. In some embodiments, a guide RNA is encompassed wherein the modification pattern is least 50%, 55%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, and 99% identical over the upper stem. In some embodiments, a guide RNA is encompassed wherein the modification pattern is least 50%, 55%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, and 99% identical over the nexus. In some embodiments, a guide RNA is encompassed wherein the modification pattern is least 50%, 55%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, and 99% identical over the hairpin 1. In some embodiments, a guide RNA is encompassed wherein the modification pattern is least 50%, 55%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, and 99% identical over the hairpin 2. In some embodiments, a guide RNA is encompassed wherein the modification pattern is least 50%, 55%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, and 99% identical over the 3' terminus. In some embodiments, the modification pattern differs from the modification pattern of a sequence of Table 4, or a region (e.g. 5' terminus, lower stem, bulge, upper stem, nexus, hairpin 1, hairpin 2, 3' terminus) of such a sequence, at 0, 1, 2, 3, 4, 5, or 6 nucleotides. In some embodiments, the gRNA comprises modifications that differ from the modifications of a sequence of Table 4, at 0, 1, 2, 3, 4, 5, or 6 nucleotides. In some embodiments, the gRNA comprises modifications that differ from modifications of a region (e.g. 5' terminus, lower stem, bulge, upper stem, nexus, hairpin 1, hairpin 2, 3' terminus) of a sequence of Table 4, at 0, 1, 2, 3, 4, 5, or 6 nucleotides.

[0088] In some embodiments, the gRNA comprises a 2'-O-methyl (2'-O-Me) modified nucleotide. In some embodiments, the gRNA comprises a 2'-O-(2-methoxyethyl) (2'-O-moe) modified nucleotide. In some embodiments, the gRNA comprises a 2'-fluoro (2'-F) modified nucleotide. In some embodiments, the gRNA comprises a phosphorothioate (PS) bond between nucleotides.

[0089] In some embodiments, the gRNA comprises a 5' end modification, a 3' end modification, or 5' and 3' end modifications. In some embodiments, the 5' end modification comprises a phosphorothioate (PS) bond between nucleotides. In some embodiments, the 5' end modification comprises a 2'-O-methyl (2'-O-Me), 2'-O-(2-methoxyethyl) (2'-O-moe), and/or 2'-fluoro (2'-F) modified nucleotide. In some embodiments, the 5' end modification comprises at least one phosphorothioate (PS) bond and one or more of a 2'-O-methyl (2'-O-Me) , 2'-O-(2-methoxyethyl) (2'-O-moe), and/or 2'-fluoro (2'-F) modified nucleotide. The end modification may comprise a phosphorothioate (PS), 2'-O-methyl (2'-O-Me) , 2'-O-(2-methoxyethyl) (2'-O-moe), and/or 2'-fluoro (2'-F) modification. Equivalent end modifications are also encompassed by embodiments described herein. In some embodiments, the gRNA comprises an end modification in combination with a modification of one or more regions of the gRNA.

A. Compositions of sgRNAs

[0090] In some embodiments, the compositions and methods of the invention comprise gRNA comprising a crRNA and trRNA that direct a nuclease such as Cas9 to a target DNA sequence. In some embodiments, the gRNAs described herein may be associated on one RNA molecule (single guide RNA or sgRNA).

[0091] In some embodiments, the invention comprises a sgRNA comprising or consisting of any one of the sequences described in SEQ ID Nos: 228-332.

[0092] In some embodiments, a sgRNA comprising any one of the modified sequences of SEQ ID Nos: 235-240, 265-285, and 309-329 is provided. In some embodiments, a sgRNA comprising any one of the modified sequences of SEQ ID Nos: 235-240, 265-285, and 309-329, wherein the sgRNA further comprises a 5' "spacer" sequence ("guide sequence") that is complementary to a target sequence, and directs a Cas9 to its target for cleavage is encompassed. In some instances, the invention comprises sgRNA comprising nucleic acids having at least 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 85, 80, 75, or 70% identity to the nucleic acids of any one of SEQ ID Nos: 235-240, 265-285, and 309-329, wherein the

modification pattern is identical to the modification pattern shown in the reference sequence identifier.

1. Domains of sgRNAs

[0093] *Briner AE et al.*, *Molecular Cell* 56:333–339 (2014) describes functional domains of sgRNAs, referred to herein as "domains", including the "spacer" domain responsible for targeting, the "lower stem", the "bulge", "upper stem" (which may include a tetraloop), the "nexus", and the "hairpin 1" and "hairpin 2" domains. See, Briner et al. at page 334, Figure 1A.

[0094] **Table 1** and FIG. 21A provide a description of the domains of a sgRNA as used herein. In Table 1, the "n" between regions represents a variable number of nucleotides, for example, from 0 to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more. In some embodiments, n equals 0. In some embodiments, n equals 1.

Table 1: Regions of sgRNA (linear view, 5' to 3')

	LS1-6		B1 -2		US1-12		B3-6	
5' terminus (n)	lower stem	n	bulge	n	upper stem	n	bulge	n

(continued below)

LS7-12		N1-18		H1-1 thru H1-12		H2-1 thru H2-15	
lower stem	n	nexus	n	hairpin 1	n	hairpin 2	3' terminus

a) **5' terminus region**

[0095] In some embodiments, the sgRNA comprises nucleotides at the 5' terminus as shown in Table 1. In some embodiments, the 5' terminus of the sgRNA comprises a spacer or guide region that functions to direct a Cas protein to a target nucleotide sequence. In some embodiments, the 5' terminus does not comprise a spacer or guide region. In some embodiments, the 5' terminus comprises a spacer and additional nucleotides that do not function to direct a Cas protein to a target nucleotide region.

[0096] In some embodiments, the guide region comprises the first 1-10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides at the 5' end of the sgRNA. In some embodiments, the guide region comprises 20 nucleotides. In some embodiments, the guide region may comprise 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 or more nucleotides. In some embodiments, the guide region may comprise 17 nucleotides. In some embodiments, the guide region may comprise 18 nucleotides. In some embodiments, the guide region may comprise 19 nucleotides.

[0097] In some embodiments, the selection of the guide region is determined based on target sequences within the gene of interest for editing. For example, in some embodiments, the sgRNA comprises a guide region that is complementary to target sequences of a gene of interest.

[0098] In some embodiments, the target sequence in the gene of interest may be complementary to the guide region of the sgRNA. In some embodiments, the degree of complementarity or identity between a guide region of a sgRNA and its corresponding target sequence in the gene of interest may be about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%. In some embodiments, the guide region of a sgRNA and the target region of a gene of interest may be 100% complementary or identical. In other embodiments, the guide region of a sgRNA and the target region of a gene of interest may contain at least one mismatch. For example, the guide region of a sgRNA and the target sequence of a gene of interest may contain 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 mismatches, where the total length of the target sequence is at least about 17, 18, 19, 20 or more base pairs. In some embodiments, the guide region of a sgRNA and the target region of a gene of interest may contain 1-6 mismatches where the guide sequence comprises at least about 17, 18, 19, 20 or more nucleotides. In some embodiments, the guide region of a sgRNA and the target region of a gene of interest may contain 1, 2, 3, 4, 5, or 6 mismatches where the guide

sequence comprises about 20 nucleotides. The 5' terminus may comprise nucleotides that are not considered guide regions (i.e., do not function to direct a cas9 protein to a target nucleic acid).

b) Lower stem

[0099] In some embodiments, the sgRNA comprises a lower stem (LS) region that when viewed linearly, is separated by a bulge and upper stem regions. See Table 1.

[00100] In some embodiments, the lower stem regions comprise 1-12 nucleotides, e.g. in one embodiment the lower stem regions comprise LS1-LS12. In some embodiments, the lower stem region comprises fewer nucleotides than shown in Table 1 and FIG. 21A. In some embodiments, the lower stem region comprises more nucleotides than shown in Table 1 and FIG. 21A. When the lower stem region comprises fewer or more nucleotides than shown in the schematic of Table 1 and FIG. 21A, the modification pattern, as will be apparent to the skilled artisan, should be maintained.

[00101] In some embodiments, the lower stem region has nucleotides that are complementary in nucleic acid sequence when read in opposite directions. In some embodiments, the complementarity in nucleic acid sequence of lower stem leads to a secondary structure of a stem in the sgRNA (e.g., the regions may base pair with one another). In some embodiments, the lower stem regions may not be perfectly complimentary to each other when read in opposite directions.

c) Bulge

[00102] In some embodiments, the sgRNA comprises a bulge region comprising six nucleotides, B1-B6. When viewed linearly, the bulge region is separated into two regions. See Table 1. In some embodiments, the bulge region comprises six nucleotides, wherein the first two nucleotides are followed by an upper stem region, followed by the last four nucleotides of the bulge. In some embodiments, the bulge region comprises fewer nucleotides than shown in Table 1 and FIG. 21A. In some embodiments, the bulge region comprises more nucleotides than shown in Table 1 and FIG. 21A. When the bulge region comprises fewer or more nucleotides than shown in the schematic of Table 1 and FIG. 21A, the modification pattern, as will be apparent to the skilled artisan, should be maintained.

[00103] In some embodiments, the presence of a bulge results in a directional kink between the upper and lower stem modules in a sgRNA.

d) **Upper stem**

[00104] In some embodiments, the sgRNA comprises an upper stem region comprising 12 nucleotides. In some embodiments, the upper stem region comprises a loop sequence. In some instances, the loop is a tetraloop (loop consisting of four nucleotides).

[00105] In some embodiments, the upper stem region comprises fewer nucleotides than shown in Table 1 and FIG. 21A. In some embodiments, the upper stem region comprises more nucleotides than shown in Table 1 and FIG. 21A. When the upper stem region comprises fewer or more nucleotides than shown in the schematic of Table 1 and FIG. 21A, the modification pattern, as will be apparent to the skilled artisan, should be maintained.

[00106] In some embodiments, the upper stem region has nucleotides that are complementary in nucleic acid sequence when read in opposite directions. In some embodiments, the complementarity in nucleic acid sequence of upper stem leads to a secondary structure of a stem in the sgRNA (e.g., the regions may base pair with one another). In some embodiments, the upper stem regions may not be perfectly complimentary to each other when read in opposite directions.

e) **Nexus**

[00107] In some embodiments, the sgRNA comprises a nexus region that is located between the lower stem region and the hairpin 1 region. In some embodiments, the nexus comprises 18 nucleotides. In some embodiments, the nexus region comprises nucleotides N1 through N18 as shown in Table 1 and FIG. 21A.

[00108] In some embodiments, the nexus region comprises fewer nucleotides than shown in Table 1 and FIG. 21A. In some embodiments, the nexus region comprises more nucleotides than shown in Table 1 and FIG. 21A. When the nexus region comprises fewer or more nucleotides than shown in the schematic of Table 1 and FIG. 21A, the modification pattern, as will be apparent to the skilled artisan, should be maintained.

[00109] In some embodiments, the nexus region has nucleotides that are complementary in nucleic acid sequence when read in opposite directions. In some embodiments, the complementarity in nucleic acid sequence leads to a secondary structure of a stem and/or stem loop in the sgRNA (e.g., certain nucleotides in the nexus region may base pair with one another). In some embodiments, the nexus regions may not be perfectly complimentary to each other when read in opposite directions.

f) **Hairpin**

[00110] In some embodiments, the sgRNA comprises one or more hairpin regions. In some embodiments, the hairpin region is downstream of (e.g., 3' to) the nexus region. In some embodiments, the region of nucleotides immediately downstream of the nexus region is termed "hairpin 1" or "H1". In some embodiments, the region of nucleotides 3' to hairpin 1 is termed "hairpin 2" or "H2". In some embodiments, the hairpin region comprises hairpin 1 and hairpin 2. In some embodiments, the sgRNA comprises only hairpin 1 or hairpin 2.

[00111] In some embodiments, the hairpin 1 region comprises 12 nucleic acids immediately downstream of the nexus region. In some embodiments, the hairpin 1 region comprises nucleotides H1-1 through H1-12 as shown in Table 1 and FIG. 21A.

[00112] In some embodiments, the hairpin 2 region comprises 15 nucleic acids downstream of the hairpin 1 region. In some embodiments, the hairpin 2 region comprises nucleotides H2-1 through H2-15 as shown in Table 1 and FIG. 21A.

[00113] In some embodiments, one or more nucleotides is present between the hairpin 1 and the hairpin 2 regions. The one or more nucleotides between the hairpin 1 and hairpin 2 region may be modified or unmodified. In some embodiments, hairpin 1 and hairpin 2 are separated by one nucleotide. In some embodiments, the hairpin regions comprise fewer nucleotides than shown in Table 1 and FIG. 21A. In some embodiments, the hairpin regions comprise more nucleotides than shown in Table 1 and FIG. 21A. When a hairpin region comprises fewer or more nucleotides than shown in the schematic of Table 1 and FIG. 21A, the modification pattern, as will be apparent to the skilled artisan, should be maintained.

[00114] In some embodiments, a hairpin region has nucleotides that are complementary in nucleic acid sequence when read in opposite directions. In some embodiments, the hairpin regions may not be perfectly complimentary to each other when read in opposite directions (e.g., the top or loop of the hairpin comprises unpaired nucleotides).

[00115] In some embodiments, the sgRNA comprises replacement of hairpin 1 with nucleotides "n", wherein "n" is an integer between 1 and 50, 40, 30, 20, 15, 10, 5, 4, 3, and 2. In some embodiments, the hairpin 1 region of a sgRNA is replaced by 2 nucleotides.

g) **3' terminus region**

[00116] In some embodiments, the sgRNA comprises nucleotides after the hairpin region(s). In some embodiments, the 3' terminus region comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15 or 20 or more nucleotides, e.g. that are not associated with the secondary structure of a

hairpin. In some embodiments, the 3' terminus region comprises 1, 2, 3, or 4 nucleotides that are not associated with the secondary structure of a hairpin. In some embodiments, the 3' terminus region comprises 4 nucleotides that are not associated with the secondary structure of a hairpin. In some embodiments, the 3' terminus region comprises 1, 2, or 3 nucleotides that are not associated with the secondary structure of a hairpin.

2. Modifications of sgRNAs

[00117] In some embodiments, the invention comprises a sgRNA comprising one or more modifications within one or more of the following regions: the nucleotides at the 5' terminus; the lower stem region; the bulge region; the upper stem region; the nexus region; the hairpin 1 region; the hairpin 2 region; and the nucleotides at the 3' terminus.

[00118] In some embodiments, the modification comprises a 2'-O-methyl (2'-O-Me) modified nucleotide. In some embodiments, the modification comprises a 2'-O-(2-methoxyethyl) (2'-O-moe) modified nucleotide. In some embodiments, the modification comprises a 2'-fluoro (2'-F) modified nucleotide. In some embodiments, the modification comprises a phosphorothioate (PS) bond between nucleotides.

[00119] In some embodiments, the sgRNA comprises modifications at 1, 2, 3, or 4 of the first 4 nucleotides at its 5' end. In some embodiments, the first three or four nucleotides at the 5' terminus, and the last three or four nucleotides at the 3' terminus are modified. In some embodiments, the first four nucleotides at the 5' terminus, and the last four nucleotides at the 3' terminus are linked with phosphorothioate (PS) bonds. In some embodiments, the modification comprises 2'-O-Me. In some embodiments, the modification comprises 2'-F. In some embodiments, the modification comprises 2'-O-moe.

[00120] In some embodiments, the sgRNA comprises modifications at 1, 2, 3, or 4 of the first 4 nucleotides at the 5' end. In some embodiments, the sgRNA comprises modifications at 1, 2, 3, or 4 of the first 4 nucleotides at the 3' end. In some embodiments, the first four nucleotides at the 5' terminus and the last four nucleotides at the 3' terminus are linked with a PS bond, and the first three nucleotides at the 5' terminus and the last three nucleotides at the 3' terminus comprise 2'-O-Me or 2'-O-moe modifications.

[00121] In some embodiments, the first four nucleotides at the 5' terminus and the last four nucleotides at the 3' terminus are linked with a PS bond, and the first three nucleotides at the 5' terminus and the last three nucleotides at the 3' terminus comprise 2'-F modifications.

[00122] In some embodiments, a sgRNA is provided wherein LS1, LS6, LS7, LS8, LS11, and LS12 are modified with 2'-O-Me. In some embodiments, each of the nucleotides in

the bulge region of the sgRNA are modified with 2'-O-Me. In some embodiments, each of the nucleotides in the upper stem region of the sgRNA are modified with 2'-O-Me. In some embodiments, N16, N17, and N18 in the nexus region of the sgRNA are modified with 2'-O-Me. In some embodiments, each of the nucleotides in the hairpin 1 region of the sgRNA are modified with 2'-O-Me. In some embodiments, each of the nucleotides in the hairpin 2 region of the sgRNA are modified with 2'-O-Me.

[00123] In some embodiments, the sgRNA comprises 2'-O-Me modified nucleotides at the following nucleotides: the first three nucleotides at the 5' terminus; LS1, LS6, LS7, LS8, LS11, and LS12; B1 and B2 in the bulge region; each of the nucleotides in the upper stem region of the sgRNA; N16, N17, and N18 in the nexus region; each of the nucleotides in the hairpin 1 region; each of the nucleotides in the hairpin 2 region; and last four nucleotides at the 3' terminus.

[00124] In some embodiments, the sgRNA further comprises three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus. In some embodiments, the sgRNA further comprises 2'-O-Me or 2'-F modified nucleic acids at the first three nucleotides at the 5' terminus, and 2'-O-Me or 2'-F modified nucleic acids at the last four nucleotides at the 3' terminus. In some embodiments, LS9 and LS10 are modified with 2'-F. In some embodiments, N15, N16, N17, and N18 are modified with 2'-F. In some embodiments, H2-9, H2-10, H2-11, H2-12, H2-13, HS-14, and H2-15 are modified with 2'-F. In some embodiments, the second to last, third to last, and fourth to last nucleotides at the 3' terminus are modified with 2'-F

[00125] In some embodiments, a single guide RNA (sgRNA) is provided comprising 2'-F modified nucleic acids at the following nucleotides: LS9 and LS10 in the lower stem region; N15, N16, N17, and N18 in the nexus region; and H2-9, H2-10, H2-11, H2-12, H2-13, HS-14, and H2-15 in the hairpin 2 region. In some embodiments, the sgRNA further comprises 2'-F modified nucleotides at the second to last, third to last, and fourth to last nucleotides at the 3' terminus. In some embodiments, the sgRNA further comprises three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus. In some embodiments, the sgRNA further comprises 2'-O-Me or 2'-F modified nucleic acids at the first three nucleotides at the 5' terminus, and 2'-O-Me or 2'-F modified nucleic acids at three of the last four nucleotides at the 3' terminus.

[00126] In some embodiments, a single guide RNA (sgRNA) is provided comprising 2'-O-Me modified nucleotides at the first three nucleotides at the 5' terminus; 2'-O-Me

modified nucleotides at LS1 and LS6; 2'-O-Me modified nucleotides at US1-US12; 2'-O-Me modified nucleotides at H1-1 – H1-12; a 2'-O-Me modified nucleotide between Hairpin 1 and Hairpin 2; 2'-O-Me modified nucleotides at H2-1 – H2-15; and 2'-O-Me modified nucleotides at the last four nucleotides at the 3' terminus. In some embodiments, the sgRNA further comprises three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus.

[00127] In some embodiments, a single guide RNA (sgRNA) is provided comprising 2'-O-Me modified nucleotides at the first three nucleotides at the 5' terminus; 2'-F modified nucleotides at LS1-LS6; 2'-O-Me modified nucleotides at US1-US12; 2'-O-Me modified nucleotides at H1-1 – H1-12; a 2'-O-Me modified nucleotide at "n" between Hairpin 1 and Hairpin 2; 2'-O-Me modified nucleotides at H2-1 – H2-15; and 2'-O-Me modified nucleotides at the last four nucleotides at the 3' terminus. In some embodiments, the sgRNA further comprises three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus.

[00128] In some embodiments, a single guide RNA (sgRNA) is provided comprising 2'-O-Me modified nucleotides at the first three nucleotides at the 5' terminus; 2'-F modified nucleotides at LS2-LS5; 2'-O-Me modified nucleotides at LS1 and LS6; 2'-O-Me modified nucleotides at US1-US12; 2'-O-Me modified nucleotides at H1-1 – H1-12; a 2'-O-Me modified nucleotide at "n" between Hairpin 1 and Hairpin 2; 2'-O-Me modified nucleotides at H2-1 – H2-15; and 2'-O-Me modified nucleotides at the last four nucleotides at the 3' terminus. In some embodiments, the sgRNA further comprises three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus.

[00129] In some embodiments, a single guide RNA (sgRNA) is provided comprising 2'-O-Me modified nucleotides at the first three nucleotides at the 5' terminus; 2'-O-Me modified nucleotides at US1-US12; 2'-O-Me modified nucleotides at LS7, LS8, LS11, and LS12; 2'-O-Me modified nucleotides at H1-1 – H1-12; a 2'-O-Me modified nucleotide at "n" between Hairpin 1 and Hairpin 2; 2'-O-Me modified nucleotides at H2-1 – H2-15; and 2'-O-Me modified nucleotides at the last four nucleotides at the 3' terminus. In some embodiments, the sgRNA further comprises three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus.

[00130] In some embodiments, a single guide RNA (sgRNA) is provided comprising 2'-O-Me modified nucleotides at the first three nucleotides at the 5' terminus; 2'-O-Me

modified nucleotides at US1-US12; 2'-O-Me modified nucleotides at LS8, LS10, and LS12; 2'-O-F modified nucleotides at LS7, LS9, and LS11; 2'-O-Me modified nucleotides at H1-1 – H1-12; a 2'-O-Me modified nucleotide between Hairpin 1 and Hairpin 2; 2'-O-Me modified nucleotides at H2-1 – H2-15; and 2'-O-Me modified nucleotides at the last four nucleotides at the 3' terminus. In some embodiments, the sgRNA further comprises three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus.

[00131] In some embodiments, a single guide RNA (sgRNA) is provided comprising 2'-O-Me modified nucleotides at the first three nucleotides at the 5' terminus; 2'-O-Me modified nucleotides at LS1, LS6, LS7, LS8, LS11, and LS12; 2'-O-Me modified nucleotides at US1-US12; 2'-O-Me modified nucleotides at H1-1 – H1-12; a 2'-O-Me modified nucleotide between Hairpin 1 and Hairpin 2; 2'-O-Me modified nucleotides at H2-1 – H2-15; and 2'-O-Me modified nucleotides at the last four nucleotides at the 3' terminus. In some embodiments, the sgRNA further comprises three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus.

[00132] In some embodiments, a single guide RNA (sgRNA) is provided comprising 2'-O-Me modified nucleotides at the first three nucleotides at the 5' terminus; 2'-O-Me modified nucleotides at LS1, LS6, LS7, LS8, LS11, and LS12; 2'-F modified nucleotides at LS9 and LS10; 2'-O-Me modified nucleotides at US1-US12; 2'-O-Me modified nucleotides at H1-1 – H1-12; a 2'-O-Me modified nucleotide between Hairpin 1 and Hairpin 2; 2'-O-Me modified nucleotides at H2-1 – H2-15; and 2'-O-Me modified nucleotides at the last four nucleotides at the 3' terminus. In some embodiments, the sgRNA further comprises three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus.

[00133] In some embodiments, a single guide RNA (sgRNA) is provided comprising 2'-O-Me modified nucleotides at the first three nucleotides at the 5' terminus; 2'-O-Me modified nucleotides at US1-US12; 2'-O-Me modified nucleotides at H1-1 – H1-12; a 2'-O-Me modified nucleotide between Hairpin 1 and Hairpin 2; 2'-O-Me modified nucleotides at H2-1 – H2-8; 2'-F modified nucleotides at H2-9 – H2-15; 2'-F modified nucleotides at the second from last, third from last, and fourth from last nucleotide at the 3' terminus; and a 2'-O-Me modified nucleotide at the last nucleotide at the 3' terminus. In some embodiments, the sgRNA further comprises three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus.

[00134] In some embodiments, a single guide RNA (sgRNA) is provided comprising 2'-O-Me modified nucleotides at the first three nucleotides at the 5' terminus; 2'-O-Me modified nucleotides at US1-US12; 2'-O-Me modified nucleotides at H1-2, H1-4, H1-6, H1-8, H1-10, and H1-12; 2'-F modified nucleotides at H1-1, H1-3, H1-5, H1-7, H1-9, and H1-11; a 2'-F modified nucleotide between Hairpin 1 and Hairpin 2; 2'-F modified nucleotides at H2-2, H2-4, H2-6, H2-8, H2-10, H2-12; and H2-14; 2'-O-Me modified nucleotides at H2-1, H2-3, H2-5, H2-7, H2-9, H2-11; H2-13, and H2-15; 2'-F modified nucleotides at the second from last, and fourth from last nucleotide at the 3' terminus; and 2'-O-Me modified nucleotide at the third from last, and last nucleotide at the 3' terminus. In some embodiments, the sgRNA further comprises three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus.

[00135] Disclosed herein, in some embodiments, is a single guide RNA (sgRNA) comprising 2'-O-Me modifications at nucleotides LS8, LS10, LS12, H1-2, H1-4, H1-6, H1-8, H1-10, H1-12, H2-1, H2-3, H2-5, H2-7, H2-9, H2-11, H2-13, and H2-15; and 2'-F modifications at LS7, LS9, LS11; H1-1, H1-3, H1-5, H1-7, H1-9, H1-11, H1-13, H2-2, H2-4, H2-6, H2-8, H2-10, H2-12, and H2-14. In some embodiments, the sgRNA further comprises three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus. In some embodiments, the sgRNA further comprises 2'-O-Me modified nucleotides at the last and third to last nucleotide at the 3' terminus; and 2'-F modified nucleotides at the second to last and third to last nucleotide at the 3' terminus.

[00136] Disclosed herein, in some embodiments, is a sgRNA comprising the nucleic acids of any one of SEQ ID Nos: 228-232. Disclosed herein, in some embodiments, is a sgRNA comprising the nucleic acids of any one of SEQ ID Nos: 235-240, 265-285, and 309-329. Disclosed herein, in some embodiments, is a sgRNA comprises nucleic acids having at least 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 85, 80, 75, or 70% identity to the nucleic acids of any one of SEQ ID Nos: 235-240, 265-285, and 309-329, wherein the modification pattern is identical to the modification pattern shown in the reference sequence identifier. In some embodiments, the sgRNA further comprises three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus.

[00137] In some embodiments, a sgRNA comprising a 5' end modification and one or more modifications in one or more of: the upper stem region; the hairpin 1 region; and the

hairpin 2 region is provided, wherein the 5' end modification comprises at least two phosphorothioate linkages within the first seven nucleotides of the 5' terminus.

[00138] In some embodiments, a sgRNA comprising a 5' end modification and one or more modifications in one or more of: the upper stem region; the hairpin 1 region; and the hairpin 2 region is provided, wherein the 5' end modification comprises one or more phosphorothioate linkages at the 5' end of the RNA. In some embodiments, one or more phorphorothioate bonds link the 5' terminal nucleotides.

[00139] In some embodiments, a sgRNA comprising a 5' end modification and one or more modifications in one or more of: the upper stem region; the hairpin 1 region; and the hairpin 2 region is provided, wherein the 5' end modification comprises one or more phosphorothioate linkages within the first seven nucleotides of the 5' terminus.

[00140] In some embodiments, a sgRNA comprising any one of the modified sgRNA sequences of SEQ ID Nos: 228-332 is provided.

[00141] In some embodiments, a sgRNA comprising or consisting of any one of the modified sgRNA sequences of SEQ ID Nos: 235-240, 265-285, and 309-329 is provided.

[00142] In some embodiments, the invention comprises a sgRNA comprising any one of the modified sequences of SEQ ID Nos: 235-240, 265-285, and 309-329, wherein the sgRNA further comprises a 5' spacer sequence that is at least partially complementary to a target sequence, and directs a Cas9 to its target for cleavage.

[00143] In some embodiments, the invention comprises a sgRNA comprising nucleotides having at least 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 85, 80, 75, or 70% identity to the nucleotides of any one of SEQ ID Nos: 235-240, 265-285, and 309-329, wherein the modification pattern is identical to the modification pattern shown in the reference sequence identifier. That is, the nucleotides A, U, C, and G may differ by 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 85, 80, 75, or 70% compared to what is shown in in the sequences, but the modification remains unchanged.

[00144] In some embodiments, the invention comprises a sgRNA comprising one or more modifications within one or more of the following regions: the nucleotides at the 5' terminus; the lower stem region; the bulge region; the upper stem region; the; the nexus region; the hairpin 1 region; the hairpin 2 region; and the nucleotides at the 3' terminus.

[00145] In some embodiments, the modification comprises a 2'-O-methyl (2'-O-Me) modified nucleotide. In some embodiments, the modification comprises a 2'-fluoro (2'-F) modified nucleotide. In some embodiments, the modification comprises a phosphorothioate

(PS) bond between nucleotides. In some embodiments, the modification comprises an inverted abasic nucleotide.

[00146] In some embodiments, a sgRNA is provided comprising 2'-O-Me modified nucleotides at: the first three nucleotides in the 5' terminus; LS1, LS6, LS7, LS8, LS11, and LS12 in the lower stem; B1 and B2 in the bulge region; each of the nucleotides in the upper stem region; N16, N17, and N18 in the nexus region; each of the nucleotides in the hairpin 1 region; one nucleotide between hairpin 1 and hairpin 2; each of the nucleotides in the hairpin 2 region; and the last four nucleotides at the 3' terminus. In one embodiment, the sgRNA further comprises three PS bonds between the first four nucleotides at the 5' terminus and three PS bonds between the last four nucleotides at the 3' terminus.

[00147] In some embodiments, a sgRNA is provided comprising 2'-O-Me modified nucleotides at: the first three nucleotides in the 5' terminus; LS1, LS6, LS7, LS8, LS11, and LS12 in the lower stem; B1-B6 in the bulge region; each of the nucleotides in the upper stem region; N16, N17, and N18 in the nexus region; each of the nucleotides in the hairpin 1 region; one nucleotide between hairpin 1 and hairpin 2; each of the nucleotides in the hairpin 2 region; and the last four nucleotides at the 3' terminus. In one embodiment, the sgRNA further comprises three PS bonds between the first four nucleotides at the 5' terminus and three PS bonds between the last four nucleotides at the 3' terminus.

[00148] In some embodiments, a sgRNA is provided comprising 2'-F modified nucleotides at: LS9 and LS10 in the lower stem; 15-N18 in the nexus region; H2-9-HS-15 in the hairpin 2 region; and the second to last, third to last, and fourth to last nucleotide in the 3' terminus region.

[00149] In some embodiments, a sgRNA is provided comprising 2'-F modified nucleotides at: each nucleotide in the lower stem; 15-N18 in the nexus region; H2-9-HS-15 in the hairpin 2 region; and the second to last, third to last, and fourth to last nucleotide in the 3' terminus region.

[00150] In some embodiments, a single guide RNA (sgRNA) is provided comprising 2'-O-Me modified nucleotides at LS8, LS10, LS12, H1-2, H1-4, H1-6, H1-8, H1-10, H1-12, H2-1, H2-3, H2-5, H2-7, H2-9, H2-11, H2-13, H2-15, and the last and third to last nucleotides at the 3' terminus; and 2'-F modifications at LS7, LS9, LS11; H1-1, H1-3, H1-5, H1-7, H1-9, H1-11, H1-13, H2-2, H2-4, H2-6, H2-8, H2-10, H2-12, H2-14, and the second to last and fourth to last nucleotide at the 3' terminus.

[00151] Each of the following embodiments are encompassed:

Embodiment 01. A single guide RNA (sgRNA) comprising one or more modifications in one or more of the following regions:

a. the 5' terminus;
b. the lower stem region;
c. the bulge region;
d. the upper stem region;
e. the nexus region;
f. the hairpin 1 region;
g. the hairpin 2 region; and
h. the 3' terminus.

Embodiment 02. The sgRNA of embodiment 1, wherein the modification comprises a 2'-O-methyl (2'-O-Me) modified nucleotide.

Embodiment 03. The sgRNA of embodiment 1, wherein the modification comprises a 2'-fluoro (2'-F) modified nucleotide.

Embodiment 04. The sgRNA of embodiment 1, wherein the modification comprises a phosphorothioate (PS) bond between nucleotides.

Embodiment 05. The sgRNA of any one of embodiments 1-3, wherein the first three or four nucleotides at the 5' terminus, and the last three or four nucleotides at the 3' terminus are modified.

Embodiment 06. The sgRNA of any one of embodiments 1-5, wherein the first four nucleotides at the 5' terminus, and the last four nucleotides at the 3' terminus are linked with phosphorothioate (PS) bonds.

Embodiment 07. The sgRNA of embodiment 5, wherein the modification comprises 2'-O-Me.

Embodiment 08. The sgRNA of embodiment 5, wherein the modification comprises 2'-F.

Embodiment 09. The sgRNA of any one of embodiments 1-7, wherein the first four nucleotides at the 5' terminus and the last four nucleotides at the 3'terminus are linked with a PS bond, and wherein the first three nucleotides at the 5' terminus and the last three nucleotides at the 3' terminus comprise 2'-O-Me modifications.

Embodiment 10. The sgRNA of any one of embodiments 1-8, wherein the first four nucleotides at the 5' terminus and the last four nucleotides at the 3' terminus are linked with a PS bond, and wherein the first three nucleotides at the 5' terminus and the last three nucleotides at the 3' terminus comprise 2'-F modifications.

Embodiment 11. The sgRNA of any one of embodiments 1-10, wherein LS1, LS6, LS7, LS8, LS11, and LS12 are modified with 2'-O-Me.

Embodiment 12. The sgRNA of any one of embodiments 1-11, wherein each of the nucleotides in the bulge region are modified with 2'-O-Me.

Embodiment 13. The sgRNA of any one of embodiments 1-12, wherein each of the nucleotides in the upper stem region are modified with 2'-O-Me.

Embodiment 14. The sgRNA of any one of embodiments 1-13, wherein N16, N17, and N18 in the nexus region are modified with 2'-O-Me.

Embodiment 15. The sgRNA of any one of embodiments 1-14, wherein each of the nucleotides in the hairpin 1 region are modified with 2'-O-Me.

Embodiment 16. The sgRNA of any one of embodiments 1-15, wherein each of the nucleotides in the hairpin 2 region are modified with 2'-O-Me.

Embodiment 17. A single guide RNA (sgRNA) comprising 2'-O-Me modified nucleic acids at the following nucleotides:

a. the first three nucleotides at the 5' terminus;
b. LS1, LS6, LS7, LS8, LS11, and LS12 in the lower stem region;
c. B1 and B2 in the bulge region;
d. each nucleotide in the upper stem region;
e. N16, N17, and N18 in the nexus region;
f. each nucleotide in the hairpin 1 region;
g. each nucleotide in the hairpin 2 region; and
h. the last four nucleotides at the 3' terminus.

Embodiment 18. The sgRNA of embodiment 17, wherein B3-B6 are modified with 2'-O-Me.

Embodiment 19. The sgRNA of embodiment 17, further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus.

Embodiment 20. The sgRNA of any one of embodiments 1-10, wherein LS9 and LS10 are modified with 2'-F.

Embodiment 21. The sgRNA of any one of embodiments 1-10 and 20, wherein N15, N16, N17, and N18 are modified with 2'-F.

Embodiment 22. The sgRNA of any one of embodiments 1-10 and 20-21, wherein H2-9, H2-10, H2-11, H2-12, H2-13, H2-14, and H2-15 are modified with 2'-F.

Embodiment 23. The sgRNA of any one of embodiments 1-10 and 21-22, wherein the second to last, third to last, and fourth to last nucleotides at the 3' terminus are modified with 2'-F.

Embodiment 24. A single guide RNA (sgRNA) comprising 2'-F modified nucleotides at the following positions:

a. LS9 and LS10 in the lower stem region;
b. N15, N16, N17, and N18 in the nexus region; and
c. H2-9, H2-10, H2-11, H2-12, H2-13, H2-14, and H2-15 in the hairpin 2 region.

Embodiment 25. The sgRNA of embodiment 24, further comprising 2'-F modified nucleotides at the second to last, third to last, and fourth to last nucleotides at the 3' terminus.

Embodiment 26. The sgRNA of any one of embodiments 24 or 25, further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus.

Embodiment 27. The sgRNA of any one of embodiments 24 -26, further comprising 2'-O-Me or 2'-F modified nucleotides at the first three nucleotides at the 5' terminus, and 2'-O-Me or 2'-F modified nucleotides at the three of the last four nucleotides at the 3' terminus.

Embodiment 28. A single guide RNA (sgRNA) comprising

a. 2'-O-Me modified nucleotides at the first three nucleotides at the 5' terminus;
b. 2'-O-Me modified nucleotides at LS1 and LS6;
c. 2'-O-Me modified nucleotides at US1-US12;
d. 2'-O-Me modified nucleotides at H1-1 – H1-12;
e. a 2'-O-Me modified nucleotide between Hairpin 1 and Hairpin 2;
f. 2'-O-Me modified nucleotides at H2-1 – H2-15; and
g. 2'-O-Me modified nucleotides at the last four nucleotides at the 3' terminus.

Embodiment 29. The sgRNA of embodiment 28 further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus.

Embodiment 30. A single guide RNA (sgRNA) comprising

a. 2'-O-Me modified nucleotides at the first three nucleotides at the 5' terminus;
b. 2'-F modified nucleotides at LS1-LS6;
c. 2'-O-Me modified nucleotides at US1-US12;
d. 2'-O-Me modified nucleotides at H1-1 – H1-12;

e. a 2'-O-Me modified nucleotide between Hairpin 1 and Hairpin 2;
f. 2'-O-Me modified nucleotides at H2-1 – H2-15; and
g. 2'-O-Me modified nucleotides at the last four nucleotides at the 3' terminus.

Embodiment 31. The sgRNA of embodiment 30 further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus.

Embodiment 32. A single guide RNA (sgRNA) comprising

a. 2'-O-Me modified nucleotides at the first three nucleotides at the 5' terminus;
b. 2'-F modified nucleotides at LS2-LS5;
c. 2'-O-Me modified nucleotides at LS1 and LS6;
d. 2'-O-Me modified nucleotides at US1-US12;
e. 2'-O-Me modified nucleotides at H1-1 – H1-12;
f. a 2'-O-Me modified nucleotide between Hairpin 1 and Hairpin 2;
g. 2'-O-Me modified nucleotides at H2-1 – H2-15; and
h. 2'-O-Me modified nucleotides at the last four nucleotides at the 3' terminus.

Embodiment 33. The sgRNA of embodiment 32 further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus.

Embodiment 34. A single guide RNA (sgRNA) comprising

a. 2'-O-Me modified nucleotides at the first three nucleotides at the 5' terminus;
b. 2'-O-Me modified nucleotides at US1-US12;
c. 2'-O-Me modified nucleotides at LS7, LS8, LS11, and LS12;
d. 2'-O-Me modified nucleotides at H1-1 – H1-12;
e. a 2'-O-Me modified nucleotide between Hairpin 1 and Hairpin 2;
f. 2'-O-Me modified nucleotides at H2-1 – H2-15; and
g. 2'-O-Me modified nucleotides at the last four nucleotides at the 3' terminus.

Embodiment 35. The sgRNA of embodiment 34 further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus.

Embodiment 36. A single guide RNA (sgRNA) comprising

a. 2'-O-Me modified nucleotides at the first three nucleotides at the 5' terminus;
b. 2'-O-Me modified nucleotides at US1-US12;
c. 2'-O-Me modified nucleotides at LS7, LS8, LS11, and LS12;
d. 2'-F modified nucleotides at LS9 and LS10;

e. 2'-O-Me modified nucleotides at H1-1 – H1-12;

f. a 2'-O-Me modified nucleotide between Hairpin 1 and Hairpin 2;

g. 2'-O-Me modified nucleotides at H2-1 – H2-15; and

h. 2'-O-Me modified nucleotides at the last four nucleotides at the 3' terminus.

Embodiment 37. The sgRNA of embodiment 36 further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus.

Embodiment 38. A single guide RNA (sgRNA) comprising

a. 2'-O-Me modified nucleotides at the first three nucleotides at the 5' terminus;

b. 2'-O-Me modified nucleotides at US1-US12;

c. 2'-O-Me modified nucleotides at LS8, LS10, and LS12;

d. 2'-O-F modified nucleotides at LS7, LS9, and LS11;

e. 2'-O-Me modified nucleotides at H1-1 – H1-12;

f. a 2'-O-Me modified nucleotide between Hairpin 1 and Hairpin 2;

g. 2'-O-Me modified nucleotides at H2-1 – H2-15; and

h. 2'-O-Me modified nucleotides at the last four nucleotides at the 3' terminus.

Embodiment 39. The sgRNA of embodiment 32 further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus.

Embodiment 40. A single guide RNA (sgRNA) comprising

a. 2'-O-Me modified nucleotides at the first three nucleotides at the 5' terminus;

b. 2'-O-Me modified nucleotides at LS1, LS6, LS7, LS8, LS11, and LS12

c. 2'-O-Me modified nucleotides at US1-US12;

d. 2'-O-Me modified nucleotides at H1-1 – H1-12;

e. a 2'-O-Me modified nucleotide between Hairpin 1 and Hairpin 2;

f. 2'-O-Me modified nucleotides at H2-1 – H2-15; and

g. 2'-O-Me modified nucleotides at the last four nucleotides at the 3' terminus.

Embodiment 41. The sgRNA of embodiment 40 further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus

Embodiment 42. A single guide RNA (sgRNA) comprising

a. 2'-O-Me modified nucleotides at the first three nucleotides at the 5' terminus;

b. 2'-O-Me modified nucleotides at LS1, LS6, LS7, LS8, LS11, and LS12;

c. 2'-F modified nucleotides at LS9 and LS10;

d. 2'-O-Me modified nucleotides at US1-US12;
e. 2'-O-Me modified nucleotides at H1-1 – H1-12;
f. a 2'-O-Me modified nucleotide between Hairpin 1 and Hairpin 2;
g. 2'-O-Me modified nucleotides at H2-1 – H2-15; and
h. 2'-O-Me modified nucleotides at the last four nucleotides at the 3' terminus.

Embodiment 43. The sgRNA of embodiment 43 further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus.

Embodiment 44. A single guide RNA (sgRNA) comprising

a. 2'-O-Me modified nucleotides at the first three nucleotides at the 5' terminus;
b. 2'-O-Me modified nucleotides at US1-US12;
c. 2'-O-Me modified nucleotides at H1-1 – H1-12;
d. a 2'-O-Me modified nucleotide between Hairpin 1 and Hairpin 2;
e. 2'-O-Me modified nucleotides at H2-1 – H2-8;
f. 2'-F modified nucleotides at H2-9 – H2-15;
g. 2'-F modified nucleotides at the second from last, third from last, and fourth from last nucleotide at the 3' terminus; and
h. a 2'-O-Me modified nucleotide at the last nucleotide at the 3' terminus.

Embodiment 45. The sgRNA of embodiment 44 further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus.

Embodiment 46. A single guide RNA (sgRNA) comprising

a. 2'-O-Me modified nucleotides at the first three nucleotides at the 5' terminus;
b. 2'-O-Me modified nucleotides at US1-US12;
c. 2'-O-Me modified nucleotides at H1-2, H1-4, H1-6, H1-8, H1-10, and H1-12;
d. 2'-F modified nucleotides at H1-1, H1-3, H1-5, H1-7, H1-9, and H1-11;
e. a 2'-F modified nucleotide between Hairpin 1 and Hairpin 2;
f. 2'-F modified nucleotides at H2-2, H2-4, H2-6, H2-8, H2-10, H2-12; and H2-14;
g. 2'-O-Me modified nucleotides at H2-1, H2-3, H2-5, H2-7, H2-9, H2-11; H2-13, and H2-15;
h. 2'-F modified nucleotides at the second from last, and fourth from last nucleotide at the 3' terminus; and

i. 2'-O-Me modified nucleotide at the third from last, and last nucleotide at the 3' terminus.

Embodiment 47. The sgRNA of embodiment 46 further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus.

Embodiment 48. A single guide RNA (sgRNA) comprising

a. 2'-O-Me modified nucleotides LS8, LS10, LS12, H1-2, H1-4, H1-6, H1-8, H1-10, H1-12, H2-1, H2-3, H2-5, H2-7, H2-9, H2-11, H2-13, and H2-15; and
b. 2'-F modified nucleotides at LS7, LS9, LS11; H1-1, H1-3, H1-5, H1-7, H1-9, H1-11, H1-13, H2-2, H2-4, H2-6, H2-8, H2-10, H2-12, and H2-14.

Embodiment 49. The sgRNA of embodiment 48, further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus.

Embodiment 50. The sgRNA of any one of embodiments 48-49, further comprising

a. 2'-O-Me modified nucleotides at the last and third to last nucleotide at the 3' terminus; and
b. 2'-F modified nucleotides at the second to last and third to last nucleotide at the 3' terminus.

Embodiment 51. A sgRNA comprising the nucleic acids of any one of SEQ ID Nos: 228-332.

Embodiment 52. A sgRNA comprising the nucleic acids of any one of SEQ ID Nos: 235-240, 265-285, and 309-329.

Embodiment 53. A sgRNA comprising nucleic acids having at least 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 85, 80, 75, or 70% identity to the nucleic acids of any one of SEQ ID Nos: 235-240, 265-285, and 309-329, wherein the modification pattern is identical to the modification pattern shown in the reference sequence identifier.

Embodiment 54. The sgRNA of any one of embodiments 51-53, further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus.

B. Compositions of dgRNAs

[00152] In some embodiments, the compositions and methods of the invention comprise gRNA comprising a crRNA and trRNA that direct a nuclease such as Cas9 to a

target DNA sequence. In some embodiments, the gRNAs are associated, but on two separate RNA molecules (dual guide RNA or dgRNA).

[00153] **Table 2** and FIG. 21C provides a description of domains of a crRNA as used herein. The 5' terminus region may comprise a spacer region at or near the 5' terminus of the crRNA and functions to direct a Cas9 to a target region in the DNA, e.g., as described herein. In Table 2, the "n" between regions represents a variable number of nucleotides, for example, from 0 to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more. In some embodiments, n equals 0. Any of the dgRNAs described herein may include an "n" between any domain.

[00154] **Table 3** and FIG. 21C provide a description of domains of a trRNA as used herein. In Table 3, the "n" between regions represents a variable number of nucleotides, for example, from 0 to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more. In some embodiments, n equals 0. Any of the dgRNAs described herein may include an "n" between any domain.

1. Domains of dgRNAs

[00155] As described in *Briner 2014*, dgRNAs can be developed based on specific functional domains, referred to herein as "domains", including the spacer responsible for targeting, the lower stem, the bulge, the upper stem, the nexus, and the hairpin domains. In dgRNAs, the crRNA comprises some components of the gRNA and the trRNA comprises some components of the gRNA.

[00156] Regions of crRNAs are provided in **Table 2** and **FIG 21C**. Regions of trRNAs are provided in **Table 3** and **FIG 21C**. FIG 21C shows a schematic of an exemplary dgRNA.

Table 2: Regions of crRNA (linear view, 5' to 3')

	LS1-6		B1 -2		US1-14	
5' terminus (n)	lower stem	n	bulge	n	upper stem	3' terminus

Table 3: Regions of trRNA (linear view, 5' to 3')

	US1-11		B1 -4		LS1-6		N1-18		H1-1 thru H1-12		H2-1 thru H2-15	
5' terminus (n)	upper stem	n	bulge	n	lower stem	n	nexus	n	hairpin 1	n	hairpin 2	3' terminus

a) **5' Terminus Region**

[00157] In some embodiments, the dgRNA comprises nucleotides at the 5' terminus of the crRNA and trRNA as shown in Tables 2-3 and FIG 21C.

[00158] In some embodiments, the 5' terminus of the crRNA comprises a spacer or guide region that functions to direct a Cas protein to a target nucleotide sequence. In some embodiments, the 5' terminus does not comprise a spacer or guide region. In some embodiments, the 5' terminus comprises a spacer and additional nucleotides that do not function to direct a Cas protein to a target nucleotide region.

[00159] In some embodiments, the guide region comprises the first 1-10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides at the 5' end of the crRNA. In some embodiments, the guide region comprises 20 nucleotides. In some embodiments, the guide region may comprise 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 or more nucleotides. In some embodiments, the guide region may comprise 17 nucleotides. In some embodiments, the guide region may comprise 18 nucleotides. In some embodiments, the guide region may comprise 19 nucleotides.

[00160] In some embodiments, the selection of the guide region is determined based on target sequences within the gene of interest for editing. For example, in some embodiments, the crRNA comprises a guide region that is complementary to target sequences of a gene of interest.

[00161] In some embodiments, the target sequence in the gene of interest may be complementary to the guide region of the crRNA. In some embodiments, the degree of complementarity or identity between a guide region of a crRNA and its corresponding target sequence in the gene of interest may be about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%. In some embodiments, the guide region of a crRNA and the target region of a gene of interest may be 100% complementary or identical. In other embodiments, the guide region of a crRNA and the target region of a gene of interest may contain at least one mismatch. For example, the guide region of a crRNA and the target sequence of a gene of interest may contain 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 mismatches, where the total length of the target sequence is at least about 17, 18, 19, 20 or more base pairs. In some embodiments, the guide region of a crRNA and the target region of a gene of interest may contain 1-6 mismatches where the guide sequence comprises at least about 17, 18, 19, 20 or more nucleotides. In some embodiments, the guide region of a crRNA and the target

region of a gene of interest may contain 1, 2, 3, 4, 5, or 6 mismatches where the guide sequence comprises about 20 nucleotides.

[00162] In some embodiments, the trRNA comprises a 5' terminus. In some embodiments, the trRNA comprises a 5' terminus which forms, in part, the upper stem of a dgRNA. The 5' terminus of the trRNA is not complementary to a region of the target gene.

b) Lower stem

[00163] In some embodiments, the dgRNA comprises a lower stem (LS) region. The lower stem region comprises a crRNA lower stem region and a trRNA lower stem region that associate as depicted in FIG 21C. In some embodiments, the lower stem region of the crRNA is at least partially complementary to the lower stem region of the trRNA. In some embodiments, the lower stem region of the crRNA is fully complementary to the lower stem region of the trRNA.

[00164] In some embodiments, the lower stem region of the crRNA and trRNA each comprise 6 nucleotides. In some embodiments, the lower stem region of the crRNA and trRNA each comprise fewer nucleotides than shown in Tables 2 and 3 and FIG. 21C. In some embodiments, the lower stem region comprises more nucleotides than shown in Tables 2 and 3 and FIG. 21C. When the lower stem region comprises fewer or more nucleotides than shown in the schematic of Tables 2 and 3 and FIG. 21C, the modification patterns, as will be apparent to the skilled artisan, are maintained. In some embodiments, the number of nucleotides in the lower stem of the crRNA differs from the number of nucleotides in the lower stem of the trRNA.

c) Bulge

[00165] In some embodiments, the dgRNA comprises a bulge (B) region. In some embodiments, the crRNA comprises one bulge region and the trRNA comprises one bulge region. In some embodiments, each bulge region comprises 1-4 nucleotides. In some embodiments, the bulge region of the crRNA comprises two nucleotides, and the bulge region of the trRNA comprises four nucleotides.

[00166] In some embodiments, the crRNA bulge region is located between the lower stem region and the upper stem region of the crRNA. In some embodiments, the bulge region of the crRNA comprises two nucleotides. In some embodiments, the bulge region of the crRNA comprises nucleotides B1 and B2 as shown Table 2 and FIG 21C.

[00167] In some embodiments, the trRNA bulge region is located between the upper stem region and the lower stem region of the trRNA. In some embodiments, the bulge region

of the trRNA comprises four nucleotides. In some embodiments, the bulge region of the trRNA comprises nucleotides B1 through B4 as shown Table 3 and FIG 21C.

[00168] In some embodiments, the presence of a bulge results in a directional kink between the upper and lower stems modules in a dgRNA. The crRNA bulge and trRNA bulge may be partially complementary. The crRNA bulge and trRNA bulge may have no complementary.

[00169] In some embodiments, the bulge regions of the crRNA and trRNA comprise more nucleotides than shown in Tables 2 and 3 and FIG. 21C. When the bulge region comprises fewer or more nucleotides than shown in the schematic of Tables 2 and 3 and FIG. 21C, the modification patterns, as will be apparent to the skilled artisan, are maintained. In some embodiments, the number of nucleotides in the bulge of the crRNA differs from the number of nucleotides in the bulge of the trRNA.

d) Upper stem

[00170] In some embodiments, the dgRNA comprises an upper stem (US) region. The upper stem region comprises a crRNA upper stem region and a trRNA upper stem region that associate as depicted in FIG 21C. In some embodiments, the upper stem region of the crRNA is at least partially complementary to the upper stem region of the trRNA. In some embodiments, the upper stem region of the crRNA is fully complementary to the upper stem region of the trRNA.

[00171] In some embodiments, the upper stem region of the crRNA comprises fourteen nucleotides. In some embodiments, the upper stem region of the trRNA comprises eleven nucleotides. In some embodiments, the upper stem regions of the crRNA and trRNA each comprise fewer nucleotides than shown in Tables 2 and 3 and FIG. 21C. In some embodiments, the upper stem regions of the crRNA and trRNA comprise more nucleotides than shown in Tables 2 and 3 and FIG. 21C. When the upper stem region comprises fewer or more nucleotides than shown in the schematic of Tables 2 and 3 and FIG. 21C, the modification patterns, as will be apparent to the skilled artisan, are maintained.

[00172] In some embodiments, the upper stem of the crRNA comprises nucleotides US1 through US14 as shown in Table 2 and FIG. 21C.

[00173] In some embodiments, the upper stem of the trRNA comprises nucleotides US1 through US11 as shown in Table 3 and FIG. 21C.

e) **Nexus**

[00174] In some embodiments, the dgRNA comprises a trRNA comprising a nexus region. In some embodiments, the nexus is between the lower stem region and the hairpin 1 region of the trRNA. In some embodiments, the nexus is located immediately downstream of the lower stem of the trRNA. In some embodiments, the nexus comprises eighteen nucleotides. In some embodiments, the nexus region of the trRNA comprises nucleotides N1-N18 as shown in Table 3 and FIG 21C. In some embodiments, the nexus comprises fewer nucleotides than shown in Table 3 and FIG 21C. In some embodiments, the nexus region of the trRNA comprises more nucleotides than shown in Table 3 and FIG. 21C. When the nexus region comprises fewer or more nucleotides than shown in Table 3 and FIG. 21C, the modification patterns, as will be apparent to the skilled artisan, are maintained.

[00175] In some embodiments, the nexus region has nucleotides that are complementary in nucleic acid sequence when read in opposite directions. In some embodiments, the complementarity in nucleic acid sequence leads to a secondary structure of a stem and/or stem loop in the sgRNA (e.g., certain nucleotides in the nexus region may base pair with one another). In some embodiments, the nexus regions may not be perfectly complimentary to each other when read in opposite directions.

f) **Hairpin**

[00176] In some embodiments, the hairpin region of the trRNA is downstream of the nexus region. In some embodiments, the region of nucleotides immediately downstream of the nexus region is termed "hairpin 1." In some embodiments, the region of nucleotides immediately downstream of the hairpin 1 region is termed "hairpin 2." In some embodiments, the hairpin region comprises hairpin 1 and hairpin 2. In some instances, hairpin 1 and hairpin 2 are separated by one or more nucleotide "n." In some embodiments, n=1. In some embodiments, the trRNA comprises only hairpin 1 or hairpin 2.

[00177] Replacement of the hairpin 1 region of a trRNA with 2 nucleotides has been shown to allow editing activity of a Cas RNP (see US20150376586, Fig. 16). In some embodiments, the trRNA comprises replacement of hairpin 1 with nucleotides "n", wherein "n" is an integer between 1 and 50, 40, 30, 20, 15, 10, 5, 4, 3, and 2. In some embodiments, the hairpin 1 region of a trRNA is replaced by 2 nucleotides.

[00178] In some embodiments, hairpin 1 of the trRNA comprises twelve nucleotides immediately downstream of the nexus region. In some embodiments, the hairpin 1 region of the trRNA comprises nucleotides H1-1 through H1-12 as shown in Table 3 and FIG 21C.

[00179] In some embodiments, non-hairpin nucleotides are present between the hairpin 1 and the hairpin 2 regions of the trRNA. In some embodiments, one to two non-hairpin nucleotides reside between hairpin 1 and hairpin 2.

[00180] In some embodiments, hairpin 2 of the trRNA comprises fifteen nucleotides after (3' to) hairpin 1. In some embodiments, the hairpin 2 region of the trRNA comprises nucleotides H2-1 through H2-15 as shown in Table 3 and FIG 21C. In some embodiments, the hairpin 2 region of the trRNA comprises nucleotides H2-1 through H2-15 as shown in Table 3, and the "n" between hairpin 1 and hairpin 2 is 1 or 2.

[00181] In some embodiments, a hairpin region of the trRNA comprises more nucleotides than shown in Table 3 and FIG. 21C. When a hairpin region comprises fewer or more nucleotides than shown in Table 3 and FIG. 21C, the modification patterns, as will be apparent to the skilled artisan, are maintained.

[00182] In some embodiments, a hairpin region has nucleotides that are complementary in nucleic acid sequence when read in opposite directions. In some embodiments, the hairpin regions may not be perfectly complimentary to each other when read in opposite directions (e.g., the top or loop of the hairpin comprises unpaired nucleotides).

[00183] In some embodiments, the trRNA comprises replacement of hairpin 1 with nucleotides "n", wherein "n" is an integer between 1 and 50, 40, 30, 20, 15, 10, 5, 4, 3, and 2. In some embodiments, the hairpin 1 region of a trRNA is replaced by 2 nucleotides.

g) 3' terminus

[00184] In some embodiments, the dgRNA comprises a trRNA comprising a 3' terminus region comprising additional nucleotides after (3' to) the hairpin region(s). In some embodiments, the 3' terminus region comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15 or 20 or more nucleotides that are not associated with the secondary structure of a hairpin. In some embodiments, the 3' terminus region comprises 1, 2, 3, or 4 nucleotides that are not associated with the secondary structure of a hairpin. In some embodiments, the 3' terminus region comprises 4 nucleotides that are not associated with the secondary structure of a hairpin. In some embodiments, the 3' terminus region comprises 1, 2, or 3 nucleotides that are not associated with the secondary structure of a hairpin.

2. Modifications of dgRNAs

[00185] In some embodiments, a dgRNA comprises a modified crRNA and an unmodified trRNA. In some embodiments, a dgRNA comprises an unmodified crRNA and a

modified trRNA. In some embodiments, both the crRNA and trRNA of a dgRNA comprise modifications.

[00186] In some embodiments, the gRNAs described herein are in two separate RNA molecules (dual guide or dgRNA). See, Tables 2, 3, and FIG. 21C.

[00187] In some embodiments, the invention comprises a dgRNA comprising or consisting of a) any one of the crRNA sequences of SEQ ID Nos: 1-187; and b) any one of the trRNA sequences described in SEQ ID Nos: 188-227.

[00188] In some embodiments, a dgRNA comprising any one of the modified crRNA sequences of 1-187 is provided.

[00189] In some embodiments, a dgRNA comprising any one of the modified trRNA sequences of 188-227 is provided.

[00190] In some embodiments, a dgRNA comprising any one of the modified crRNA sequences of SEQ ID Nos: 19-31, 53-73, and 104-130 is provided. In some embodiments, the invention comprises a dgRNA comprising any one of the modified sequences of SEQ ID Nos: 19-31, 53-73, and 104-130, wherein the crRNA further comprises a 5' spacer sequence that is at least partially complementary to a target sequence, and directs a Cas9 to its target for cleavage.

[00191] In some embodiments, the invention comprises a crRNA comprising any one of the sequences described in SEQ ID Nos: 1-187. In some embodiments, the invention comprises a crRNA comprising or consisting of any one of the sequences described in SEQ ID Nos: 19-31, 53-73, and 104-130. In some embodiments, the invention comprises a crRNA comprising any one of the sequences described in SEQ ID Nos: 19-31, 53-73, and 104-130 and a spacer region.

[00192] In some embodiments, the invention comprises a trRNA comprising or consisting of any one of the sequences described in SEQ ID Nos:188-277.

[00193] In some embodiments, the invention comprises a crRNA comprising nucleotides having at least 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 85, 80, 75, or 70% identity to the nucleotides of any one of SEQ ID Nos: 1-187, wherein the modification pattern is identical to the modification pattern shown in the reference sequence identifier. That is, the nucleotides A, U, C, and G may differ by 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 85, 80, 75, or 70% compared to what is shown in in the sequences, but the modification remains unchanged.

[00194] In some embodiments, the invention comprises a trRNA comprising nucleotides having at least 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 85, 80, 75, or 70% identity to

the nucleotides of any one of SEQ ID Nos: 188-277, wherein the modification pattern is identical to the modification pattern shown in the reference sequence identifier. That is, the nucleotides A, U, C, and G may differ by 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 85, 80, 75, or 70% compared to what is shown in the sequences, but the modification on each nucleotide remains unchanged.

3. crRNAs, trRNAs, and dgRNAs with modifications

[00195] In some embodiments, the crRNA comprises one or more modified nucleotides within one or more of the 5' terminus, lower stem, bulge, upper stem, and 3' terminus.

[00196] In some embodiments, the modification comprises 2'-O-Me.

[00197] In some embodiments, the modification comprises 2'-F.

[00198] In some embodiments, the modification comprises a phosphorothioate (PS) bond linking one or more nucleotides. In some embodiments, the modification is three PS bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus.

[00199] In some embodiments, the modification comprises an inverted abasic nucleotide.

[00200] In some embodiments, a crRNA is provided comprising 2'-O-Me modified nucleotides at each nucleotide in the upper stem. In some embodiments, US-1 through US-14 of the crRNA are each modified with 2'-O-Me. In some embodiments, LS1 and LS6 of the crRNA are modified with 2'-O-Me. In some embodiments, LS5 of the crRNA is modified with 2'-O-Me.

[00201] In some embodiments, a crRNA comprising 2'-O-Me modified nucleotides at each of the nucleotides in the upper stem, and LS1 and LS6 in the lower stem is provided. In some embodiments, the crRNA further comprises one or more 2'-O-Me or 2'-O-moe modified nucleotides in the 5' and/or 3' terminus region, e.g. in a 5' and/or 3' end modification.

[00202] In some embodiments, a crRNA comprising 2'-O-Me modified nucleotides at each of the nucleotides in the upper stem, LS1, LS5, and LS6 in the lower stem is provided. In some embodiments, the crRNA further comprises one or more 2'-O-Me or 2'-O-moe modified nucleotides in the 5' and/or 3' terminus region, e.g. in a 5' and/or 3' end modification.

[00203] In some embodiments, the invention comprises a crRNA comprising 2'-F modified nucleotides at LS1, LS2, and LS6 in the lower stem. In some embodiments, the crRNA further comprises 2'-F modified nucleotides at each of B1 and B2 in the bulge region. In some embodiments, the invention comprises a crRNA comprising 2'-F modified nucleotides at LS1, LS2, and LS6 in the lower stem, and at each of B1 and B2 in the bulge region. In some embodiments, the crRNA further comprises one or more 2'-O-Me or 2'-O-moe modified nucleotides in the 5' and/or 3' terminus region, e.g. in a 5' and/or 3' end modification.

[00204] In some embodiments, the crRNA comprises 2'-O-Me modified nucleotides at nucleotides LS1 and LS6 in the lower stem region; each of the nucleic acids in the bulge region; and each of the nucleic acids in the upper stem region. In some embodiments, the LS5 nucleotide of the crRNA is also modified with 2'-O-Me. In some embodiments, LS2, LS3, and LS4 of the crRNA are not modified. In some embodiments, the crRNA further comprises one or more 2'-O-Me or 2'-O-moe modified nucleotides in the 5' and/or 3' terminus region, e.g. in a 5' and/or 3' end modification.

[00205] In some embodiments, the crRNA comprises 2'-fluoro (2'-F) modified nucleotides at LS1, LS2, and LS6 in the lower stem region, and each of the nucleotides in the bulge region. In some embodiments, the crRNA comprises 2'-fluoro (2'-F) modified nucleotides at LS1, LS2, and LS6 in the lower stem region, and at B2 and B2 in the bulge region. In some embodiments, the crRNA comprises 2'-fluoro (2'-F) modified nucleotides at LS1- LS6 in the lower stem region, and each of the nucleotides in the bulge region. In some embodiments, the crRNA further comprises one or more 2'-O-Me or 2'-O-moe modified nucleotides in the 5' and/or 3' terminus region, e.g. in a 5' and/or 3' end modification.

[00206] In some embodiments, the invention comprises a trRNA comprising one or more modified nucleotides within one or more of the following regions: the 5' terminus, the upper stem region; the bulge region; the lower stem region; the nexus region; the hairpin 1 region; the intervening region between the hairpin 1 and hairpin 2 regions; the hairpin 2 region; and the 3' terminus region.

[00207] In some embodiments, the modification comprises 2'-O-Me.

[00208] In some embodiments, the modification comprises 2'-F.

[00209] In some embodiments, the modification comprises a phosphorothioate (PS) bond linking one or more nucleotides. In some embodiments, the modification is three PS bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus.

[00210] In some embodiments, the modification comprises an inverted abasic nucleotide.

[00211] In some embodiments, the trRNA comprises 2'-O-Me modified nucleotides at each nucleic acid in the upper stem; B1 and B2 in the bulge region; LS1 and LS2 in the lower stem region; N3, N4, N5, N15, N16, N17, and N18 in the nexus region; each nucleotide in the hairpin 1 region; one nucleotide between the hairpin 1 and hairpin 2 region; and each nucleotide in the hairpin 2 region. In some embodiments, the trRNA further comprises one or more 2'-O-Me or 2'-O-moe modified nucleotides in the 5' and/or 3' terminus region, e.g. in a 5' and/or 3' end modification.

[00212] In some embodiments, the trRNA comprises 2'-O-Me modified nucleotides at each nucleic acid in the upper stem; each nucleotide in the bulge region; LS1, LS2, LS5, and LS6 in the lower stem region; N3- N5, N10-N18 in the nexus region; each nucleotide in the hairpin 1 region; one nucleotide between the hairpin 1 and hairpin 2 region; and each nucleotide in the hairpin 2 region. In some embodiments, the crRNA further comprises one or more 2'-O-Me or 2'-O-moe modified nucleotides in the 5' and/or 3' terminus region, , e.g. in a 5' and/or 3' end modification.

[00213] In some embodiments, the trRNA comprises 2'-F modified nucleotides at N15 through N18 in the nexus region. In some embodiments, the trRNA further comprises one or more 2'-F modified nucleotides in the 5' and/or 3' terminus region, e.g. in a 5' and/or 3' end modification.

[00214] In some embodiments, the trRNA comprises 2'-F modified nucleotides at LS4 and LS5 in the lower stem region, and N13-N18 in the nexus region. In some embodiments, the trRNA further comprises one or more 2'-F modified nucleotides in the 5' and/or 3' terminus region, e.g. in a 5' and/or 3' end modification.

[00215] In some embodiments, the trRNA comprises 2'-F modified nucleotides at LS1, LS3, and LS5 in the lower stem, and 2'-O-Me modified nucleotides at LS2, LS4, and LS6 in the lower stem.

[00216] Disclosed herein, in some embodiments, is a crispr RNA (crRNA) comprising one or more modifications within one or more of the following regions: the first five nucleotides at the 5' terminus; the lower stem region; the bulge region; the upper stem region; and the last five nucleotides at the 3' terminus. In some embodiments, the modification comprises a 2'-O-methyl (2'-O-Me) modified nucleotide. In some embodiments, the modification comprises a 2'-fluoro (2'-F) modified nucleotide. In some embodiments, the modification comprises a phosphorothioate (PS) bond between nucleotides. In some

embodiments, the first three nucleotides at the 5' terminus, and the last three nucleotides at the 3' terminus are modified. In some embodiments, the first four nucleotides at the 5' terminus, and the last four nucleotides at the 3' terminus are linked with phosphorothioate (PS) bonds. In some embodiments, the modification comprises 2'-O-Me. In some embodiments, the modification comprises 2'-F. In some embodiments, the first four nucleotides at the 5' terminus and the last four nucleotides at the 3' terminus are linked with a PS bond, and wherein the first three nucleotides at the 5' terminus and the last three nucleotides at the 3' terminus comprise 2'-O-Me modifications. In some embodiments, the first four nucleotides at the 5' terminus and the last four nucleotides at the 3' terminus are linked with a PS bond, and wherein the first three nucleotides at the 5' terminus and the last three nucleotides at the 3' terminus comprise 2'-F modifications. In some embodiments, LS1 and LS6 are modified with 2'-O-Me. In some embodiments, each of the nucleotides in the upper stem region are modified with 2'-O-Me.

[00217] In some embodiments, the invention comprises a crispr RNA (crRNA) comprising 2'-O-Me modified nucleic acids at the following nucleotides: LS1 and LS6 in the lower stem region; and each nucleotide in the upper stem region. In some embodiments, the crRNA further comprises three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus. In some embodiments, the crRNA further comprises 2'-O-Me or 2'-F modified nucleic acids at the first three nucleotides at the 5' terminus, and 2'-O-Me or 2'-F modified nucleic acids at the last three nucleotides at the 3' terminus. In some embodiments, LS1, LS2, and LS6 are modified with 2'-F. In some embodiments, each nucleotide in the bulge region is modified with 2'-F.

[00218] Disclosed herein, in some embodiments, is a crispr RNA (crRNA) comprising 2'-F modified nucleic acids at the following nucleotides: LS1, LS2, and LS6 in the lower stem region; and each nucleotide in the bulge region. In some embodiments, the crRNA further comprises three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus. In some embodiments, the crRNA further comprises 2'-O-Me or 2'-F modified nucleic acids at the first three nucleotides at the 5' terminus, and 2'-O-Me or 2'-F modified nucleic acids at the last three nucleotides at the 3' terminus.

[00219] In some embodiments, a crRNA comprising the nucleic acids of any one of SEQ ID Nos: 1 – 187 is provided. In some embodiments, a crRNA comprising the nucleic acids of any one of SEQ ID Nos: 19-31, 53-73, 104-130, and 161-187 is provided. In some

embodiments, a crRNA comprising nucleic acids having at least 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 85, 80, 75, or 70% identity to the nucleic acids of any one of SEQ ID Nos: 19-31, 53-73, 104-130, and 161-187, wherein the modification pattern is identical to the modification pattern shown in the reference sequence identifier, is provided. In some embodiments, the crRNA further comprises three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus.

[00220] Also encompassed is a tracr RNA (trRNA) comprising one or more modifications within one or more of the following regions: the first five nucleotides at the 5' terminus; the upper stem region; the bulge region; the lower stem region; the nexus region; the hairpin 1 region; the hairpin 2 region; and the last five nucleotides at the 3' terminus. In some embodiments, the modification comprises a 2'-O-methyl (2'-O-Me) modified nucleotide. In some embodiments, the modification comprises a 2'-fluoro (2'-F) modified nucleotide. In some embodiments, the modification comprises a phosphorothioate (PS) bond between nucleotides. In some embodiments, the first four nucleotides at the 5' terminus, and the last four nucleotides at the 3' terminus are linked with phosphorothioate (PS) bonds. In some embodiments, the first three nucleotides at the 5' terminus, and the last three nucleotides at the 3' terminus are modified. In some embodiments, the modification comprises 2'-O-Me. In some embodiments, the modification comprises 2'-F. In some embodiments, the first four nucleotides at the 5' terminus and the last four nucleotides at the 3' terminus are linked with a PS bond, and wherein the first three nucleotides at the 5' terminus and the last three nucleotides at the 3' terminus comprise 2'-O-Me modifications. In some embodiments, the first four nucleotides at the 5' terminus and the last four nucleotides at the 3' terminus are linked with a PS bond, and wherein the first three nucleotides at the 5' terminus and the last three nucleotides at the 3' terminus comprise 2'-F modifications. In some embodiments, each nucleotide in the upper stem region is modified with 2'-O-Me. In some embodiments, B1 and B2 within the bulge region are modified with 2'-O-Me. In some embodiments, N3, N4, N5, N15, N16, N17, and N18 in the nexus region are modified with 2'-O-Me. In some embodiments, each nucleotide in the hairpin 1 region is modified with 2'-O-Me. In some embodiments, each nucleotide in the hairpin 2 region is modified with 2'-O-Me.

[00221] In some embodiments, the invention comprises a tracr RNA (trRNA) comprising 2'-O-Me modified nucleic acids at the following nucleotides: each nucleotide in the upper stem; B1 and B2 within the bulge region; N3, N4, N5, N15, N16, N17, and N18 in

the nexus region; each nucleotide in the hairpin 1 region; and each nucleotide in the hairpin 2 region. In some embodiments, the trRNA further comprises three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus. In some embodiments, the trRNA further comprises 2'-O-Me or 2'-F modified nucleotides at the first three nucleotides at the 5' terminus, and 2'-O-Me or 2'-F modified nucleic acids at the last three nucleotides at the 3' terminus. In some embodiments, N15, N16, N17, and N18 are modified with 2'-F. In some embodiments, LS1, LS3, and LS5 are modified with 2'-F, and LS2, LS4, and LS6 are modified with 2'-O-Me. In some embodiments, the trRNA further comprises three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus. In some embodiments, the trRNA further comprises 2'-O-Me or 2'-F modified nucleic acids at the first three nucleotides at the 5' terminus, and 2'-O-Me or 2'-F modified nucleic acids at the last three nucleotides at the 3' terminus.

[00222] In some embodiments, a trRNA comprising the nucleic acids of any one of SEQ ID Nos: 188-227 is provided. In some embodiments, a trRNA comprising nucleic acids having at least 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 85, 80, 75, or 70% identity to the nucleic acids of any one of SEQ ID Nos: 188-227, wherein the modification pattern is identical to the modification pattern shown in the reference sequence identifier, is provided. In some embodiments, the trRNA further comprises three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus.

[00223] In some instances, a dual guide comprising a crRNA and a trRNA is provided, wherein the crRNA comprises the nucleic acids of any one of SEQ ID Nos: 1-187, and wherein the trRNA comprises the nucleic acids of any one of SEQ ID Nos: 188-227.

[00224] A dual guide comprising a crRNA disclosed herein and a trRNA disclosed herein is encompassed, as is a dual guide comprising a crRNA disclosed herein and an unmodified trRNA. In some embodiments, a dual guide comprising an unmodified crRNA and a modified trRNA disclosed herein is provided.

[00225] In some embodiments, and of the following are encompassed:

Embodiment 55. A crispr RNA (crRNA) comprising one or more modifications within one or more of the following regions:

a. the first five nucleotides at the 5’ terminus;
b. the lower stem region;
c. the bulge region;

d. the upper stem region; and

e. the last five nucleotides at the 3' terminus.

Embodiment 56. The crRNA of embodiment 55, wherein the modification comprises a 2'-O-methyl (2'-O-Me) modified nucleotide.

Embodiment 57. The crRNA of embodiment 55, wherein the modification comprises a 2'-fluoro (2'-F) modified nucleotide.

Embodiment 58. The crRNA of embodiment 55, wherein the modification comprises a phosphorothioate (PS) bond between nucleotides.

Embodiment 59. The crRNA of any one of embodiments 55-58, wherein the first three nucleotides at the 5' terminus and the last three nucleotides at the 3' terminus are modified.

Embodiment 60. The crRNA of any one of embodiments 55-58, wherein the first four nucleotides at the 5' terminus, and the last four nucleotides at the 3' terminus are linked with phosphorothioate (PS) bonds.

Embodiment 61. The crRNA of embodiment 59, wherein the modification comprises 2'-O-Me.

Embodiment 62. The crRNA of embodiment 59, wherein the modification comprises 2'-F.

Embodiment 63. The crRNA of any one of embodiments 55-62, wherein the first four nucleotides at the 5' terminus and the last four nucleotides at the 3'terminus are linked with a PS bond, and wherein the first three nucleotides at the 5' terminus and the last three nucleotides at the 3' terminus comprise 2'-O-Me modifications.

Embodiment 64. The crRNA of any one of embodiments 55-62, wherein the first four nucleotides at the 5' terminus and the last four nucleotides at the 3'terminus are linked with a PS bond, and wherein the first three nucleotides at the 5' terminus and the last three nucleotides at the 3' terminus comprise 2'-F modifications.

Embodiment 65. The crRNA of any one of embodiments 55-60, wherein LS1 and LS6 are modified with 2'-O-Me.

Embodiment 66. The crRNA of any one of embodiments 55-60and 65, wherein each of the nucleotides in the upper stem region are modified with 2'-O-Me.

Embodiment 67. A crispr RNA (crRNA) comprising 2'-O-Me modified nucleotides at:

a. LS1 and LS6 in the lower stem region; and

b. each nucleotide in the upper stem region.

Embodiment 68. The crRNA of embodiment 67, further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus.

Embodiment 69. The crRNA of embodiment 67 or 68, further comprising 2'-O-Me or 2'-F modified nucleotides at the first three nucleotides at the 5' terminus, and 2'-O-Me or 2'-F modified nucleotides at the last three nucleotides at the 3' terminus.

Embodiment 70. The crRNA of any of embodiments 55-60, wherein LS1, LS2, and LS6 are modified with 2'-F.

Embodiment 71. The crRNA of any of embodiments 55-60 and 70, wherein each nucleotide in the bulge region is modified with 2'-F.

Embodiment 72. A crispr RNA (crRNA) comprising 2'-F modified nucleotides at:

a. LS1, LS2, and LS6 in the lower stem region; and
b. each nucleotide in the bulge region.

Embodiment 73. The crRNA of any one of embodiments 70-72, further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus.

Embodiment 74. The crRNA of embodiment 72 or 73, further comprising 2'-O-Me or 2'-F modified nucleotides at the first three nucleotides at the 5' terminus, and 2'-O-Me or 2'-F modified nucleotides at the last three nucleotides at the 3' terminus.

Embodiment 75. A crRNA comprising the nucleic acids of any one of SEQ ID Nos: 1 – 187.

Embodiment 76. A crRNA comprising the nucleic acids of any one of SEQ ID Nos: 19-31, 53-73, 104-130, and 161-187.

Embodiment 77. A crRNA comprising nucleic acids having at least 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 85, 80, 75, or 70% identity to the nucleic acids of any one of SEQ ID Nos: 19-31, 53-73, 104-130, and 161-187, wherein the modification pattern is identical to the modification pattern shown in the reference sequence identifier.

Embodiment 78. The crRNA of any one of embodiments 75-77, further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus.

Embodiment 79. A tracr RNA (trRNA) comprising one or more modifications within one or more of the following regions:

a. the first five nucleotides at the 5' terminus;
b. the upper stem region;

c. the bulge region;
d. the lower stem region;
e. the nexus region;
f. the hairpin 1 region;
g. the hairpin 2 region; and
h. the last five nucleotides at the 3' terminus.

Embodiment 80. The trRNA of embodiment 79, wherein the modification comprises a 2'-O-methyl (2'-O-Me) modified nucleotide.

Embodiment 81. The trRNA of embodiment 79, wherein the modification comprises a 2'-fluoro (2'-F) modified nucleotide.

Embodiment 82. The trRNA of embodiment 79, wherein the modification comprises a phosphorothioate (PS) bond between nucleotides.

Embodiment 83. The trRNA of any one of embodiments 79-82, wherein the first four nucleotides at the 5' terminus, and the last four nucleotides at the 3' terminus are linked with phosphorothioate (PS) bonds.

Embodiment 84. The trRNA of any one of embodiments 79-82, wherein the first three nucleotides at the 5' terminus, and the last three nucleotides at the 3' terminus are modified.

Embodiment 85. The trRNA of embodiment 84, wherein the modification comprises 2'-O-Me.

Embodiment 86. The trRNA of embodiment 84, wherein the modification comprises 2'-F.

Embodiment 87. The trRNA of any one of embodiments 79-86, wherein the first four nucleotides at the 5' terminus and the last four nucleotides at the 3'terminus are linked with a PS bond, and wherein the first three nucleotides at the 5' terminus and the last three nucleotides at the 3' terminus comprise 2'-O-Me modifications.

Embodiment 88. The trRNA of any one of embodiments 79-86, wherein the first four nucleotides at the 5' terminus and the last four nucleotides at the 3'terminus are linked with a PS bond, and wherein the first three nucleotides at the 5' terminus and the last three nucleotides at the 3' terminus comprise 2'-F modifications.

Embodiment 89. The trRNA of any one of embodiments 79-84, wherein each nucleotide in the upper stem region is modified with 2'-O-Me.

Embodiment 90. The trRNA of any one of embodiments 79-84 and 89, wherein B1 and B2 within the bulge region are modified with 2'-O-Me.

Embodiment 91. The trRNA of any one of embodiments 79-84 and 89-90, wherein N3, N4, N5, N15, N16, N17, and N18 in the nexus region are modified with 2'-O-Me.

Embodiment 92. The trRNA of any one of embodiments 79-84 and 89-91, wherein each nucleotide in the hairpin 1 region is modified with 2'-O-Me.

Embodiment 93. The trRNA of any one of embodiments 79-84 and 89-92, wherein each nucleotide in the hairpin 2 region is modified with 2'-O-Me.

Embodiment 94. A tracr RNA (trRNA) comprising 2'-O-Me modified nucleotides at:

a. each nucleotide in the upper stem;
b. B1 and B2 within the bulge region;
c. N3, N4, N5, N15, N16, N17, and N18 in the nexus region;
d. each nucleotide in the hairpin 1 region; and
e. each nucleotide in the hairpin 2 region.

Embodiment 95. The trRNA of embodiment 94, further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus.

Embodiment 96. The crRNA of embodiment 94 or 95, further comprising 2'-O-Me or 2'-F modified nucleotides at the first three nucleotides at the 5' terminus, and 2'-O-Me or 2'-F modified nucleic acids at the last three nucleotides at the 3' terminus.

Embodiment 97. The trRNA of any of embodiments 79-84, wherein N15, N16, N17, and N18 are modified with 2'-F.

Embodiment 98. The trRNA of any of embodiments 79-84 and 97, wherein LS1, LS3, and LS5 are modified with 2'-F, and LS2, LS4, and LS6 are modified with 2'-O-Me.

Embodiment 99. The trRNA of any one of embodiments 87-98, further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus.

Embodiment 100. The trRNA of embodiment 98 or 99, further comprising 2'-O-Me or 2'-F modified nucleotides at the first three nucleotides at the 5' terminus, and 2'-O-Me or 2'-F modified nucleotides at the last three nucleotides at the 3' terminus.

Embodiment 101. A trRNA comprising the nucleic acids of any one of SEQ ID Nos: 188-227.

Embodiment 102. A trRNA comprising nucleic acids having at least 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 85, 80, 75, or 70% identity to the nucleic acids of any one of SEQ ID Nos: 188-227, wherein the modification pattern is identical to the modification pattern shown in the reference sequence identifier.

Embodiment 103. The trRNA of any one of embodiments 101 - 102, further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus.

Embodiment 104. A dual guide comprising a crRNA and a trRNA, wherein the crRNA comprises the nucleotides of any one of SEQ ID Nos: 1-187, and wherein the trRNA comprises the nucleic acids of any one of SEQ ID Nos: 188-227.

Embodiment 105. A dual guide comprising a crRNA of any one of embodiments 55-78 and a trRNA of any one of embodiments 79-103.

Embodiment 106. A dual guide comprising a crRNA of any one of embodiments 55-78 and an unmodified trRNA.

Embodiment 107. A dual guide comprising an unmodified crRNA and a trRNA of any one of embodiments 79-103.

C. Modifications to terminal nucleotides

[00226] In some embodiments, the 5' or 3' terminal nucleotides of any of the guide RNAs described herein are modified. In some embodiments, the terminal (i.e., last) 1, 2, 3, 4, 5, 6, or 7 nucleotides in 3' terminus region of guide RNA, including, for example, the sgRNA, the dgRNA, the crRNA, trRNA, or both crRNA and trRNA are modified. In some embodiments, the terminal (i.e., last) 1, 2, 3, 4, 5, 6, or 7 nucleotides in 3' terminus region of guide RNA comprise more than one modification. In some embodiments, at least one of the terminal (i.e., last) 1, 2, 3, 4, 5, 6, or 7 nucleotides at the 3' terminus region are modified. In some embodiments, at least two of the terminal (i.e., last) 1, 2, 3, 4, 5, 6, or 7 nucleotides in 3' terminus region are modified. In some embodiments, at least three of the terminal (i.e., last) 1, 2, 3, 4, 5, 6, or 7 nucleotides in 3' terminus region are modified. some embodiments, the modification comprises a PS linkage.

[00227] In some embodiments, the 5' end of the 5' terminus region is modified, for example, the first 1, 2, 3, 4, 5, 6, or 7 nucleotides of the sgRNA, the dgRNA, crRNA, trRNA, or both crRNA and trRNA are modified. In some embodiments, the first 1, 2, 3, 4, 5, 6, or 7 nucleotides in 3' terminus region of guide RNA comprise more than one modification. In some embodiments, at least one of the terminal (i.e., first) 1, 2, 3, 4, 5, 6, or 7 nucleotides at the 5' end are modified. In some embodiments, at least two of the terminal 1, 2, 3, 4, 5, 6, or 7 nucleotides at the 5' end are modified. In some embodiments, at least three of the terminal 1, 2, 3, 4, 5, 6, or 7 nucleotides at the 5' end are modified. some embodiments, the modification comprises a PS linkage.

[00228] In some embodiments, both the 5' and 3' termini (e.g., ends) of the guide RNA, e.g., sgRNA, dgRNA, crRNA, trRNA, or both crRNA and trRNA are modified. In some embodiments, only the 5' terminus of the guide RNA, e.g., sgRNA, dgRNA, crRNA, trRNA, or both crRNA and trRNA is modified. In some embodiments, only the 3' terminus of the guide RNA, e.g., sgRNA, dgRNA, crRNA, trRNA, or both crRNA and trRNA is modified.

[00229] In some embodiments, the gRNA comprises modifications at 1, 2, 3, 4, 5, 6, or 7 of the first 7 nucleotides at a 5' end of the gRNA. In some embodiments, the gRNA comprises modifications at 1, 2, 3, 4, 5, 6, or 7 of the 7 terminal nucleotides at a 3' end. In some embodiments, 2, 3, or 4 of the first 4 nucleotides at the 5' end, and/or 2, 3, or 4 of the terminal 4 nucleotides at the 3' end are modified. In some embodiments, 2, 3, or 4 of the first 4 nucleotides at the 5' end are linked with phosphorothioate (PS) bonds.

[00230] In some embodiments, the modification to the 5' terminus and/or 3' terminus comprises a 2'-O-methyl (2'-O-Me) or 2'-O-(2-methoxyethyl) (2'-O-moe) modification to a nucleotide. In some embodiments, the modification comprises a 2'-fluoro (2'-F) modification to a nucleotide. In some embodiments, the modification comprises a phosphorothioate (PS) linkage between nucleotides. In some embodiments, the modification comprises an inverted abasic nucleotide. In some embodiments, the modification comprises a more than one modification selected from 2'-O-Me, 2'-O-moe, 2'-fluoro (2'-F), a phosphorothioate (PS) linkage between nucleotides, and an inverted abasic nucleotide. In some embodiments, an equivalent modification is encompassed.

[00231] In some embodiments, the guide RNA, e.g., sgRNA, dgRNA, crRNA, trRNA, or both crRNA and trRNA comprises one or more phosphorothioate (PS) linkages between the first one, two, three, four, five, six, or seven nucleotides at the 5' terminus. In some embodiments, the guide RNA, e.g., sgRNA, dgRNA, crRNA, trRNA, or both crRNA and trRNA comprises one or more PS linkages between the last one, two, three, four, five, six, or seven nucleotides at the 3' terminus. In some embodiments, the guide RNA, e.g., sgRNA, dgRNA, crRNA, trRNA, or both crRNA and trRNA comprises one or more PS linkages between the last one, two, three, four, five, six, or seven nucleotides at both the 5' terminus and the 3' terminus. In some embodiments, in addition to PS linkages, the 5' and 3' terminal nucleotides may comprise 2'-O-Me, 2'-O-moe, or 2'-F modified nucleotides.

[00232] In some embodiments, the guide RNA, e.g., sgRNA, dgRNA, crRNA, trRNA, or both crRNA and trRNA comprises modified nucleotides at the 5' and 3' terminus, and

modified nucleotides in one or more other regions described in Tables 1-3 and FIG 21A or 21C.

[00233] In some embodiments, the crRNA, trRNA, or both crRNA and trRNA comprises modified nucleotides that are not at the 5' or 3' ends. Specific patterns of modifications are described below and in Table 4.

3. Delivery of gRNAs and Cas Protein

[00234] In some embodiments, in addition to the at least one gRNA, the compositions provided herein further comprise a nuclease. In some embodiments, the nuclease is a Cas protein. In some embodiments, the gRNA together with a Cas protein is called a Cas RNP. In some embodiments, the Cas protein is from the Type-II CRISPR/Cas system. In some embodiments, the Cas protein is Cas9. In some embodiments, the Cas9 protein is a wild type Cas9. In some embodiments, the Cas9 protein is derived from the *Streptococcus pyogenes* Cas9 protein, e.g., a S. *pyogenes* Cas9. In some embodiments, the Cas9 protein is not derived from S. *pyogenes*, but functions in the same way as S. *pyogenes* Cas9 such that gRNA that is specific to S. *pyogenes* Cas9 will direct the non-S. *pyogenes* Cas9 to its target site. In some embodiments, the Cas induces a double strand break in target DNA. Equivalents of S. *pyogenes* Cas9 protein are encompassed by the embodiments described herein.

[00235] Cas9 encompasses modified and variants thereof. Modified versions of Cas9 having one catalytic domain, either RuvC or HNH, that is inactive are termed "nickases." Nickases cut only one strand on the target DNA, thus creating a single-strand break. A single-strand break may also be known as a "nick." In some embodiments, the compositions and methods comprise nickases. In some embodiments, the compositions and methods comprise a nickase Cas9 that induces a nick rather than a double strand break in the target DNA.

[00236] In some embodiments, the Cas protein may be modified to contain only one functional nuclease domain. For example, the Cas protein may be modified such that one of the nuclease domains is mutated or fully or partially deleted to reduce its nucleic acid cleavage activity. In some embodiments, a nickase Cas is used having a RuvC domain with reduced activity. In some embodiments, a nickase Cas is used having an inactive RuvC domain. In some embodiments, a nickase Cas is used having an HNH domain with reduced activity. In some embodiments, a nickase Cas is used having an inactive HNH domain.

[00237] In some embodiments, a conserved amino acid within a Cas protein nuclease domain is substituted to reduce or alter nuclease activity. In some embodiments, a Cas protein

may comprise an amino acid substitution in the RuvC or RuvC-like nuclease domain. Exemplary amino acid substitutions in the RuvC or RuvC-like nuclease domain include D10A (based on the S. *pyogenes* Cas9 protein). In some embodiments, the Cas protein may comprise an amino acid substitution in the HNH or HNH-like nuclease domain. Exemplary amino acid substitutions in the HNH or HNH-like nuclease domain include E762A, H840A, N863A, H983A, and D986A (based on the S. *pyogenes* Cas9 protein).

[00238] In some embodiments, the RNP complex described herein comprises a nickase and a pair of guide RNAs that are complementary to the sense and antisense strands of the target sequence, respectively. In this embodiment, the guide RNAs direct the nickase to a target sequence and introduce a double stranded break (DSB) by generating a nick on opposite strands of the target sequence (i.e., double nicking). In some embodiments, use of double nicking may improve specificity and reduce off-target effects. In some embodiments, a nickase Cas is used together with two separate guide RNAs targeting opposite strands of DNA to produce a double nick in the target DNA. In some embodiments, a nickase Cas is used together with two separate guide RNAs that are selected to be in close proximity to produce a double nick in the target DNA.

[00239] In some embodiments, chimeric Cas proteins are used, where one domain or region of the protein is replaced by a portion of a different protein. In some embodiments, a Cas nuclease domain may be replaced with a domain from a different nuclease such as Fok1. In some embodiments, a Cas protein may be a modified nuclease.

[00240] In some embodiments, the Cas protein comprises a fusion protein comprising a catalytically inactive Cas9 linked to a heterologous functional domain (see, e.g., WO2014152432). In some embodiments, the catalytically inactive Cas9 is from *S. pyogenes*. In some embodiments, the catalytically inactive Cas9 comprises mutations that inactivate the Cas9. In some embodiments, the heterologous functional domain is a domain that modifies gene expression, histones, or DNA. In some embodiments, the heterologous functional domain is a transcriptional activation domain or a transcriptional repressor domain.

A. PAM

[00241] In some embodiments, the target sequence may be adjacent to the PAM. In some embodiments, the PAM may be adjacent to or within 1, 2, 3, or 4, nucleotides of the 3' end of the target sequence. The length and the sequence of the PAM may depend on the Cas protein used. For example, the PAM may be selected from a consensus or a particular PAM sequence for a specific Cas9 protein or Cas9 ortholog, including those disclosed in Figure 1

of Ran et al., Nature 520:186-191 (2015), which is incorporated herein by reference. In some embodiments, the PAM may comprise 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides in length. Non-limiting exemplary PAM sequences include NGG, NAG, NGA, NGAG, NGCG, NNGRRT, TTN, NGGNG, NG, NAAAAN, NNAAAAW, NNNNACA, GNNNCNNA, and NNNNGATT (wherein N is defined as any nucleotide, and W is defined as either A or T, and R is defined as either A or G). In some embodiments, the PAM sequence may be NGG. In some embodiments, the PAM sequence may be NGGNG. In some embodiments, the PAM sequence may be NNAAAAW.

B. Delivery of Modified gRNA

[00242] Lipid nanoparticles (LNPs) are a well-known means for delivery of nucleotide and protein cargo, and may be used for delivery of the gRNA, mRNA, Cas9, and RNPs disclosed herein. In some embodiments, the LNPs deliver nucleic acid, protein, or nucleic acid together with protein.

[00243] In some embodiments, the invention comprises a method for delivering any one of the gRNAs disclosed herein to a subject, wherein the gRNA is associated with an LNP. In some embodiments, the gRNA/LNP is also associated with a Cas9 or an mRNA encoding Cas9.

[00244] In some embodiments, the invention comprises a composition comprising any one of the gRNAs disclosed and an LNP. In some embodiments, the composition further comprises a Cas9 or an mRNA encoding Cas9.

[00245] In some embodiments, the LNPs comprise cationic lipids. In some embodiments, the LNPs comprise (9Z,12Z)-3-((4,4-bis(octyloxy)butanoyl)oxy)-2-((((3-(diethylamino)propoxy)carbonyl)oxy)methyl)propyl octadeca-9,12-dienoate, also called 3-((4,4-bis(octyloxy)butanoyl)oxy)-2-((((3-(diethylamino)propoxy)carbonyl)oxy)methyl)propyl (9Z,12Z)-octadeca-9,12-dienoate). In some embodiments, the LNPs comprise molar ratios of a cationic lipid amine to RNA phosphate (N:P) of about 4.5.

[00246] In some embodiments, LNPs associated with the gRNAs disclosed herein are for use in preparing a medicament for treating a disease or disorder.

[00247] Electroporation is a well-known means for delivery of cargo, and any electroporation methodology may be used for delivery of any one of the gRNAs disclosed herein. In some embodiments, electroporation may be used to deliver any one of the gRNAs disclosed herein and Cas9 or an mRNA encoding Cas9.

[00248] In some embodiments, the invention comprises a method for delivering any one of the gRNAs disclosed herein to an ex vivo cell, wherein the gRNA is associated with an LNP or not associated with an LNP. In some embodiments, the gRNA/LNP or gRNA is also associated with a Cas9 or an mRNA encoding Cas9.

4. Methods of Gene Modulation

[00249] In some embodiments, the invention comprises a pharmaceutical formulation comprising any one of the gRNAs disclosed herein together with a pharmaceutically acceptable carrier. In some embodiments, the invention comprises a pharmaceutical formulation comprising any one of the gRNAs disclosed herein and an LNP together with a pharmaceutically acceptable carrier. In some embodiments, the invention comprises a pharmaceutical formulation comprising any one of the gRNAs disclosed herein, a Cas9 protein or an mRNA encoding a Cas9 protein, and a LNP together with a pharmaceutically acceptable carrier. In some embodiments, the pharmaceutical formulation is for use in preparing a medicament for treating a disease or disorder. In some embodiments, the invention comprises a method of treating a human patient comprising administering any one of the gRNAs or pharmaceutical formulations described herein.

[00250] In some embodiments, the invention comprises a method or use of modifying a target DNA comprising, administering or delivering a Cas protein or Cas mRNA and any one or more of the gRNAs disclosed herein.

[00251] In some embodiments, the invention comprises a method or use for modulation of a target gene comprising, administering or delivering a Cas protein or Cas mRNA and any one or more of the gRNAs disclosed herein. In some embodiments, the modulation is editing of the target gene. In some embodiments, the modulation is a change in expression of the protein encoded by the target gene.

[00252] In some embodiments, the method or use results in gene editing. In some embodiments, the method or use results in a double-stranded break within the target gene. In some embodiments, the method or use results in formation of indel mutations during non-homologous end joining of the DSB. In some embodiments, the method or use results in an insertion or deletion of nucleotides in a target gene. In some embodiments, the insertion or deletion of nucleotides in a target gene leads to a frameshift mutation or premature stop codon that results in a non-functional protein. In some embodiments, the insertion or deletion of nucleotides in a target gene leads to a knockdown or elimination of target gene expression. In some embodiments, the method or use comprises homology directed repair of a DSB. In

some embodiments, the method or use further comprises delivering to the cell a template, wherein at least a part of the template incorporates into a target DNA at or near a double strand break site induced by the Cas protein.

[00253] In some embodiments, the method or use results in gene modulation. In some embodiments, the gene modulation is an increase or decrease in gene expression, a change in methylation state of DNA, or modification of a histone subunit. In some embodiments, the method or use results in increased or decreased expression of the protein encoded by the target gene.

[00254] In some embodiments, any of the gRNAs disclosed herein may be useful in preparing a medicament for treating a disease or disorder.

A. Measures of Gene Modulation

[00255] The efficacy of modified gRNAs can be tested in vitro and in vivo. In some embodiments, the invention comprises one or more of the gRNAs disclosed herein, wherein the gRNA results in gene modulation when provided to a cell together with Cas9. In some embodiments, the efficacy of gRNA can be measured in *in vitro* or *in vivo* assays.

1. In vitro measurement of Cas efficacy

[00256] In some embodiments, the activity of a Cas RNP comprising a modified sgRNA is compared to the activity of a Cas RNP comprising an unmodified sgRNA.

[00257] In some embodiments, the activity of a Cas RNP comprising a dgRNA comprising a modified trRNA is compared to the activity of a Cas RNP comprising a dgRNA comprising an unmodified trRNA.

[00258] In some embodiments, the activity of a Cas RNP comprising a dgRNA comprising a modified crRNA is compared to the activity of a Cas RNP comprising a dgRNA comprising an unmodified crRNA.

[00259] In some embodiments, the activity of a Cas RNP comprising a dgRNA comprising a modified crRNA and a modified trRNA is compared to the activity of a Cas RNP comprising an unmodified crRNA and an unmodified trRNA.

[00260] In some embodiments, the efficiency of a gRNA in increasing or decreasing target protein expression is determined by measuring the amount of target protein. In some embodiments, the invention comprises any one of the gRNAs described herein, wherein the gRNA increases or decreases the amount of protein produced from the targeted gene. In some embodiments, the invention comprises a method of modulating protein expression

comprising administering any one of the gRNAs disclosed herein to a subject, wherein the gRNA directs Cas9 to the gene encoding the target protein, and the target protein expression is increased or decreased as compared to a gRNA control that does not target Cas9 to that gene.

[00261] In some embodiments, the efficiency of editing with specific gRNAs is determined by the editing present at the target location in the genome following delivery of Cas9 and the gRNA (either sgRNA or dgRNA comprising a crRNA and trRNA). In some embodiments, the efficiency of editing with specific gRNAs is measured by next-generation sequencing. In some embodiments, the editing percentage of the target region of interest is determined. In some embodiments, the total number of sequence reads with insertions or deletions of nucleotides into the target region of interest over the total number of sequence reads is measured following delivery of a gRNA and Cas9. In some embodiments, the invention comprises a method of increasing the efficiency of gene editing comprising, administering or delivering any one of the modified gRNAs described herein to a cell, wherein the percentage of gene editing is increased as compared to a control gRNA that is not similarly modified.

[00262] In some embodiments, the efficiency of editing with specific gRNAs is measured by the presence of insertions or deletions of nucleotides introduced by successful gene editing. In some embodiments, the invention comprises a method of creating insertions or deletions of nucleotides in genes comprising, administering or delivering any one of the modified gRNAs described herein to a cell, wherein the nucleotides are inserted or deleted as compared to a control gRNA that is not similarly modified. In some embodiments, activity of a Cas9 and gRNAs is tested in biochemical assays. In some embodiments, activity of a Cas9 and gRNAs is tested in a cell-free cleavage assay. In some embodiments, activity of a Cas9 and gRNAs is tested in Neuro2A cells.

[00263] In some embodiments, Cas 9 and sgRNA or dgRNA comprising modified crRNA and/or trRNA shows similar, greater, or reduced activity compared to the unmodified sgRNA or dgRNA comprising unmodified crRNA and trRNA. In some embodiments, Cas9 and modified sgRNA or dgRNA comprising modified crRNA and/or trRNA shows enhanced activity compared to the unmodified sgRNA or dgRNA comprising unmodified crRNA and trRNA.

2. In vivo measurement of Cas efficacy

[00264] In some embodiments, the activity of modified gRNAs is measured after in vivo dosing of LNPs comprising modified gRNAs and Cas protein or mRNA encoding Cas protein.

[00265] In some embodiments, in vivo efficacy of a gRNA or composition provided herein is determined by editing efficacy measured in DNA extracted from tissue (e.g., liver tissue) after administration of gRNA and Cas9.

3. In vivo measurement of immune system activation

[00266] Modifications to gRNA as disclosed herein may reduce the subject's immune response to in vivo dosing of gRNAs. In some embodiments, activation of the subject's immune response is measured by serum concentrations of cytokine(s) following in vivo dosing of sgRNA or dgRNA comprising trRNA and crRNA together with Cas9 mRNA or protein (e.g., formulated in a LNP). In some embodiments, the cytokine is interferon-alpha (IFN-alpha), interleukin 6 (IL-6), monocyte chemotactic protein 1 (MCP-1), and/or tumor necrosis factor alpha (TNF-alpha). In some embodiments, the invention comprises a method of reducing a subject's immune response to delivery of a gRNA comprising, administering any one of the gRNAs disclosed herein, wherein the gRNA produces a reduced response by the subject's immune system following administration. In some embodiments, the invention comprises a method of reducing activation of the subject's immune system following administration as compared to a control gRNA that is not similarly modified.

[00267] In some embodiments, administration of Cas RNP or Cas9 mRNA together with the modified gRNA (e.g., sgRNA or dgRNA) produces lower serum concentration(s) of immune cytokines compared to administration of unmodified sgRNA. In some embodiments, the invention comprises a method of reducing a subject's serum concentration of immune cytokines comprising, administering any one of the gRNAs disclosed herein, wherein the gRNA produces a lower concentration of immune cytokines in a subject's serum as compared to a control gRNA that is not similarly modified.

[00268] This description and exemplary embodiments should not be taken as limiting. For the purposes of this specification and appended claims, unless otherwise indicated, all numbers expressing quantities, percentages, or proportions, and other numerical values used in the specification and claims, are to be understood as being modified in all instances by the term "about," to the extent they are not already so modified. Accordingly, unless indicated to the contrary, the numerical parameters set forth in the following specification and attached

claims are approximations that may vary depending upon the desired properties sought to be obtained. At the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques.

[00269] It is noted that, as used in this specification and the appended claims, the singular forms "a," "an," and "the," and any singular use of any word, include plural referents unless expressly and unequivocally limited to one referent. As used herein, the term "include" and its grammatical variants are intended to be non-limiting, such that recitation of items in a list is not to the exclusion of other like items that can be substituted or added to the listed items.

EXAMPLES

[00270] The following examples are provided to illustrate certain disclosed embodiments and are not to be construed as limiting the scope of this disclosure in any way.

Example 1 – Materials and Methods

A. Synthetic guide RNA (gRNA)

[00271] gRNA in both dual (dgRNA, i.e., crRNA and trRNA) and single guide (sgRNA) format were chemically synthesized by commercial vendors with modified nucleotides and linkages as provided in Table 4.

B. In vitro transcription ("IVT") of Cas9 mRNA

[00272] Capped and polyadenylated Cas9 mRNA containing N1-methyl pseudo-U was generated by *in vitro* transcription using a linearized plasmid DNA template and T7 RNA polymerase. Plasmid DNA containing a T7 promoter and a 100 nucleotide (nt) poly(A/T) region was linearized by XbaI and obtained from a commercial manufacturer. The IVT reaction to generate Cas9 modified mRNA was incubated at 37 °C for 4 hours in the following conditions: 50 ng/μL linearized plasmid; 2 mM each of GTP, ATP, CTP, and N1-methyl pseudo-UTP (Trilink); 10 mM ARCA (Trilink); 5 U/μL T7 RNA polymerase (NEB); 1 U/μL Murine RNase inhibitor (NEB); 0.004 U/μL Inorganic *E. coli* pyrophosphatase (NEB); and 1x reaction buffer. After the 4 hr incubation, TURBO DNase (ThermoFisher) was added to a final concentration of 0.01 U/μL, and the reaction was incubated for an additional 30 minutes to remove the DNA template. The Cas9 mRNA was purified from enzyme and nucleotides using standard protocols, including silica binding columns such as a

MegaClear Transcription Clean-up kit (ThermoFisher) or precipitation steps using LiCl followed by EtOH with NaOAc. The transcript concentration was determined by measuring the light absorbance at 260 nm (Nanodrop), and the transcript was analyzed by capillary electrophoresis by Bioanlayzer (Agilent).

C. Cas9 mRNA and gRNA Transfections in Neuro2A Cells

[00273] The mouse cell line Neuro2A was cultured in DMEM media supplemented with 10% fetal bovine serum and was plated at a density of 15,000 cells/well in a 96-well plate 24 hours prior to transfection. On the day of transfection, the media was aspirated from cells and replaced with fresh media. Lipofectamine-2000 (Invitrogen) was diluted 1:50 (v/v) in Opti-MEM (Invitrogen). Cas9 mRNA and single guide RNA were diluted separately in Opti-MEM. For the dual guide format, crRNA and trRNA were diluted together in 1:1 molar ratio in Opti-MEM. Both Cas9 mRNA and gRNA were mixed separately 1:1 (v/v) with diluted Lipofectamine-2000, producing two lipoplexes. After 5 minutes of incubation, lipoplexes were added in succession to cells, for a final concentration of 100 ng Cas9 mRNA/well and 0.4 µL total lipofection reagent. Guides were tested at two dose levels for each experiment, including 25 nM and 2.5 nM, 16.7 nM and 1.67 nM, 10 nM and 1 nM, 8.3 nM and 0.83 nM, and 3 nM and 0.3 nM. For dual guide, this concentration includes equimolar amounts of crRNA and trRNA, such that, for example, 25 nM crRNA and 25 nM trRNA produce 25 nM total dual guide. Cells were lysed 24 hours post transfection, and lysates were used directly in the PCR reaction that was analyzed for editing by NGS.

[00274] Cas9 mRNA with 1xNLS (SEQ ID NO: 359):

GGGUCCCGCAGUCGGCGUCCAGCGGCUCUGCUUGUUCGUGUGUGUGUCGUUGCAGGC
CUUAUUCGGAUCCAUGGAUAAGAAGUACUCAAUCGGGCUGGAUAUCGGAACUAAUUC
CGUGGGUUGGGCAGUGAUCACGGAUGAAUACAAAGUGCCGUCCAAGAAGUUCAAGGU
CCUGGGGAACACCGAUAGACACAGCAUCAAGAAAAAUCUCAUCGGAGCCCUGCUGUU
UGACUCCGGCGAAACCGCAGAAGCGACCCGGCUCAAACGUACCGCGAGGCGACGCUA
CACCCGGCGGAAGAAUCGCAUCUGCUAUCUGCAAGAGAUCUUUUCGAACGAAAUGGC
AAAGGUCGACGACAGCUUCUUCCACCGCCUGGAAGAAUCUUUCCUGGUGGAGGAGGA
CAAGAAGCAUGAACGGCAUCCUAUCUUUGGAAACAUCGUCGACGAAGUGGCGUACCA
CGAAAAGUACCCGACCAUCUACCAUCUGCGGAAGAAGUUGGUUGACUCAACUGACAA
GGCCGACCUCAGAUUGAUCUACUUGGCCCUCGCCCAUAUGAUCAAAUUCCGCGGACA
CUUCCUGAUCGAAGGCGAUCUGAACCCUGAUAACUCCGACGUGGAUAAGCUUUUCAU
UCAACUGGUGCAGACCUACAACCAACUGUUCGAAGAAAACCCAAUCAAUGCUAGCGG
CGUCGAUGCCAAGGCCAUCCUGUCCGCCCGGCUGUCGAAGUCGCGGCGCCUCGAAAA
CCUGAUCGCACAGCUGCCGGGAGAGAAAAAGAACGGACUUUUCGGCAACUUGAUCGC
UCUCUCACUGGGACUCACUCCCAAUUUCAAGUCCAAUUUUGACCUGGCCGAGGACGC
GAAGCUGCAACUCUCAAAGGACACCUACGACGACGACUUGGACAAUUUGCUGGCACA
AAUUGGCGAUCAGUACGCGGAUCUGUUCCUUGCCGCUAAGAACCUUUCGGACGCAAU
CUUGCUGUCCGAUAUCCUGCGCGUGAACACCGAAAUAACCAAAGCGCCGCUUAGCGC
CUCGAUGAUUAAGCGGUACGACGAGCAUCACCAGGAUCUCACGCUGCUCAAAGCGCU

CGUGAGACAGCAACUGCCUGAAAAGUACAAGGAGAUCUUCUUCGACCAGUCCAAGAA
UGGGUACGCAGGGUACAUCGAUGGAGGCGCUAGCCAGGAAGAGUUCUAUAAGUUCAU
CAAGCCAAUCCUGGAAAAGAUGGACGGAACCGAAGAACUGCUGGUCAAGCUGAACAG
GGAGGAUCUGCUCCGGAAACAGAGAACCUUUGACAACGGAUCCAUUCCCCACCAGAU
CCAUCUGGGUGAGCUGCACGCCAUCUUGCGGCGCCAGGAGGACUUUUACCCAUUCCU
CAAGGACAACCGGGAAAAGAUCGAGAAAAUUCUGACGUUCCGCAUCCCGUAUUACGU
GGGCCCACUGGCGCGCGGCAAUUCGCGCUUCGCGUGGAUGACUAGAAAAUCAGAGGA
AACCAUCACUCCUUGGAAUUUCGAGGAAGUUGUGGAUAAGGGAGCUUCGGCACAAAG
CUUCAUCGAACGAAUGACCAACUUCGACAAGAAUCUCCCAAACGAGAAGGUGCUUCC
UAAGCACAGCCUCCUUUACGAAUACUUCACUGUCUACAACGAACUGACUAAAGUGAA
AUACGUUACUGAAGGAAUGAGGAAGCCGGCCUUUCUGUCCGGAGAACAGAAGAAAGC
AAUUGUCGAUCUGCUGUUCAAGACCAACCGCAAGGUGACCGUCAAGCAGCUUAAAGA
GGACUACUUCAAGAAGAUCGAGUGUUUCGACUCAGUGGAAAUCAGCGGGGUGGAGGA
CAGAUUCAACGCUUCGCUGGGAACCUAUCAUGAUCUCCUGAAGAUCAUCAAGGACAA
GGACUUCCUUGACAACGAGGAGAACGAGGACAUCCUGGAAGAUAUCGUCCUGACCUU
GACCCUUUUCGAGGAUCGCGAGAUGAUCGAGGAGAGGCUUAAGACCUACGCUCAUCU
CUUCGACGAUAAGGUCAUGAAACAACUCAAGCGCCGCCGGUACACUGGUUGGGGCCG
CCUCUCCCGCAAGCUGAUCAACGGUAUUCGCGAUAAACAGAGCGGUAAAACUAUCCU
GGAUUUCCUCAAAUCGGAUGGCUUCGCUAAUCGUAACUUCAUGCAAUUGAUCCACGA
CGACAGCCUGACCUUUAAGGAGGACAUCCAAAAAGCACAAGUGUCCGGACAGGGAGA
CUCACUCCAUGAACACAUCGCGAAUCUGGCCGGUUCGCCGGCGAUUAAGAAGGGAAU
UCUGCAAACUGUGAAGGUGGUCGACGAGCUGGUGAAGGUCAUGGGACGGCACAAACC
GGAGAAUAUCGUGAUUGAAAUGGCCCGAGAAAACCAGACUACCCAGAAGGGCCAGAA
AAACUCCCGCGAAAGGAUGAAGCGGAUCGAAGAAGGAAUCAAGGAGCUGGGCAGCCA
GAUCCUGAAAGAGCACCCGGUGGAAAACACGCAGCUGCAGAACGAGAAGCUCUACCU
GUACUAUUUGCAAAAUGGACGGGACAUGUACGUGGACCAAGAGCUGGACAUCAAUCG
GUUGUCUGAUUACGACGUGGACCACAUCGUUCCACAGUCCUUUCUGAAGGAUGACUC
GAUCGAUAACAAGGUGUUGACUCGCAGCGACAAGAACAGAGGGAAGUCAGAUAAUGU
GCCAUCGGAGGAGGUCGUGAAGAAGAUGAAGAAUUACUGGCGGCAGCUCCUGAAUGC
GAAGCUGAUUACCCAGAGAAAGUUUGACAAUCUCACUAAAGCCGAGCGCGGCGGACU
CUCAGAGCUGGAUAAGGCUGGAUUCAUCAAACGGCAGCUGGUCGAGACUCGGCAGAU
UACCAAGCACGUGGCGCAGAUCUUGGACUCCCGCAUGAACACUAAAUACGACGAGAA
CGAUAAGCUCAUCCGGGAAGUGAAGGUGAUUACCCUGAAAAGCAAACUUGUGUCGGA
CUUUCGGAAGGACUUUCAGUUUUACAAAGUGAGAGAAAUCAACAACUACCAUCACGC
GCAUGACGCAUACCUCAACGCUGUGGUCGGUACCGCCCUGAUCAAAAAGUACCCUAA
ACUUGAAUCGGAGUUUGUGUACGGAGACUACAAGGUCUACGACGUGAGGAAGAUGAU
AGCCAAGUCCGAACAGGAAAUCGGGAAAGCAACUGCGAAAUACUUCUUUUACUCAAA
CAUCAUGAACUUUUUCAAGACUGAAAUUACGCUGGCCAAUGGAGAAAUCAGGAAGAG
GCCACUGAUCGAAACUAACGGAGAAACGGGCGAAAUCGUGUGGGACAAGGGCAGGGA
CUUCGCAACUGUUCGCAAAGUGCUCUCUAUGCCGCAAGUCAAUAUUGUGAAGAAAAC
CGAAGUGCAAACCGGCGGAUUUUCAAAGGAAUCGAUCCUCCCAAAGAGAAAUAGCGA
CAAGCUCAUUGCACGCAAGAAAGACUGGGACCCGAAGAAGUACGGAGGAUUCGAUUC
GCCGACUGUCGCAUACUCCGUCCUCGUGGUGGCCAAGGUGGAGAAGGGAAAGAGCAA
AAAGCUCAAAUCCGUCAAAGAGCUGCUGGGGAUUACCAUCAUGGAACGAUCCUCGUU
CGAGAAGAACCCGAUUGAUUUCCUCGAGGCGAAGGGUUACAAGGAGGUGAAGAAGGA
UCUGAUCAUCAAACUCCCCAAGUACUCACUGUUCGAACUGGAAAAUGGUCGGAAGCG
CAUGCUGGCUUCGGCCGGAGAACUCCAAAAAGGAAAUGAGCUGGCCUUGCCUAGCAA
GUACGUCAACUUCCUCUAUCUUGCUUCGCACUACGAAAAACUCAAAGGGUCACCGGA
AGAUAACGAACAGAAGCAGCUUUUCGUGGAGCAGCACAAGCAUUAUCUGGAUGAAAU
CAUCGAACAAAUCUCCGAGUUUUCAAAGCGCGUGAUCCUCGCCGACGCCAACCUCGA
CAAAGUCCUGUCGGCCUACAAUAAGCAUAGAGAUAAGCCGAUCAGAGAACAGGCCGA
GAACAUUAUCCACUUGUUCACCCUGACUAACCUGGGAGCCCCAGCCGCCUUCAAGUA
CUUCGAUACUACUAUCGAUCGCAAAAGAUACACGUCCACCAAGGAAGUUCUGGACGC
GACCCUGAUCCACCAAAGCAUCACUGGACUCUACGAAACUAGGAUCGAUCUGUCGCA
GCUGGGUGGCGAUGGCGGUGGAUCUCCGAAAAAGAAGAGAAAGGUGUAAUGAGCUAG
CCAUCACAUUUAAAAGCAUCUCAGCCUACCAUGAGAAUAAGAGAAAGAAAAUGAAGA

UCAAUAGCUUAUUCAUCUCUUUUUCUUUUUCGUUGGUGUAAAGCCAACACCCUGUCU
AAAAAACAUAAAAUUUCUUUAAUCAUUUUGCCUCUUUUCUCUGUGCUUCAAUUAAUAA
AAAAUGGAAAGAACCUCGAGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAUCUAG

[00275] Cas9 mRNA with 2xNLS and HA tag (SEQ ID NO: 360):

GGGUCCCGCAGUCGGCGUCCAGCGGCUCUGCUUGUUCGUGUGUGUGUCGUUGCAGGC
CUUAUUCGGAUCCAUGGAUAAGAAGUACUCAAUCGGGCUGGAUAUCGGAACUAAUUC
CGUGGGUUGGGCAGUGAUCACGGAUGAAUACAAAGUGCCGUCCAAGAAGUUCAAGGU
CCUGGGGAACACCGAUAGACACAGCAUCAAGAAAAAUCUCAUCGGAGCCCUGCUGUU
UGACUCCGGCGAAACCGCAGAAGCGACCCGGCUCAAACGUACCGCGAGGCGACGCUA
CACCCGGCGGAAGAAUCGCAUCUGCUAUCUGCAAGAGAUCUUUUCGAACGAAAUGGC
AAAGGUCGACGACAGCUUCUUCCACCGCCUGGAAGAAUCUUUCCUGGUGGAGGAGGA
CAAGAAGCAUGAACGGCAUCCUAUCUUUGGAAACAUCGUCGACGAAGUGGCGUACCA
CGAAAAGUACCCGACCAUCUACCAUCUGCGGAAGAAGUUGGUUGACUCAACUGACAA
GGCCGACCUCAGAUUGAUCUACUUGGCCCUCGCCCAUAUGAUCAAAUUCCGCGGACA
CUUCCUGAUCGAAGGCGAUCUGAACCCUGAUAACUCCGACGUGGAUAAGCUUUUCAU
UCAACUGGUGCAGACCUACAACCAACUGUUCGAAGAAAACCCAAUCAAUGCUAGCGG
CGUCGAUGCCAAGGCCAUCCUGUCCGCCCGGCUGUCGAAGUCGCGGCGCCUCGAAAA
CCUGAUCGCACAGCUGCCGGGAGAGAAAAAGAACGGACUUUUCGGCAACUUGAUCGC
UCUCUCACUGGGACUCACUCCCAAUUUCAAGUCCAAUUUUGACCUGGCCGAGGACGC
GAAGCUGCAACUCUCAAAGGACACCUACGACGACGACUUGGACAAUUUGCUGGCACA
AAUUGGCGAUCAGUACGCGGAUCUGUUCCUUGCCGCUAAGAACCUUUCGGACGCAAU
CUUGCUGUCCGAUAUCCUGCGCGUGAACACCGAAAUAACCAAAGCGCCGCUUAGCGC
CUCGAUGAUUAAGCGGUACGACGAGCAUCACCAGGAUCUCACGCUGCUCAAAGCGCU
CGUGAGACAGCAACUGCCUGAAAAGUACAAGGAGAUCUUCUUCGACCAGUCCAAGAA
UGGGUACGCAGGGUACAUCGAUGGAGGCGCUAGCCAGGAAGAGUUCUAUAAGUUCAU
CAAGCCAAUCCUGGAAAAGAUGGACGGAACCGAAGAACUGCUGGUCAAGCUGAACAG
GGAGGAUCUGCUCCGGAAACAGAGAACCUUUGACAACGGAUCCAUUCCCCACCAGAU
CCAUCUGGGUGAGCUGCACGCCAUCUUGCGGCGCCAGGAGGACUUUUACCCAUUCCU
CAAGGACAACCGGGAAAAGAUCGAGAAAAUUCUGACGUUCCGCAUCCCGUAUUACGU
GGGCCCACUGGCGCGCGGCAAUUCGCGCUUCGCGUGGAUGACUAGAAAAUCAGAGGA
AACCAUCACUCCUUGGAAUUUCGAGGAAGUUGUGGAUAAGGGAGCUUCGGCACAAAG
CUUCAUCGAACGAAUGACCAACUUCGACAAGAAUCUCCCAAACGAGAAGGUGCUUCC
UAAGCACAGCCUCCUUUACGAAUACUUCACUGUCUACAACGAACUGACUAAAGUGAA
AUACGUUACUGAAGGAAUGAGGAAGCCGGCCUUUCUGUCCGGAGAACAGAAGAAAGC
AAUUGUCGAUCUGCUGUUCAAGACCAACCGCAAGGUGACCGUCAAGCAGCUUAAAGA
GGACUACUUCAAGAAGAUCGAGUGUUUCGACUCAGUGGAAAUCAGCGGGGUGGAGGA
CAGAUUCAACGCUUCGCUGGGAACCUAUCAUGAUCUCCUGAAGAUCAUCAAGGACAA
GGACUUCCUUGACAACGAGGAGAACGAGGACAUCCUGGAAGAUAUCGUCCUGACCUU
GACCCUUUUCGAGGAUCGCGAGAUGAUCGAGGAGAGGCUUAAGACCUACGCUCAUCU
CUUCGACGAUAAGGUCAUGAAACAACUCAAGCGCCGCCGGUACACUGGUUGGGGCCG
CCUCUCCCGCAAGCUGAUCAACGGUAUUCGCGAUAAACAGAGCGGUAAAACUAUCCU
GGAUUUCCUCAAAUCGGAUGGCUUCGCUAAUCGUAACUUCAUGCAAUUGAUCCACGA
CGACAGCCUGACCUUUAAGGAGGACAUCCAAAAAGCACAAGUGUCCGGACAGGGAGA
CUCACUCCAUGAACACAUCGCGAAUCUGGCCGGUUCGCCGGCGAUUAAGAAGGGAAU
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D. Primary liver hepatocytes

[00276] Primary mouse liver hepatocytes (PMH) (Gibco) were cultured per the manufacturer's protocol (Invitrogen, protocol 11.28.2012). In brief, the cells were thawed and resuspended in hepatocyte thawing medium with supplements (Gibco, Cat. CM7000) followed by centrifugation at 100 g for 10 minutes. The supernatant was discarded and the pelleted cells resuspended in hepatocyte plating medium plus supplement pack (Invitrogen, Cat. A1217601 and CM3000). Cells were counted and plated on Bio-coat collagen I coated 96-well plates (ThermoFisher, Cat. 877272) at a density of 15,000 cells/well and incubated for 5 hours at 37°C and 5% CO2 atmosphere to allow for monolayer formation. After 5 hours, the plating media was removed and replaced with supplemented hepatocyte culture medium (Invitrogen, Cat. A1217601 and CM4000) containing LNP formulated Cas9 mRNA and guide RNA plus

3% mouse serum. LNPs were diluted from a starting dose level of 100ng Cas9 mRNA and approximately 30nM guide RNA per well, carrying out serial dilutions down to 0.1ng mRNA and 0.03nM guide per well. Cells were incubated for approximately 48 hours at 37°C and 5% CO_2 atmosphere before cell lysis and NGS analysis as described herein.

E. Lipid Nanoparticle ("LNP") Formulation

[00277] LNPs were formulated with a cationic lipid amine to RNA phosphate (N:P) molar ratio of about 4.5. The lipid nanoparticle components were dissolved in 100% ethanol with the following molar ratios: 45 mol-% (12.7 mM) cationic lipid (*e.g.*, (9Z,12Z)-3-((4,4-bis(octyloxy)butanoyl)oxy)-2-((((3-(diethylamino)propoxy)carbonyl)oxy)methyl)propyl octadeca-9,12-dienoate, also called 3-((4,4-bis(octyloxy)butanoyl)oxy)-2-((((3-(diethylamino)propoxy)carbonyl)oxy)methyl)propyl (9Z,12Z)-octadeca-9,12-dienoate); 44 mol-% (12.4 mM) helper lipid (*e.g.*, cholesterol); 9 mol-% (2.53 mM) neutral lipid (*e.g.*, DSPC); and 2 mol-% (.563 mM) PEG (*e.g.*, PEG2k-DMG). The RNA cargo were prepared in 25 mM sodium acetate buffer, pH 4.5, resulting in a concentration of RNA cargo of approximately 0.45 mg/mL.

[00278] The LNPs were formed by microfluidic mixing of the lipid and RNA solutions using a Precision Nanosystems NanoAssemblr™ Benchtop Instrument, according to the manufacturer's protocol. A 2:1 ratio of aqueous to organic solvent was maintained during mixing using differential flow rates.

[00279] LNP Formulation Procedure A: After mixing, the LNPs were collected, diluted in phosphate buffered saline (PBS, approximately 1:1), and then remaining buffer was exchanged into PBS (100-fold excess of sample volume), overnight at 4°C under gentle stirring using a 10 kDa Slide-a-LyzerTM G2 Dialysis Cassette (ThermoFisher Scientific). The LNPs were concentrated using 10kDa Amicon spin filter (centrifugation at 4000g at 4°C) to achieve the desired concentration. The resulting mixture was then filtered using a 0.2 μm sterile filter. The resulting filtrate was stored at 2-8 °C.

[00280] LNP Formulation Procedure B: After mixing, the LNPs were collected, diluted in 50mM Tris at pH 7.5 (approximately 1:1), and then LNPs were exchanged into 50mM Tris at pH 7.5 (100-fold excess of sample volume), overnight at 4°C under gentle stirring using a 10 kDa Slide-a-LyzerTM G2 Dialysis Cassette (ThermoFisher Scientific). The LNPs were concentrated using 10kDa Amicon spin filter (centrifugation at 4000g at 4°C) to achieve twice the desired concentration. These concentrated LNPs were mixed 1:1 with 50mM Tris, 90mM

NaCl, 10% sucrose at pH 7.5 (2X TSS). The resulting mixture was then filtered using a 0.2 μM sterile filter. The resulting filtrate was stored at -80°C.

[00281] LNP Formulation Procedure C: The RNA cargo were prepared in 25mM sodium citrate, 100mM sodium chloride at pH 5 resulting in a concentration of RNA cargo of approximately 0.45 mg/mL. After mixing, the LNPs were collected in water at the ratio of 3:1. The LNPs were incubated for an hour at room temperature and mixed 1:1 with water. Then they were buffer-exchanged into 1X TSS (50mM Tris, 45mM NaCl, 5% sucrose at pH 7.5) on PD-10 columns (GE Healthcare), using manufacturer's protocol. The LNPs were concentrated using 10kDa Amicon spin filter (centrifugation at 4000g at 4°C) to achieve the desired concentration. The resulting mixture was then filtered using a 0.2 μm sterile filter. The resulting filtrate was stored at -80°C.

F. Next-Generation Sequencing ("NGS") and Analysis for On-Target Cleavage Efficiency

[00282] To quantitatively determine the efficiency of editing at the target location in the genome, deep sequencing was utilized to identify the presence of insertions and deletions introduced by gene editing.

[00283] PCR primers were designed around the target site (*e.g.*, TTR, FVII), and the genomic area of interest was amplified. Primer sequences are provided below in Table 5.

Table 5

Guide	Gene	Forward Primer (5'-3')	SEQ ID	Reverse Primer (5'-3')	SEQ ID
For experiments with guides based on CR000686/G000209 targeting domains	TTR	AGTCAATAATCAGAATCAGCAGGT	333	AGAAGGCACTTCTTCTTTATCTAAGGT	337
For experiments with guides based on CR000705/G000211 targeting domains	TTR	GTTTTGTTCCAGAGTCTATCACCG	334	ACACGAATAAGAGCAAATGGGAAC	338
For experiments with guides based on G000269/ G000285 targeting domains	TTR	ATTACCAGCTTAGCATCCTGTGAA	335	ACACGGTTTATAGAGCAAGAACAC	339
For experiments with guides based on CR000657/G000208 targeting domains	FVII	AGCACATGAGACCTTCTGTTTCTC	336	GACATAGGTGTGACCCTCACAATC	340

Additional PCR was performed according to the manufacturer's protocols (Illumina) to add the necessary chemistry for sequencing. The amplicons were sequenced on an Illumina MiSeq instrument. The reads were aligned to the human reference genome (*e.g.*, hg38) after eliminating those having low quality scores. The resulting files containing the reads were mapped to the reference genome (BAM files), where reads that overlapped the target region

of interest were selected and the number of wild type reads versus the number of reads which contain an insertion, substitution, or deletion was calculated.

[00284] The editing percentage (*e.g.*, the "editing efficiency" or "percent editing") is defined as the total number of sequence reads with insertions or deletions over the total number of sequence reads, including wild type.

G. LNP Delivery in vivo

[00285] CD-1 female mice, ranging 6-10 weeks of age were used in each study. Animals were weighed and grouped according to body weight for preparing dosing solutions based on group average weight. LNPs were dosed via the lateral tail vein in a volume of 0.2 mL per animal (approximately 10 mL per kilogram body weight). The animals were observed at approximately 6 hours post dose for adverse effects. Body weight was measured at twenty-four hours post-administration, and animals were euthanized at various time points by exsanguination via cardiac puncture under isoflourane anesthesia. Blood was collected into serum separator tubes or into tubes containing buffered sodium citrate for plasma as described herein. For studies involving *in vivo* editing, liver tissue was collected from the median lobe from each animal for DNA extraction and analysis.

H. Cytokine induction analysis

[00286] For this analysis, approximately 50-100 μL of blood was collected by tail vein nick for serum cytokine measurements. Blood was allowed to clot at room temperature for approximately 2 hours, and then centrifuged at 1000xg for 10 minutes before collecting the serum. A Luminex based magnetic bead multiplex assay (Affymetrix ProcartaPlus, catalog number Exp040-00000-801) measuring IL-6, TNF-alpha, IFN-alpha, and MCP-1 was used for cytokine analysis in collected in samples. Kit reagents and standards were prepared as directed in the manufacturer's protocol. 25 μL of mouse serum was added to wells containing 25 μL of the diluted antibody coated magnetic beads. The plate was incubated for 2 hours at room temperature and then washed. Diluted biotin antibody (50 μL) was added to the beads and incubated for 1 hour at room temperature. The beads were washed again before adding 50 μL of diluted streptavidin-PE to each well, followed by incubation for 30 minutes. The beads were washed once again and then suspended in 100 μL of wash buffer and read on the Bio-Plex 200 instrument (Bio-Rad). The data was analyzed using Bioplex Manager ver. 6.1 analysis package with cytokine concentrations calculated off a standard curve using a five parameter logistic curve fit.

I. Genomic DNA Isolation

[00287] For the *in vivo* studies, genomic DNA was extracted from 10 mg of tissue using a bead based extraction kit, MagMAX-96 DNA Multi-Sample Kit (ThermoFisher, Cat #4413020) according to manufacturer's protocol, which includes homogenizing the tissue in lysis buffer (approximately 400 µL/10 mg tissue). All DNA samples were normalized to 100 ng/µL concentration for PCR and subsequent NGS analysis, as described herein.

J. Transthyretin (TTR) ELISA analysis

[00288] Blood was collected and the serum was isolated as indicated. The total TTR serum levels were determined using a Mouse Prealbumin (Transthyretin) ELISA Kit (Aviva Systems Biology, Cat. OKIA00111). Kit reagents and standards were prepared according to the manufacture's protocol. Mouse serum was diluted to a final dilution of 10,000-fold with 1x assay diluent. This was done by carrying out two sequential 50-fold dilutions resulting in a 2500-fold dilution. A final 4-fold dilution step was carried out for a total sample dilution of 10,000-fold. Both standard curve dilutions (100 µL each) and diluted serum samples were added to each well of the ELISA plate pre-coated with capture antibody. The plate was incubated at room temperature for 30 minutes before washing. Enzyme-antibody conjugate (100 µL per well) was added for a 20-minute incubation. Unbound antibody conjugate was removed and the plate was washed again before the addition of the chromogenic substrate solution. The plate was incubated for 10 minutes before adding 100 µL of the stop solution, *e.g.*, sulfuric acid (approximately 0.3 M). The plate was read on a SpectraMax M5 plate reader at an absorbance of 450 nm. Serum TTR levels were calculated by SoftMax Pro software ver. 6.4.2 using a four parameter logistic curve fit off the standard curve. Final serum values were adjusted for the assay dilution.

Example 2 – Engineering modified gRNA and in vitro testing

[00289] Modified gRNAs were designed in the dual guide format (dgRNA), as shown in Table 4. Accordingly, both modified crRNAs and trRNAs were designed and chemically synthesized to allow for the pairing of modified and unmodified components forming dgRNA. These pairings were transfected into Neuro2A cells at concentrations as indicated in the figures and editing efficiency (e.g., percent editing) was measured by NGS, as described in Example 1.

[00290] Certain modified crRNAs from Table 4 targeting the mouse TTR gene were transfected with Cas9 mRNA and unmodified trRNA (TR000002). Tested guides included

SEQ ID Nos: 1- 18. As shown in Figure 1, some of the modified crRNAs (together with unmodified trRNA) conferred similar or enhanced activity as compared to the unmodified control, while other modified crRNAs decreased activity.

[00291] In parallel, modified trRNAs from Table 4 were transfected with Cas9 mRNA along with an unmodified crRNA (CR000686) targeting the same sequence of the mouse TTR gene. Tested guides included SEQ ID Nos: 188 - 200, and 204. As shown in Figure 2, many of the modified trRNAs (together with unmodified crRNA) conferred similar or enhanced activity as compared to the unmodified control, while some of the modified trRNAs decreased activity.

[00292] In addition to substituting chemically modified nucleotides, some of the crRNA and trRNA pairings tested were also engineered with sequence substitutions, e.g., resulting in G-C pairings not found in the parental sequences. Tested guides included SEQ ID Nos: 15 and 201; 16 and 202; 1 and 188. As shown in Figure 3, one such pairing (SEQ ID Nos: 16 and 202) resulted in similar or enhanced activity as compared to the unmodified control, while two of the pairings decreased activity.

[00293] Next, pairings of modified crRNAs and modified trRNAs from Table 4 were tested. As shown in Figure 4, some of the pairings of modified crRNA with modified trRNA conferred similar or enhanced activity as compared to the unmodified controls, while some of the pairings decreased activity. In Figure 4, the column headings depict different trRNA used in the experiment, and the row headings depict different crRNA used. To determine the combination used in the experiment, you match column to row. TR000002 and CR000686 are the unmodified controls (see lower right cells).

[00294] Based on the dgRNA designs, corresponding single guide RNAs (sgRNAs) were engineered featuring aspects of some of the modified crRNAs and trRNAs, as depicted in Table 4 and Figure 15D. These sgRNAs, SEQ ID Nos: 228 - 234, were also tested in Neuro2A cells, and as shown in Figure 5, each of the modified sgRNAs displayed activities comparable to the controls containing only 5' and 3' end modifications (G0000209; SEQ ID NO: 228).

[00295] A similar set of experiments were conducted for additional dgRNAs guides depicted in Table 4 and Figure 6. Tested guides included SEQ ID Nos: 32 – 47, and 1. Modified crRNAs also targeting the mouse TTR gene were transfected with Cas9 mRNA and unmodified trRNA (TR000002). As shown in Figure 6, some of the modified crRNAs (together with unmodified trRNA) conferred similar or enhanced activity as compared to the unmodified control (CR000686), while other modified crRNAs decreased activity.

[00296] In parallel, as shown in Figure 7, modified trRNAs from Table 4 were transfected with Cas9 mRNA along with an unmodified crRNA (CR000686) targeting the same sequence of the mouse TTR gene. Tested guides included SEQ ID Nos: 205 - 222, and 1. As shown in Figure 7, many of the modified trRNAs (together with unmodified crRNA) conferred similar or enhanced activity as compared to the unmodified control (TR000002), while some of the modified trRNAs decreased activity.

[00297] In addition to substituting chemically modified nucleotides, some of the crRNA and trRNA pairings tested from Table 4 were also engineered with sequence substitutions, e.g., resulting in G-C pairings or G-U mismatches ("GU wobbles") not found in the parental sequences. As shown in Figure 8, some of the modifications and pairings conferred similar or enhanced activity as compared to the unmodified control, while some (e.g., the "GU wobble" or mismatch pairings) decreased activity. Figure 8 shows results using trRNA guides shown in SEQ ID Nos: 223-227 and 188 with crRNA guides shown in SEQ ID Nos: 48-52, and 1.

[00298] Next, select pairings of the modified crRNAs and modified trRNAs from Table 4 were tested as shown in Figure 9. Some of the pairings of modified crRNA with modified trRNA conferred similar or enhanced activity as compared to the unmodified controls, while some of the pairings decreased activity. In Figure 9, the column headings depict different trRNA used in the experiment, and the row headings depict different crRNA used. To determine the combination used in the experiment, you match column to row. Unmodified controls are TR000002, and CR000686.

[00299] Some of the modified gRNAs (dgRNAs and sgRNAs) from Table 4 were also tested in a purely biochemical assay (i.e., cell free cleavage assay). Interestingly, many of the modified gRNAs that were largely inactive in the Neuro2A cells were active in the biochemical assay, indicating that such biochemical assays may not be predictive of modified gRNA activity in cells (data not shown).

Example 3. Further testing of modified gRNAs to other targets

[00300] Having established that certain modifications affected gRNA activity, it was next tested whether these modifications would affect the activity when targeting (1) a separate sequence in the same gene or (2) a sequence in a different gene. Accordingly, gRNAs targeting another sequence in the mouse TTR gene as well as a sequence in the mouse Factor-VII (FVII) gene were engineered and synthesized having certain modification patterns tested in Example 2 (see Table 4). These gRNAs were transfected into Neuro2A

cells at the concentrations indicated in the figures and editing efficiency (e.g., percent editing) was measured by NGS, as described in Example 1.

[00301] Modified crRNAs from Table 4 targeting either the mouse TTR gene (different sequence as targeted in Example 2) or the mouse FVII gene, were transfected with Cas9 mRNA and unmodified trRNA (TR000002). Tested guides included those shown in Figures 12A and 12B. Some of the modified crRNAs (together with unmodified trRNA) conferred similar or enhanced activity as compared to the unmodified controls, while other modified crRNAs decreased activity.

[00302] In parallel, modified trRNAs from Table 4 were transfected with Cas9 mRNA along with an unmodified crRNA targeting the same sequence of the mouse TTR gene (CR000705; different sequence as targeted in Example 2) or the same sequence as the mouse FVII gene (CR000657). As shown in Figures 13A and 13B, many of the modified trRNAs (together with unmodified crRNAs) conferred similar or enhanced activity as compared to the unmodified controls, while some of the modified trRNAs decreased activity. This data shows that certain modification patterns tended to have similar effects over the different sequences.

[00303] Based on the dgRNA designs described above, corresponding single guide RNAs (sgRNAs) were engineered featuring aspects of some of the modified crRNAs and trRNAs. *See*, Table 4. These sgRNAs were also tested in Neuro2A cells. Results are shown in Figure 10 (mouse TTR) and Figure 11 (mouse FVII). These experiments show that some modification patterns result in similar effects even when targeting different genes.

Example 4. Testing of modified gRNA *in vivo*

[00304] Following the *in vitro* testing, modified sgRNAs were delivered to animals in six separate studies in order to determine whether the modifications conferred any benefits for editing *in vivo*.

[00305] LNPs were formulated with IVT Cas9 mRNA together with chemically modified sgRNA (targeting TTR or FVII), as described in Example 1. The ratio of mRNA:sgRNA was approximately 1:1, by weight of the RNA components. Unless otherwise indicated, the Cas9 mRNA used in the studies described in this example had the sequence of SEQ ID NO: 360 and the LNPs were formulated using LNP Formulation Procedure A described above.

[00306] In one experiment, mice (n=5 per group) were administered a single dose of LNP at 2mg/kg and blood was collected four hours post dose for serum cytokine analysis. 7 days post dose at necropsy, livers and blood were collected for NGS measurements of editing

efficiency and serum TTR analysis, respectively. Each of the sgRNAs in this experiment targeted the same sequence in the TTR gene, the only difference between the sgRNAs being the modifications made to each (See Figures 14A-D and 15A-E; Table 4 SEQ ID Nos: 228 - 234). G000209 (two lots tested) served as the less modified control, having only 2′-O-methyl modifications and phosphorothioate linkages at and between the three terminal nucleotides at both the 5′ and 3′ termini of the sgRNA, respectively. (See Figure 15D).

[00307] The results shown in Figures 14A-D, show that the more heavily modified sgRNAs tended to induce less of a response for each the cytokines assayed, as compared to the less modified G000209 controls. The more heavily modified sgRNAs also conferred larger editing efficiencies in the livers of treated animals, with percent editing reaching ~60% for two of the more heavily modified sgRNAs (e.g., G000263 and G000267) as compared to ~44-47% for the less modified controls (G000209 lots) (Figure 15A). Importantly, the editing efficiencies correlated with phenotypic changes as serum knockdown of TTR levels were comparable or significantly greater than the less modified controls (See e.g., G000263 and G000267 vs G000209 lots in Figures 15A-15B). The differences between the end-modified G000209 and highly-modified G000267 are summarized in Figure 15D and 15E (2'-O-Me modified nucleotides are shown in bold, and * represents phosphorothioate linkages).

[00308] In another *in vivo* study, three sgRNAs targeting a separate sequence in the mouse TTR gene were tested. Mice (n=5 per group) were administered a single dose of LNP at 2mg/kg, 1mg/kg, or 0.3mg/kg. Blood was collected four hours post dose for serum cytokine analysis. 7 days post dose at necropsy, livers and blood were collected for NGS measurements of editing efficiency and serum TTR analysis, respectively. In this study, each of the sgRNAs targeted the same sequence in the TTR gene (a different sequence from what was targeted in the previous *in vivo* study) with one sgRNA being completely unmodified (G000201 (SEQ ID NO: 243)), another having only end modifications (G000211 (SEQ ID NO: 241)), with 2′-O-methyl modifications and phosphorothioate linkages at and between the three terminal nucleotides at both the 5′ and 3′ termini of the sgRNA, respectively), and a third sgRNA having the same modification pattern as G000267 in the previous *in vivo* study (G000282 (SEQ ID NO: 242)).

[00309] As shown in Figures 16A-16D, each of the sgRNAs resulted in similar responses in a dose dependent manner for each of the cytokines tested. For editing efficiency, the unmodified sgRNA (G000201(SEQ ID NO: 243)) conferred little *in vivo* editing, while the heavily modified sgRNA (G000282 (SEQ ID NO: 242)) conferred levels

reaching ~60% with a dose of 2mg/kg, which was significantly greater than the levels achieved with the less modified sgRNA (G000211 (SEQ ID NO: 241)) (Figure 17A and B). As with the previous *in vivo* study, the levels of editing correlated with the amount of serum TTR knockdown (Figure 17C and D).

[00310] A similar study as the second *in vivo study* was next conducted with another set of three sgRNAs targeting yet a different TTR sequence in the mouse TTR gene (targeting a different sequence then what was targeted in the two previous in vivo studies). Mice (n=5 per group) were administered a single dose of LNP at 2mg/kg, 1mg/kg, or 0.3mg/kg. Blood was collected four hours post dose for serum cytokine analysis. 7 days post dose at necropsy, livers and blood were collected for NGS measurements of editing efficiency and serum TTR analysis, respectively. In this study, each of the sgRNAs targeted the same sequence in the TTR gene (a different sequence from what was targeted in the previous two *in vivo* studies) with one sgRNA being completely unmodified (G000285; (SEQ ID NO: 332)), another having only end modifications (G000269 (SEQ ID NO: 330)), with 2′-O-methyl modifications and phosphorothioate linkages at and between the three terminal nucleotides at both the 5′ and 3′ ends of the sgRNA, respectively), and a third sgRNA having the same modification pattern as G000267 and G000282 in the previous two *in vivo* studies (G000283 (SEQ ID NO: 331)).

[00311] In this study, the unmodified sgRNA (G000285 (SEQ ID NO: 332)) conferred little *in vivo* editing, while the heavily modified sgRNA (G000283 (SEQ ID NO: 331)) conferred levels reaching ~60% with a dose of 2mg/kg, which was significantly greater than the levels achieved with the less modified sgRNA (G000269 (SEQ ID NO: 330)) (Figures 18A-18B). As with the previous *in vivo* studies, the levels of editing correlated with the amount of serum TTR knockdown (Figure 18C).

[00312] In a fourth *in vivo* study, the effects of modifications to gRNAs was evaluated for another gene (FVII). For in-study comparison, two of the sgRNAs tested in the first *in vivo* study were included (G000209 and G000267). Mice (n=5 per group) were administered a single dose of LNP at 2mg/kg, 1mg/kg, or 0.3mg/kg, and blood was collected four hours post dose for serum cytokine analysis. 6 days post dose at necropsy, livers were collected for NGS measurements of editing efficiency. In this study, each of the sgRNAs targeted the same sequence in the TTR or FVII genes, with one sgRNA for each having only end modifications (G000208 (SEQ ID NO: 286)) for FVII, G000209 for TTR, both having 2′-O-methyl modifications and phosphorothioate linkages at and between the three terminal

nucleotides at both the 5′ and 3′ ends of the sgRNA, respectively), and a second sgRNA having the same modification patterns as G000267, G000282, and G000283 in the previous *in vivo* studies (G000373 (SEQ ID NO: 287) for FVII; G000267 (SEQ ID NO: 234) for TTR).

[00313] As shown in Figures 19A-19D, each of the sgRNAs resulted in similar responses in a dose dependent manner for each of the cytokines tested. For editing efficiency, the more heavily modified sgRNA targeting FVII (G000373 (SEQ ID NO: 287)) had an increase in editing efficiency as compared to the less modified version (G000208 (SEQ ID NO: 286)) across each of the doses tested (Figure 18A). These results were also observed for the sgRNAs targeting TTR (Figures 20A-20B).

[00314] In another *in vivo* study, ten additional sgRNAs targeting the same sequence in the mouse TTR gene as G000282 were tested. G000282 was also included in the study for comparative purposes. Mice (n=5 per group) were administered a single dose of LNP at 1mg/kg or 0.5mg/kg. The LNPs used in this study were formulated using LNP Formulation Procedure B described above. Seven (7) days post dose at necropsy, livers and blood were collected for NGS measurements of editing efficiency and serum TTR analysis, respectively. In this study, each of the sgRNAs targeted the same sequence in the TTR gene. The modification pattern of each sgRNA tested varied and included 2'-OMe, 2'-F, and PS modifications in the 5' terminus, 3' terminus, hairpin 1, hairpin 2, nexus, lower stem, bulge, and upper stem of the sgRNA. The results of this study are shown in Figures 22A-22C, including % editing (Figure 22A), average editing and standard deviation (Figure 22B), and serum TTR levels (Figure 22C). These same sgRNAs were tested in primary mouse hepatocytes as per the methods described herein. The results of this dose response TTR editing study are shown in Figures 24A-24C, including % editing (Figure 24A), dose response curves (Figure 24B), and EC50 values (Figure 24C).

[00315] In another *in vivo* study, thirteen sgRNAs targeting the same sequence in the mouse TTR gene as G000282 were tested. G000282 was also included in the study for comparative purposes. Mice (n=5 per group) were administered a single dose of LNP at 1mg/kg. The LNPs used in this study were formulated using LNP Formulation Procedure C described above. The Cas9 mRNA used in this study had the sequence of SEQ ID NO: 359. Blood was collected four hours post dose for serum cytokine analysis. 7 days post dose at necropsy, livers and blood were collected for NGS measurements of editing efficiency and serum TTR analysis, respectively. In this study, each of the sgRNAs targeted the same

sequence in the TTR gene. The sgRNAs tested include additional 2'-OMe and PS modifications in the 5' terminus, 3' terminus, hairpin 1, hairpin 2, and upper stem of the sgRNA. The results of this study are shown in Figures 23A-23C, including % editing (Figure 23A), average % editing (Figure 23B), and serum TTR levels (Figure 23C).

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ggcaccgagu cggugcuuuu 80

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<210> 331
<211> 100
<212> RNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
polynucleotide"

<400> 331
cccauacucc uacagcacca guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuaucaac uugaaaaagu ggcaccgagu cggugcuuuu 100

<210> 332
<211> 100
<212> RNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
polynucleotide"

<400> 332
cccauacucc uacagcacca guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuaucaac uugaaaaagu ggcaccgagu cggugcuuuu 100

<210> 333
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
primer"

<400> 333
agtcaataat cagaatcagc aggt 24

<210> 334
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
primer"

<400> 334
gttttgttcc agagtctatc accg 24

<210> 335
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
primer"

<400> 335
attaccagct tagcatcctg tgaa 24

<210> 336
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
primer"

<400> 336
agcacatgag accttctgtt tctc 24

<210> 337
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
primer"

<400> 337
agaaggcact tcttctttat ctaaggt 27

<210> 338
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
primer"

<400> 338
acacgaataa gagcaaatgg gaac 24

<210> 339
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
primer"

<400> 339
acacggttta tagagcaaga acac 24

<210> 340
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
primer"

<400> 340
gacataggtg tgaccctcac aatc 24

<210> 341
<211> 100
<212> RNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic

polynucleotide"

<400> 341
ccaguccagc gaggcaaagg guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuaucaac uugaaaaagu ggcaccgagu cggugcuuuu 100

<210> 342
<211> 100
<212> RNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic polynucleotide"

<400> 342
uuacagccac gucuacagca guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuaucaac uugaaaaagu ggcaccgagu cggugcuuuu 100

<210> 343
<211> 100
<212> RNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic polynucleotide"

<400> 343
uuacagccac gucuacagca guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuaucaac uugaaaaagu ggcaccgagu cggugcuuuu 100

<210> 344
<211> 100
<212> RNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic polynucleotide"

<400> 344
uuacagccac gucuacagca guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuaucaac uugaaaaagu ggcaccgagu cggugcuuuu 100

<210> 345
<211> 100
<212> RNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
polynucleotide"

<400> 345
uuacagccac gucuacagca guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuaucaac uugaaaaagu ggcaccgagu cggugcuuuu 100

<210> 346
<211> 100
<212> RNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
polynucleotide"

<400> 346
uuacagccac gucuacagca guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuaucaac uugaaaaagu ggcaccgagu cggugcuuuu 100

<210> 347
<211> 100
<212> RNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
polynucleotide"

<400> 347
uuacagccac gucuacagca guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuaucaac uugaaaaagu ggcaccgagu cggugcuuuu 100

<210> 348
<211> 100
<212> RNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
polynucleotide"

<400> 348
uuacagccac gucuacagca guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuaucaac uugaaaaagu ggcaccgagu cggugcuuuu 100

<210> 349
<211> 100
<212> RNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
polynucleotide"

<400> 349
uuacagccac gucuacagca guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuaucaac uugaaaaagu ggcaccgagu cggugcuuuu 100

<210> 350
<211> 100
<212> RNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
polynucleotide"

<400> 350
uuacagccac gucuacagca guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuaucaac uugaaaaagu ggcaccgagu cggugcuuuu 100

<210> 351
<211> 100
<212> RNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
polynucleotide"

<400> 351
uuacagccac gucuacagca guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuaucaac uugaaaaagu ggcaccgagu cggugcuuuu 100

<210> 352
<211> 100
<212> RNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic polynucleotide"

<400> 352
uuacagccac gucuacagca guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuaucaac uugaaaaagu ggcaccgagu cggugcuuuu 100

<210> 353
<211> 100
<212> RNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic polynucleotide"

<400> 353
uuacagccac gucuacagca guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuaucaac uugaaaaagu ggcaccgagu cggugcuuuu 100

<210> 354
<211> 100
<212> RNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic polynucleotide"

<220>
<221> misc_feature
<222> (1)..(20)
<223> n is a, c, g, or u

<400> 354
nnnnnnnnnn nnnnnnnnnn guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuaucaac uugaaaaagu ggcaccgagu cggugcuuuu 100

<210> 355
<211> 80
<212> RNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic polynucleotide"

<400> 355
guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc cguuaucaac uugaaaaagu 60

ggcaccgagu cggugcuuuu 80

<210> 356
<211> 80
<212> RNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic polynucleotide"

<400> 356
guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc cguuaucaac uugaaaaagu 60

ggcaccgagu cggugcuuuu 80

<210> 357
<211> 100
<212> RNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic polynucleotide"

<220>
<221> misc_feature
<222> (1)..(20)
<223> n is a, c, g, or u

<400> 357
nnnnnnnnnn nnnnnnnnnn guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuaucaac uugaaaaagu ggcaccgagu cggugcuuuu 100

<210> 358
<211> 100
<212> RNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
polynucleotide"

<220>
<221> modified_base
<222> (1)..(3)
<223> 2'-O-Me modified nucleotides

<220>
<221> misc_feature
<222> (1)..(2)
<223> Phosphorothioate linkage

<220>
<221> modified_base
<222> (1)..(20)
<223> N = any nucleotide

<220>
<221> misc_feature
<222> (2)..(3)
<223> Phosphorothioate linkage

<220>
<221> misc_feature
<222> (3)..(4)
<223> Phosphorothioate linkage

<220>
<221> misc_feature
<222> (5)..(20)
<223> n is a, c, g, or u

<220>
<221> modified_base
<222> (29)..(40)
<223> 2'-O-Me modified nucleotides

<220>
<221> modified_base

<222> (69)..(100)
<223> 2'-O-Me modified nucleotides

<220>
<221> misc_feature
<222> (97)..(98)
<223> Phosphorothioate linkage

<220>
<221> misc_feature
<222> (98)..(99)
<223> Phosphorothioate linkage

<220>
<221> misc_feature
<222> (99)..(100)
<223> Phosphorothioate linkage

<400> 358
nnnnnnnnnn nnnnnnnnnn guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuaucaac uugaaaaagu ggcaccgagu cggugcuuuu 100

<210> 359
<211> 4514
<212> RNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: mRNA transcript"

<400> 359
gggucccgca gucggcgucc agcggcucug cuuguucgug ugugugucgu ugcaggccuu 60

auucggaucc auggauaaga aguacucaau cgggcuggau aucggaacua auuccguggg 120

uugggcagug aucacggaug aauacaaagu gccguccaag aaguucaagg uccuggggaa 180

caccgauaga cacagcauca agaaaaaucu caucggagcc cugcuguuug acuccggcga 240

aaccgcagaa gcgacccggc ucaaacguac cgcgaggcga cgcuacaccc ggcggaagaa 300

ucgcaucugc uaucugcaag agaucuuuuc gaacgaaaug gcaaaggucg acgacagcuu 360

cuuccaccgc cuggaagaau cuuuccuggu ggaggaggac aagaagcaug aacggcaucc 420

uaucuuugga aacaucgucg acgaaguggc guaccacgaa aaguacccga ccaucuacca 480

ucugcggaag aaguugguug acucaacuga caaggccgac cucagauuga ucuacuuggc 540

ccucgcccau augaucaaau uccgcggaca cuuccugauc gaaggcgauc ugaacccuga 600

uaacuccgac guggauaagc uuuucauuca acuggugcag accuacaacc aacuguucga 660

agaaaaccca aucaaugcua gcggcgucga ugccaaggcc auccuguccg cccggcuguc 720

gaagucgcgg cgccucgaaa accugaucgc acagcugccg ggagagaaaa agaacggacu 780

uuucggcaac uugaucgcuc ucucacuggg acucacuccc aauuucaagu ccaauuuuga 840

ccuggccgag gacgcgaagc ugcaacucuc aaaggacacc uacgacgacg acuuggacaa 900

uuugcuggca caaauuggcg aucaguacgc ggaucuguuc cuugccgcua agaaccuuuc 960

ggacgcaauc uugcuguccg auauccugcg cgugaacacc gaaauaacca aagcgccgcu 1020

uagcgccucg augauuaagc gguacgacga gcaucaccag gaucucacgc ugcucaaagc 1080

gcucgugaga cagcaacugc cugaaaagua caaggagauc uucuucgacc aguccaagaa 1140

uggguacgca ggguacaucg auggaggcgc uagccaggaa gaguucuaua aguucaucaa 1200

gccaauccug gaaaagaugg acggaaccga agaacugcug gucaagcuga acagggagga 1260

ucugcuccgg aaacagagaa ccuuugacaa cggauccauu ccccaccaga uccaucuggg 1320

ugagcugcac gccaucuugc ggcgccagga ggacuuuuac ccauuccuca aggacaaccg 1380

ggaaaagauc gagaaaauuc ugacguuccg caucccguau uacgugggcc cacuggcgcg 1440

cggcaauucg cgcuucgcgu ggaugacuag aaaaucagag gaaaccauca cuccuuggaa 1500

uuucgaggaa guuguggaua agggagcuuc ggcacaaagc uucaucgaac gaaugaccaa 1560

cuucgacaag aaucucccaa acgagaaggu gcuuccuaag cacagccucc uuuacgaaua 1620

cuucacuguc uacaacgaac ugacuaaagu gaaauacguu acugaaggaa ugaggaagcc 1680

ggccuuucug uccggagaac agaagaaagc aauugucgau cugcuguuca agaccaaccg 1740

caaggugacc gucaagcagc uuaaagagga cuacuucaag aagaucgagu guuucgacuc 1800

aguggaaauc agcggggugg aggacagauu caacgcuucg cugggaaccu aucaugaucu 1860

ccugaagauc aucaaggaca aggacuuccu ugacaacgag gagaacgagg acauccugga 1920

agauaucguc cugaccuuga cccuuuucga ggaucgcgag augaucgagg agaggcuuaa 1980

gaccuacgcu caucucuucg acgauaaggu caugaaacaa cucaagcgcc gccgguacac 2040

ugguuggggc cgccucuccc gcaagcugau caacgguauu cgcgauaaac agagcgguaa 2100

aacuauccug gauuuccuca aaucggaugg cuucgcuaau cguaacuuca ugcaauugau 2160

ccacgacgac agccugaccu uuaaggagga cauccaaaaa gcacaagugu ccggacaggg 2220

agacucacuc caugaacaca ucgcgaaucu ggccgguucg ccggcgauua agaagggaau 2280

ucugcaaacu gugaaggugg ucgacgagcu ggugaagguc augggacggc acaaaccgga 2340

gaauaucgug auugaaaugg cccgagaaaa ccagacuacc cagaagggcc agaaaaacuc 2400

ccgcgaaagg augaagcgga ucgaagaagg aaucaaggag cugggcagcc agauccugaa 2460

agagcacccg guggaaaaca cgcagcugca gaacgagaag cucuaccugu acuauuugca 2520

aaauggacgg gacauguacg uggaccaaga gcuggacauc aaucgguugu cugauuacga 2580

cguggaccac aucguuccac aguccuuucu gaaggaugac ucgaucgaua acaagguguu 2640

gacucgcagc gacaagaaca gagggaaguc agauaaugug ccaucggagg aggucgugaa 2700

gaagaugaag aauuacuggc ggcagcuccu gaaugcgaag cugauuaccc agagaaaguu 2760

ugacaaucuc acuaaagccg agcgcggcgg acucucagag cuggauaagg cuggauucau 2820

caaacggcag cuggucgaga cucggcagau uaccaagcac guggcgcaga ucuuggacuc 2880

ccgcaugaac acuaaauacg acgagaacga uaagcucauc cgggaaguga aggugauuac 2940

ccugaaaagc aaacuugugu cggacuuucg gaaggacuuu caguuuuaca aagugagaga 3000

aaucaacaac uaccaucacg cgcaugacgc auaccucaac gcuguggucg guaccgcccu 3060

gaucaaaaag uacccuaaac uugaaucgga guuuguguac ggagacuaca aggucuacga 3120

cgugaggaag augauagcca aguccgaaca ggaaaucggg aaagcaacug cgaaauacuu 3180

cuuuuacuca aacaucauga acuuuuucaa gacugaaauu acgcuggcca auggagaaau 3240

caggaagagg ccacugaucg aaacuaacgg agaaacgggc gaaaucgugu gggacaaggg 3300

cagggacuuc gcaacuguuc gcaaagugcu cucuaugccg caagucaaua uugugaagaa 3360

aaccgaagug caaaccggcg gauuuucaaa ggaaucgauc cucccaaaga gaaauagcga 3420

caagcucauu gcacgcaaga aagacuggga cccgaagaag uacggaggau ucgauucgcc 3480

gacugucgca uacuccgucc ucgugguggc caagguggag aagggaaaga gcaaaaagcu 3540

caaauccguc aaagagcugc uggggauuac caucauggaa cgauccucgu ucgagaagaa 3600

cccgauugau uuccucgagg cgaaggguua caaggaggug aagaaggauc ugaucaucaa 3660

acucccccaag uacucacugu ucgaacugga aaauggucgg aagcgcaugc uggcuucggc 3720

cggagaacuc caaaaaggaa augagcuggc cuugccuagc aaguacguca acuuccucua 3780

ucuugcuucg cacuacgaaa aacucaaagg gucaccggaa gauaacgaac agaagcagcu 3840

uuucguggag cagcacaagc auuaucugga ugaaaucauc gaacaaaucu ccgaguuuuc 3900

aaagcgcgug auccucgccg acgccaaccu cgacaaaguc cugucggccu acaauaagca 3960

uagagauaag ccgaucagag aacaggccga gaacauuauc cacuuguuca cccugacuaa 4020

ccugggagcc ccagccgccu ucaaguacuu cgauacuacu aucgaucgca aaagauacac 4080

guccaccaag gaaguucugg acgcgacccu gauccaccaa agcaucacug gacucuacga 4140

aacuaggauc gaucugucgc agcugggugg cgauggcggu ggaucuccga aaaagaagag 4200

aaagguguaa ugagcuagcc aucacauuua aaagcaucuc agccuaccau gagaauaaga 4260

gaaagaaaau gaagaucaau agcuuauuca ucucuuuuuc uuuuucguug guguaaagcc 4320

aacacccugu cuaaaaaaca uaaauuucuu uaaucauuuu gccucuuuuc ucugugcuuc 4380

aauuaauaaa aaauggaaag aaccucgaga aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 4440

aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 4500

aaaaaaaaau cuag 4514

<210> 360
<211> 4603
<212> RNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: mRNA transcript"

<400> 360
gggucccgca gucggcgucc agcggcucug cuuguucgug ugugugucgu ugcaggccuu 60

auucggaucc auggauaaga aguacucaau cgggcuggau aucggaacua auuccguggg 120

uugggcagug aucacggaug aauacaaagu gccguccaag aaguucaagg uccuggggaa 180

caccgauaga cacagcauca agaaaaaucu caucggagcc cugcuguuug acuccggcga 240

aaccgcagaa gcgacccggc ucaaacguac cgcgaggcga cgcuacaccc ggcggaagaa 300

ucgcaucugc uaucugcaag agaucuuuuc gaacgaaaug gcaaaggucg acgacagcuu 360

cuuccaccgc cuggaagaau cuuuccuggu ggaggaggac aagaagcaug aacggcaucc 420

uaucuuugga aacaucgucg acgaaguggc guaccacgaa aaguacccga ccaucuacca 480

ucugcggaag aaguugguug acucaacuga caaggccgac cucagauuga ucuacuuggc 540

ccucgcccau augaucaaau uccgcggaca cuuccugauc gaaggcgauc ugaacccuga 600

uaacuccgac guggauaagc uuuucauuca acuggugcag accuacaacc aacuguucga 660

agaaaaccca aucaaugcua gcggcgucga ugccaaggcc auccuguccg cccggcuguc 720

gaagucgcgg cgccucgaaa accugaucgc acagcugccg ggagagaaaa agaacggacu 780

uuucggcaac uugaucgcuc ucucacuggg acucacuccc aauuucaagu ccaauuuuga 840

ccuggccgag gacgcgaagc ugcaacucuc aaaggacacc uacgacgacg acuuggacaa 900

uuugcuggca caaauuggcg aucaguacgc ggaucuguuc cuugccgcua agaaccuuuc 960

ggacgcaauc uugcuguccg auauccugcg cgugaacacc gaaauaacca aagcgccgcu 1020

uagcgccucg augauuaagc gguacgacga gcaucaccag gaucucacgc ugcucaaagc 1080

gcucgugaga cagcaacugc cugaaaagua caaggagauc uucuucgacc aguccaagaa 1140

uggguacgca ggguacaucg auggaggcgc uagccaggaa gaguucuaua aguucaucaa 1200

gccaauccug gaaaagaugg acggaaccga agaacugcug gucaagcuga acagggagga 1260

ucugcuccgg aaacagagaa ccuuugacaa cggauccauu ccccaccaga uccaucuggg 1320

ugagcugcac gccaucuugc ggcgccagga ggacuuuuac ccauuccuca aggacaaccg 1380

ggaaaagauc gagaaaauuc ugacguuccg caucccguau uacgugggcc cacuggcgcg 1440

cggcaauucg cgcuucgcgu ggaugacuag aaaaucagag gaaaccauca cuccuuggaa 1500

uuucgaggaa guuguggaua agggagcuuc ggcacaaagc uucaucgaac gaaugaccaa 1560

cuucgacaag aaucucccaa acgagaaggu gcuuccuaag cacagccucc uuuacgaaua 1620

cuucacuguc uacaacgaac ugacuaaagu gaaauacguu acugaaggaa ugaggaagcc 1680

ggccuuucug uccggagaac agaagaaagc aauugucgau cugcuguuca agaccaaccg 1740

caaggugacc gucaagcagc uuaaagagga cuacuucaag aagaucgagu guuucgacuc 1800

aguggaaauc agcggggugg aggacagauu caacgcuucg cugggaaccu aucaugaucu 1860

ccugaagauc aucaaggaca aggacuuccu ugacaacgag gagaacgagg acauccugga 1920

agauaucguc cugaccuuga cccuuuucga ggaucgcgag augaucgagg agaggcuuaa 1980

gaccuacgcu caucucuucg acgauaaggu caugaaacaa cucaagcgcc gccgguacac 2040

ugguuggggc cgccucuccc gcaagcugau caacgguauu cgcgauaaac agagcgguaa 2100

aacuauccug gauuuccuca aaucggaugg cuucgcuaau cguaacuuca ugcaauugau 2160

ccacgacgac agccugaccu uuaaggagga cauccaaaaa gcacaagugu ccggacaggg 2220

agacucacuc caugaacaca ucgcgaaucu ggccgguucg ccggcgauua agaagggaau 2280

ucugcaaacu gugaaggugg ucgacgagcu ggugaagguc augggacggc acaaaccgga 2340

gaauaucgug auugaaaugg cccgagaaaa ccagacuacc cagaagggcc agaaaaacuc 2400

ccgcgaaagg augaagcgga ucgaagaagg aaucaaggag cugggcagcc agauccugaa 2460

agagcacccg guggaaaaca cgcagcugca gaacgagaag cucuaccugu acuauuugca 2520

aaauggacgg gacauguacg uggaccaaga gcuggacauc aaucgguugu cugauuacga 2580

cguggaccac aucguuccac aguccuuucu gaaggaugac ucgaucgaua acaagguguu 2640

gacucgcagc gacaagaaca gagggaaguc agauaaugug ccaucggagg aggucgugaa 2700

gaagaugaag aauuacuggc ggcagcuccu gaaugcgaag cugauuaccc agagaaaguu 2760

ugacaaucuc acuaaagccg agcgcggcgg acucucagag cuggauaagg cuggauucau 2820

caaacggcag cuggucgaga cucggcagau uaccaagcac guggcgcaga ucuuggacuc 2880

ccgcaugaac acuaaauacg acgagaacga uaagcucauc cgggaaguga aggugauuac 2940

ccugaaaagc aaacuugugu cggacuuucg gaaggacuuu caguuuuaca aagugagaga 3000

aaucaacaac uaccaucacg cgcaugacgc auaccucaac gcuguggucg guaccgcccu 3060

gaucaaaaag uacccuaaac uugaaucgga guuuguguac ggagacuaca aggucuacga 3120

cgugaggaag augauagcca aguccgaaca ggaaaucggg aaagcaacug cgaaauacuu 3180

cuuuuacuca aacaucauga acuuuuucaa gacugaaauu acgcuggcca auggagaaau 3240

caggaagagg ccacugaucg aaacuaacgg agaaacgggc gaaaucgugu gggacaaggg 3300

cagggacuuc gcaacuguuc gcaaagugcu cucuaugccg caagucaaua uugugaagaa 3360

aaccgaagug caaaccggcg gauuuucaaa ggaaucgauc cucccaaaga gaaauagcga 3420

caagcucauu gcacgcaaga aagacuggga cccgaagaag uacggaggau ucgauucgcc 3480

gacugucgca uacuccgucc ucgugguggc caagguggag aagggaaaga gcaaaaagcu 3540

caaauccguc aaagagcugc uggggauuac caucauggaa cgauccucgu ucgagaagaa 3600

cccgauugau uuccucgagg cgaaggguua caaggaggug aagaaggauc ugaucaucaa 3660

acucccccaag uacucacugu ucgaacugga aaauggucgg aagcgcaugc uggcuucggc 3720

cggagaacuc caaaaaggaa augagcuggc cuugccuagc aaguacguca acuuccucua 3780

ucuugcuucg cacuacgaaa aacucaaagg gucaccggaa gauaacgaac agaagcagcu 3840

uuucguggag cagcacaagc auuaucugga ugaaaucauc gaacaaaucu ccgaguuuuc 3900

aaagcgcgug auccucgccg acgccaaccu cgacaaaguc cugucggccu acaauaagca 3960

uagagauaag ccgaucagag aacaggccga gaacauuauc cacuuguuca cccugacuaa 4020

ccugggagcc ccagccgccu ucaaguacuu cgauacuacu aucgaucgca aaagauacac 4080

guccaccaag gaaguucugg acgcgacccu gauccaccaa agcaucacug gacucuacga 4140

aacuaggauc gaucugucgc agcugggugg cgauggcucg gcuuacccau acgacgugcc 4200

ugacuacgcc ucgcucggau cgggcucccc caaaaagaaa cggaaggugg acggaucccc 4260

gaaaaagaag agaaaggugg acuccggaug agaauuaugc agucuagcca ucacauuuaa 4320

aagcaucuca gccuaccaug agaauaagag aaagaaaaug aagaucaaua gcuuauucau 4380

cucuuuuucu uuuucguugg uguaaagcca acacccuguc uaaaaaacau aaauuucuuu 4440

aaucauuuug ccucuuuucu cugugcuuca auuaauaaaa aauggaaaga accucgagaa 4500

aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 4560

aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaauc uag 4603

We claim:

1. A single guide RNA (sgRNA) comprising a 5' end modification and one or more modification in one or more of:
    a. the upper stem region;
    b. the hairpin 1 region; and
    c. the hairpin 2 region,

    wherein the 5' end modification comprises at least two phosphorothioate (PS) linkages within the first seven nucleotides at the 5' end of the 5' terminus.
2. The sgRNA of claim 1, wherein at least one modification comprises a 2'-O-methyl (2'-O-Me) modified nucleotide.
3. The sgRNA of claim 1 or claim 2, wherein at least one modification comprises a 2'-fluoro (2'-F) modified nucleotide.
4. The sgRNA of any one of claims 1-3, wherein at least one modification comprises a phosphorothioate (PS) bond between nucleotides.
5. The sgRNA of any one of claims 1-4, wherein the sgRNA comprises one or more modifications in the upper stem region.
6. The sgRNA of claim 5, comprising modifications at US1 to US12.
7. The sgRNA of any one of claims 1-6, wherein the sgRNA comprises one or more modifications in the hairpin 1 region.
8. The sgRNA of claim 7, wherein the sgRNA comprises a modification at H1-1.
9. The sgRNA of any one of claims 1-8, wherein the sgRNA comprises one or more modifications in the hairpin 2 region.
10. The sgRNA of claim 9, wherein the sgRNA comprises a modification at H2-1.
11. The sgRNA of any one of claims 1-10, wherein the sgRNA comprises modifications at H1-1 to H1-12.
12. The sgRNA of any one of claims 1-11, wherein the sgRNA comprises modifications at H2-1 to H2-15.
13. The sgRNA of any one of claims 1-12, wherein the sgRNA comprises one or more modifications in each of the upper stem region, the hairpin 1 region, and the hairpin 2 region.
14. The sgRNA of any one of claims 1-13, wherein the sgRNA comprises a modified nucleotide between hairpin 1 and hairpin 2 regions.

15. The sgRNA of any one of claims 1-14, further comprising a lower stem region comprising a modification.
16. The sgRNA of any one of claims 1-15, further comprising a 3' terminus region comprising a modification.
17. The sgRNA of claim 16, further comprising a 3' end modification in the 3' terminus.
18. The sgRNA of claim 17, wherein at least two of the last four nucleotides at the 3' end of the 3' terminus are modified.
19. The sgRNA of claim 17, wherein at least two of the last four nucleotides at the 3' end of the 3' terminus are modified with 2'-O-Me, 2'-F, or 2'-O-moe.
20. The sgRNA of any one of claims 17-19, further comprising phosphorothioate (PS) bonds between one or more of the last four nucleotides at the 3' end of the 3' terminus.
21. The sgRNA of any one of claims 1-20, further comprising a bulge region comprising a modification.
22. The sgRNA of any one of claims 1-21, further comprising a nexus region comprising a modification.
23. The sgRNA of any one of claims 1-22, wherein at least the first three nucleotides at the 5' end of the 5' terminus, and the last three nucleotides at the 3' end of the 3' terminus are modified.
24. The sgRNA of any one of claims 1-23, wherein the first four nucleotides at the 5' end of the 5' terminus, and the last four nucleotides at the 3' end of the 3' terminus are linked with phosphorothioate (PS) bonds.
25. The sgRNA of claim 24, wherein the end modifications comprise 2'-O-Me.
26. The sgRNA of claim 24, wherein the end modifications comprise 2'-F.
27. The sgRNA of any one of claims 1-26, wherein the first four nucleotides at the 5' end of the 5' terminus and the last four nucleotides at the 3' end of the 3'terminus are linked with a PS bond, and wherein the first three nucleotides at the 5' end of the 5' terminus and the last three nucleotides at the 3' end of the 3' terminus comprise 2'-O-Me modifications.
28. The sgRNA of any one of claims 1-26, wherein the first four nucleotides at the 5' terminus and the last four nucleotides at the 3' terminus are linked with a PS bond, and wherein the first three nucleotides at the 5' terminus and the last three nucleotides at the 3' terminus comprise 2'-O-Me, 2'-F, and/or 2'-O-moe modifications.
29. The sgRNA of any one of claims 1-28, wherein LS1, LS6, LS7, LS8, LS11, and/or LS12 are modified with 2'-O-Me.

30. The sgRNA of any one of claims 1-29, wherein each of the nucleotides in the bulge region are modified with 2'-O-Me.
31. The sgRNA of any one of claims 1-29, wherein at least 50% of the nucleotides in the bulge region are modified with 2'-O-Me.
32. The sgRNA of any one of claims 1-31, wherein each of the nucleotides in the upper stem region are modified with 2'-O-Me.
33. The sgRNA of any one of claims 1-32, wherein N16, N17, and/or N18 in the nexus region are modified with 2'-O-Me.
34. The sgRNA of any one of claims 1-32, wherein N15, N16, N17, and/or N18 in the nexus region are modified.
35. The sgRNA of claim 33 or claim 34, wherein the modifications in the nexus region are selected from 2'-O-Me and 2'F.
36. The sgRNA of any one of claims 32-35, wherein N16, N17, and N18 are linked with PS bonds.
37. The sgRNA of any one of claims 1-36, wherein each of the nucleotides in the hairpin 1 region are modified with 2'-O-Me.
38. The sgRNA of any one of claims 1-37, wherein each of the nucleotides in the hairpin 2 region are modified with 2'-O-Me.
39. A single guide RNA (sgRNA) comprising 2'-O-Me modified nucleotides at the following positions:
    a. the first three nucleotides at the 5' end of the 5' terminus;
    b. LS1, LS6, LS7, LS8, LS11, and/or LS12 in the lower stem region;
    c. B1 and/or B2 in the bulge region;
    d. each nucleotide in the upper stem region;
    e. N16, N17, and/or N18 in the nexus region;
    f. each nucleotide in the hairpin 1 region;
    g. each nucleotide in the hairpin 2 region; and
    h. the last four nucleotides at the 3' terminus.
40. The sgRNA of claim 39, wherein B3-B6 are modified with 2'-O-Me.
41. The sgRNA of claim 39, further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' end of the 5' terminus and three PS bonds linking the last four nucleotides at the 3' end of the 3' terminus.
42. The sgRNA of any one of the preceding claims, wherein LS9 and LS10 are modified, e.g. with 2'-F or 2'-OMe.

43. The sgRNA of any one of the preceding claims, wherein N15, N16, N17, and N18 are modified, e.g. with 2'-F or 2'-OMe.
44. The sgRNA of any one of the preceding claims, wherein H2-9, H2-10, H2-11, H2-12, H2-13, H2-14, and H2-15 are modified with 2'-F.
45. The sgRNA of any one of the preceding claims, wherein the second to last, third to last, and fourth to last nucleotides at the 3' terminus are modified with 2'-F.
46. A single guide RNA (sgRNA) comprising 2'-F modified nucleotides at the following positions:
    a. LS9 and LS10 in the lower stem region;
    b. N15, N16, N17, and N18 in the nexus region; and
    c. H2-9, H2-10, H2-11, H2-12, H2-13, H2-14, and H2-15 in the hairpin 2 region.
47. The sgRNA of claim 46, further comprising 2'-F modified nucleotides at the second to last, third to last, and fourth to last nucleotides at the 3' terminus.
48. The sgRNA of claim 46 or claim 47, further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' end of the 5' terminus and three PS bonds linking the last four nucleotides at the 3' end of the 3' terminus.
49. The sgRNA of any one of claims 46-48, further comprising 2'-O-Me or 2'-F modified nucleotides at the first three nucleotides at the 5' end of the 5' terminus, and 2'-O-Me or 2'-F modified nucleotides at the three of the last four nucleotides at the 3' end of the 3' terminus.
50. A single guide RNA (sgRNA) comprising:
    a. 2'-O-Me modified nucleotides at the first three nucleotides at the 5' end of the 5' terminus;
    b. 2'-O-Me modified nucleotides at US1-US12;
    c. 2'-O-Me modified nucleotides at H1-1 – H1-12;
    d. 2'-O-Me modified nucleotides at H2-1 – H2-15; and
    e. 2'-O-Me modified nucleotides at the last four nucleotides at the 3' end of the 3' terminus.
51. The sgRNA of claim 50 further comprising 2'-O-Me modified nucleotides at LS1 and/or LS6.
52. The sgRNA of claim 50 or claim 51 further comprising a 2'-O-Me modified nucleotide between Hairpin 1 and Hairpin 2.

53. A single guide RNA (sgRNA) comprising:
    a. 2'-O-Me modified nucleotides at the first three nucleotides at the 5' end of the 5' terminus;
    b. 2'-F modified nucleotides at LS1-LS6;
    c. 2'-O-Me modified nucleotides at US1-US12;
    d. 2'-O-Me modified nucleotides at H1-1 – H1-12;
    e. a 2'-O-Me modified nucleotide between hairpin 1 and hairpin 2;
    f. 2'-O-Me modified nucleotides at H2-1 – H2-15; and
    g. 2'-O-Me modified nucleotides at the last four nucleotides at the 3' end of the 3' terminus.
54. A single guide RNA (sgRNA) comprising:
    a. 2'-O-Me modified nucleotides at the first three nucleotides at the 5' end of the 5' terminus;
    b. 2'-F modified nucleotides at LS2-LS5;
    c. 2'-O-Me modified nucleotides at LS1 and LS6;
    d. 2'-O-Me modified nucleotides at US1-US12;
    e. 2'-O-Me modified nucleotides at H1-1 – H1-12;
    f. a 2'-O-Me modified nucleotide between hairpin 1 and hairpin 2;
    g. 2'-O-Me modified nucleotides at H2-1 – H2-15; and
    h. 2'-O-Me modified nucleotides at the last four nucleotides at the 3' end of the 3' terminus.
55. A single guide RNA (sgRNA) comprising
    a. 2'-O-Me modified nucleotides at the first three nucleotides at the 5' end of the 5' terminus;
    b. 2'-O-Me modified nucleotides at US1-US12;
    c. 2'-O-Me modified nucleotides at LS7, LS8, LS11, and LS12;
    d. 2'-O-Me modified nucleotides at H1-1 – H1-12;
    e. a 2'-O-Me modified nucleotide between hairpin 1 and hairpin 2;
    f. 2'-O-Me modified nucleotides at H2-1 – H2-15; and
    g. 2'-O-Me modified nucleotides at the last four nucleotides at the 3' end of the 3' terminus.
56. A single guide RNA (sgRNA) comprising
    a. 2'-O-Me modified nucleotides at the first three nucleotides at the 5' end of the 5' terminus;

b. 2'-O-Me modified nucleotides at US1-US12;
c. 2'-O-Me modified nucleotides at LS7, LS8, LS11, and LS12;
d. 2'-F modified nucleotides at LS9 and LS10;
e. 2'-O-Me modified nucleotides at H1-1 – H1-12;
f. a 2'-O-Me modified nucleotide between hairpin 1 and hairpin 2;
g. 2'-O-Me modified nucleotides at H2-1 – H2-15; and
h. 2'-O-Me modified nucleotides at the last four nucleotides at the 3' end of the 3' terminus.

57. A single guide RNA (sgRNA) comprising
a. 2'-O-Me modified nucleotides at the first three nucleotides at the 5' end of the 5' terminus;
b. 2'-O-Me modified nucleotides at US1-US12;
c. 2'-O-Me modified nucleotides at LS8, LS10, and LS12;
d. 2'-O-F modified nucleotides at LS7, LS9, and LS11;
e. 2'-O-Me modified nucleotides at H1-1 – H1-12;
f. a 2'-O-Me modified nucleotide between hairpin 1 and hairpin 2;
g. 2'-O-Me modified nucleotides at H2-1 – H2-15; and
h. 2'-O-Me modified nucleotides at the last four nucleotides at the 3' end of the 3' terminus.

58. A single guide RNA (sgRNA) comprising:
a. 2'-O-Me modified nucleotides at the first three nucleotides at the 5' end of the 5' terminus;
b. 2'-O-Me modified nucleotides at LS1, LS6, LS7, LS8, LS11, and LS12
c. 2'-O-Me modified nucleotides at US1-US12;
d. 2'-O-Me modified nucleotides at H1-1 – H1-12;
e. a 2'-O-Me modified nucleotide between hairpin 1 and hairpin 2;
f. 2'-O-Me modified nucleotides at H2-1 – H2-15; and
g. 2'-O-Me modified nucleotides at the last four nucleotides at the 3' end of the 3' terminus.

59. A single guide RNA (sgRNA) comprising
a. 2'-O-Me modified nucleotides at the first three nucleotides at the 5' end of the 5' terminus;
b. 2'-O-Me modified nucleotides at LS1, LS6, LS7, LS8, LS11, and LS12;
c. 2'-F modified nucleotides at LS9 and LS10;

d. 2'-O-Me modified nucleotides at US1-US12;
e. 2'-O-Me modified nucleotides at H1-1 – H1-12;
f. a 2'-O-Me modified nucleotide between hairpin 1 and hairpin 2;
g. 2'-O-Me modified nucleotides at H2-1 – H2-15; and
h. 2'-O-Me modified nucleotides at the last four nucleotides at the 3' end of the 3' terminus.

60. A single guide RNA (sgRNA) comprising
a. 2'-O-Me modified nucleotides at the first three nucleotides at the 5' end of the 5' terminus;
b. 2'-O-Me modified nucleotides at US1-US12;
c. 2'-O-Me modified nucleotides at H1-1 – H1-12;
d. a 2'-O-Me modified nucleotide between Hairpin 1 and Hairpin 2;
e. 2'-O-Me modified nucleotides at H2-1 – H2-8;
f. 2'-F modified nucleotides at H2-9 – H2-15;
g. 2'-F modified nucleotides at the second from last, third from last, and fourth from last nucleotide at the 3' terminus; and
h. a 2'-O-Me modified nucleotide at the last nucleotide at the 3' end of the 3' terminus.

61. A single guide RNA (sgRNA) comprising
a. 2'-O-Me modified nucleotides at the first three nucleotides at the 5' end of the 5' terminus;
b. 2'-O-Me modified nucleotides at US1-US12;
c. 2'-O-Me modified nucleotides at H1-2, H1-4, H1-6, H1-8, H1-10, and H1-12;
d. 2'-F modified nucleotides at H1-1, H1-3, H1-5, H1-7, H1-9, and H1-11;
e. a 2'-F modified nucleotide between hairpin 1 and hairpin 2;
f. 2'-F modified nucleotides at H2-2, H2-4, H2-6, H2-8, H2-10, H2-12; and H2-14;
g. 2'-O-Me modified nucleotides at H2-1, H2-3, H2-5, H2-7, H2-9, H2-11; H2-13, and H2-15;
h. 2'-F modified nucleotides at the second from last, and fourth from last nucleotide at the 3' terminus; and
i. 2'-O-Me modified nucleotide at the third from last, and last nucleotide at the 3' end of the 3' terminus.

62. The sgRNA of any of claims 50-61 further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' end of the 5' terminus and three PS bonds linking the last four nucleotides at the 3' end of the 3' terminus.
63. The sgRNA of any of claims 50-61 further comprising at least one phosphorothioate (PS) bond within the first seven nucleotides at the 5' end of the 5' terminus.
64. A single guide RNA (sgRNA) comprising
    a. 2'-O-Me modified nucleotides LS8, LS10, LS12, H1-2, H1-4, H1-6, H1-8, H1-10, H1-12, H2-1, H2-3, H2-5, H2-7, H2-9, H2-11, H2-13, and H2-15; and
    b. 2'-F modified nucleotides at LS7, LS9, LS11; H1-1, H1-3, H1-5, H1-7, H1-9, H1-11, H1-13, H2-2, H2-4, H2-6, H2-8, H2-10, H2-12, and H2-14.
65. The sgRNA of claim 64, further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' end of the 5' terminus and three PS bonds linking the last four nucleotides at the 3' end of the 3' terminus.
66. The sgRNA of claim 64 or claim 65, further comprising
    a. 2'-O-Me modified nucleotides at the last and third to last nucleotide at the 3' end of the 3' terminus; and
    b. 2'-F modified nucleotides at the second to last and fourth to last nucleotide at the 3' end of the 3' terminus.
67. An sgRNA comprising any of SEQ ID Nos: 228-332, including the modifications of Table 4.
68. An sgRNA comprising any of SEQ ID Nos: 235-240, 265-285, and 309-329, including the modifications of Table 4.
69. An sgRNA comprising SEQ ID No: 240.
70. An sgRNA comprising SEQ ID No. 240, including the modifications of Table 4.
71. An sgRNA comprising SEQ ID No: 242, including the modifications of Table 4.
72. An sgRNA comprising the modifications of SEQ ID No: 242, as depicted in Table 4.
73. An sgRNA comprising SEQ ID No: 358.
74. An sgRNA comprising nucleic acids having at least 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 85, 80, 75, or 70% identity to the nucleic acids of any one of SEQ ID Nos: 235-240, 265-285, and 309-329, wherein the modification at each nucleotide of the sgRNA that corresponds to a nucleotide of the reference sequence identifier in Table 4, is identical to or equivalent to the modification shown in the reference sequence identifier in Table 4.

75. The sgRNA of any one of claims 66-74, further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' terminus and three PS bonds linking the last four nucleotides at the 3' terminus.
75. The sgRNA of any one of claims 1-74, further comprising at least three PS bonds linking the nucleotides in the hairpin 1 region.
76. The sgRNA of any one of claims 1-75, further comprising at least three PS bonds linking the nucleotides in the hairpin 2 region.
77. The sgRNA of any one of claims 1-76, further comprising at least three PS bonds linking the nucleotides in the upper stem region.
78. The sgRNA of any one of claims 1-77, wherein the sgRNA forms a ribonucleoprotein complex with an *S. pyogenes* Cas9.
79. A guide RNA comprising 2'-O-Me modifications at each nucleotide in the upper stem region.
80. A guide RNA comprising 2'-O-Me modifications at one, two, three, or four, five, six, seven, eight, nine, ten, or eleven of the following nucleotides: US1, US2, US3, US4, US5, US6, US7, US8, US9, US10, US11, and US12.
81. A guide RNA comprising 2'-O-Me modifications at 50% or more of the nucleotides in the hairpin 1 and hairpin 2 regions.
82. A guide RNA comprising 2'-O-Me modifications at each nucleotide in the upper stem region and 2'-O-Me modifications at 50% or more of the nucleotides in the hairpin 1 and hairpin 2 regions.
83. A guide RNA comprising or consisting of 2'-O-Me modifications at each nucleotide starting at H2-1 or H2-2 through the last nucleotide of the 3' terminus.
84. A guide RNA comprising or consisting of a 2'-O-Me modification at LS1 and/or LS6 and no modification at LS2-LS5.
85. A guide RNA comprising 2'-O-Me modified nucleotides at 2 or more of LS8, LS9, LS10, LS11, and/or LS12.
86. The guide RNA of claim 85, wherein at least LS8 and LS10 are modified with 2'-O-Me.
87. The guide RNA of any of claims 79-86, further comprising 2'-O-Me modifications at 50% or more of the nucleotides in the nexus region.
88. The guide RNA of claim 87, wherein at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% of the nucleotides in the nexus region are modified with 2'-O-Me.

89. A guide RNA comprising 2'-O-Me modifications at up to 50% of the nucleotides in the nexus region.
90. A guide RNA comprising 2'-O-Me modifications at four or more nucleotides in the nexus region.
91. The guide RNA of claim 90, wherein at least one, two, three, four, or five of nucleotides N2-N6 in the nexus region are modified with 2'-O-Me.
92. A guide RNA comprising 2'-O-Me modified nucleotides at ten or more nucleotides in the nexus region.
93. The guide RNA of claim 92, wherein at least N2-N6 are modified with 2'-O-Me.
94. A guide RNA comprising 2'-O-Me modifications at 50% or more of the nucleotides in the bulge region.
95. The guide RNA of claim 94, wherein at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% of the nucleotides in the bulge region are modified with 2'-O-Me.
96. The guide RNA of claim 94, wherein B2, B3, and/or B4 are modified with 2'-O-Me.
97. A guide RNA comprising 2'-O-Me modified nucleotides at three or more nucleotides in the bulge region.
98. The guide RNA of claim 97, wherein B2, B3, and/or B4 are modified with 2'-O-Me.
99. A guide RNA comprising the nucleotides of SEQ ID NO: 357 with the modification pattern of the guide RNA of any preceding claim.
100. The guide RNA comprising the nucleotides of SEQ ID NO: 356 with the modification pattern of the guide RNA of any preceding claim.
101. The guide RNA of any one of claims 79-100, further comprising 2'-O-Me modifications at the first three, four, or five nucleotides at the 5' end of the 5' terminus region.
102. The guide RNA of any one of claims 79-100, further comprising 2'-O-Me modifications at the last three, last four, or last five nucleotides at the 3' end of the 3' terminus region.
103. The guide RNA of claim 102, wherein the last nucleotide at the 3' end of the 3' terminus region is not modified.
104. The guide RNA of claim 102, wherein only the second to last and third to last nucleotide at the 3' end of the 3' terminus region are modified.
105. The guide RNA of claim 102, wherein only the second to last, third to last, and fourth to last nucleotide in the 3' terminus region are modified.

106. The guide RNA of any one of claims 79-100, further comprising PS bonds between the first three, four, or five nucleotides at the 5' end of the 5' terminus.

107. The guide RNA of claim 106, wherein the first four nucleotides at the 5' end of the 5' terminus are linked with PS bonds.

108. The guide RNA of any one of claims 79-100, further comprising PS bonds between the last three, four, or five nucleotides at the 3' end of the 3' terminus.

109. The guide RNA of claim 108, wherein the last four nucleotides at the 3' end of the 3' terminus are linked with PS bonds.

110. The guide RNA of any one of claims 79-100, wherein the first three or four nucleotides at the 5' end of the 5' terminus, and the last three or four nucleotides at the 3' end of the 3' terminus comprise 2'-O-Me modifications.

111. The guide RNA of any one of claims 79-100, wherein the first three or four nucleotides at the 5' end of the 5' terminus are linked with PS bonds, and the last three or four nucleotides at the 3' end of the 3' terminus are linked with PS bonds.

112. The guide RNA of any one of claims 79-100, wherein the first three or four nucleotides at the 5' end of the 5' terminus comprise 2'-O-Me modifications and are linked with PS bonds, and the last three or four nucleotides at the 3' end of the 3' terminus comprise 2'-O-Me modifications and are linked with PS bonds.

113. The guide RNA of any one of claims 79-100, wherein at least two of nucleotides LS8, LS9, LS10, LS11, and LS12 comprise 2'-O-Me modifications.

114. The guide RNA of any one of claims 79-100, wherein at least LS8 and LS10 are modified with 2'-O-Me.

115. The guide RNA of any one of claims 79-100, wherein at least 50% of the nucleotides in the nexus region are modified with 2'-O-Me.

116. The guide RNA of claim 115, wherein at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% of the nucleotides in the nexus region are modified with 2'-O-Me.

117. The guide RNA of claim 115, wherein at least N2-N6 are modified with 2'-O-Me.

118. The guide RNA of any one of claims 79-100, wherein at least ten of the nucleotides in the nexus region are modified with 2'-O-Me.

119. The guide RNA of claim 115, wherein at least N2-N6 are modified with 2'-O-Me.

120. The guide RNA of any one of claims 79-100, wherein at least 50% of the nucleotides in the bulge region are modified with 2'-O-Me.

121. The guide RNA of claim 120, wherein at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% of the nucleotides in the bulge region are modified with 2'-O-Me.

122. The guide RNA of claim 121, wherein B2, B3, and/or B4 are modified with 2'-O-Me.

123. The guide RNA of any one of claims 79-100, wherein at least three of the nucleotides in the bulge region are modified with 2'-O-Me.

124. The guide RNA of claim 123, wherein B2, B3, and/or B4 are modified with 2'-O-Me.

125. The guide RNA of any one of claims 79-124, wherein the guide RNA is an sgRNA.

126. The guide RNA of any one of claims 79-125, wherein the guide RNA is a dgRNA.

127. The guide RNA of claims 81 or 82, wherein at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% of the nucleotides in the hairpin 1 and hairpin 2 regions are modified with 2'-OMe.

128. The guide RNA of any one of claims 79-127, wherein the gRNA forms a ribonucleoprotein complex with an *S. pyogenes* Cas9.

129. A single guide RNA (sgRNA) comprising or consisting of 2'-O-Me modified nucleotides at:

a. the first three nucleotides at the 5' end of the 5' terminus;
b. each nucleotide in the upper stem region;
c. each nucleotide in the hairpin 1 region;
d. the nucleotide between hairpin 1 and hairpin 2;
e. each nucleotide in the hairpin 2 region; and
f. the last four nucleotides at the 3' end of the 3' terminus.

130. The sgRNA of claim 129, further comprising PS bonds linking the first four nucleotides at the 5' end of the 5' terminus and PS bonds linking the last four nucleotides at the 3' end of the 3' terminus.

131. An sgRNA having the modification pattern shown in SEQ ID NO: 350, 351, 352, or 353, as depicted in Table 4.

132. A single guide RNA (sgRNA) comprising or consisting of 2'-O-Me modified nucleotides at:

a. the first three, four, five, or seven nucleotides at the 5' end of the 5' terminus;
b. each nucleotide in the upper stem region;
c. each nucleotide in the hairpin 1 region;
d. the nucleotide between hairpin 1 and hairpin 2;
e. each nucleotide in the hairpin 2 region; and

f. the last four nucleotides at the 3' end of the 3' terminus.

133. A single guide RNA (sgRNA) comprising or consisting of 2'-O-Me modified nucleotides at:

a. the first three, four, five, or seven nucleotides at the 5' end of the 5' terminus;
b. each of nucleotides US1, US2, US3, US4, US5, US6, US7, US8, US9, US10, US11 and US12;
c. each of nucleotides H1-1, H1-2, H1-3, H1-4, H1-5, H1-6, H1-7, H1-8, H1-9, H1-10, H1-11, and H1-12;
d. the nucleotide between hairpin 1 and hairpin 2;
e. each of nucleotides H2-1, H2-2, H2-3, H2-4, H2-5, H2-6, H2-7, H2-8, H2-9, H2-10, H2-11, H2-12, H2-13, H2-14, and H2-15; and
f. the last four nucleotides at the 3' end of the 3' terminus.

134. The sgRNA of any one of claims 129-133, further comprising PS bonds linking the first four nucleotides at the 5' end of the 5' terminus and PS bonds linking the last four nucleotides at the 3' end of the 3' terminus.

135. An sgRNA having the modification pattern of SEQ ID NO: 350, 351, 352, or 353 as shown in Table 4.

136. The sgRNA of any one of claims 129-135, wherein the sgRNA forms a ribonucleoprotein complex with an *S. pyogenes* Cas9.

137. A crispr RNA (crRNA) comprising one or more modifications within one or more of the following regions:

a. the first five nucleotides at the 5' end of the 5' terminus;
b. the lower stem region;
c. the bulge region;
d. the upper stem region; and
e. the last five nucleotides at the 3' end of the crRNA.

138. The crRNA of claim 136, further comprising a 5' end modification, wherein the 5' end modification comprises one or more phosphorothioate linkages within the first seven nucleotides at the 5' end of the 5' terminus.

139. The crRNA of claim 136, further comprising a 5' end modification, wherein the 5' end modification comprises at least two phosphorothioate linkages within the first seven nucleotides at the 5' end of the 5' terminus.

140. The crRNA of any one of claims 137-139, wherein at least one modification comprises a 2'-O-methyl (2'-O-Me) modified nucleotide.

141. The crRNA of any one of claims 137-140, wherein at least one modification comprises a 2'-fluoro (2'-F) modified nucleotide.

142. The crRNA of any one of claims 137-141, wherein at least one modification comprises a phosphorothioate (PS) bond between nucleotides.

143. The crRNA of any one of claims 137-142, wherein the first three nucleotides at the 5' end of the 5' terminus and the last three nucleotides at the 3' end of the 3' terminus are modified.

144. The crRNA of any one of claims 137-143, wherein the first four nucleotides at the 5' end of the 5' terminus, and the last four nucleotides at the 3' end of the 3' terminus are linked with phosphorothioate (PS) bonds.

145. The crRNA of claim 143, wherein the modification comprises 2'-O-Me.

146. The crRNA of claim 143, wherein the modification comprises 2'-F.

147. The crRNA of any one of claims 137-146, wherein the first four nucleotides at the 5' end of the 5' terminus and the last four nucleotides at the 3' end of the 3' terminus are linked with a PS bond, and wherein the first three nucleotides at the 5' end of the 5' terminus and the last three nucleotides at the 3' end of the 3' terminus comprise 2'-O-Me modifications.

148. The crRNA of any one of claims 137-146, wherein the first four nucleotides at the 5' end of the 5' terminus and the last four nucleotides at the 3' end of the 3' terminus are linked with a PS bond, and wherein the first three nucleotides at the 5' end of the 5' terminus and the last three nucleotides at the 3' end of the 3' terminus comprise 2'-F modifications.

149. The crRNA of any one of claims 137-148, wherein LS1 and LS6 are modified with 2'-O-Me.

150. The crRNA of any one of claims 137-149, wherein each of the nucleotides in the upper stem region are modified with 2'-O-Me.

151. A crispr RNA (crRNA) comprising 2'-O-Me modified nucleotides at:

a. LS1 and LS6 in the lower stem region; and

b. each nucleotide in the upper stem region.

152. The crRNA of claim 151, further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' end of the 5' terminus and three PS bonds linking the last four nucleotides at the 3' end of the 3' terminus.

153. The crRNA of claim 151 or claim 152, further comprising 2'-O-Me or 2'-F modified nucleotides at the first three nucleotides at the 5' end of the 5' terminus, and 2'-O-Me or 2'-F modified nucleotides at the last three nucleotides at the 3' end of the 3' terminus.

154. The crRNA of any of claims 137-153, wherein LS1, LS2, and LS6 are modified with 2'-F.

155. The crRNA of any of claims 137-154, wherein each nucleotide in the bulge region is modified with 2'-F.

156. A crispr RNA (crRNA) comprising 2'-F modified nucleotides at:

a. LS1, LS2, and LS6 in the lower stem region; and

b. each nucleotide in the bulge region.

157. The crRNA of any one of claims 137-156, further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' end of the 5' terminus and three PS bonds linking the last four nucleotides at the 3' end of the 3' terminus.

158. The crRNA of any one of claims 137-157, further comprising 2'-O-Me or 2'-F modified nucleotides at the first three nucleotides at the 5' end of the 5' terminus, and 2'-O-Me or 2'-F modified nucleotides at the last three nucleotides at the 3' end of the 3' terminus.

159. A crRNA comprising any one of SEQ ID Nos: 1–187, including the modifications of Table 4.

160. A crRNA comprising any one of SEQ ID Nos: 19-31, 53-73, 104-130, and 161-187, including the modifications of Table 4.

161. A crRNA comprising nucleic acids having at least 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 85, 80, 75, or 70% identity to any one of SEQ ID Nos: 19-31, 53-73, 104-130, and 161-187, wherein the modification at each nucleotide of the crRNA that corresponds to a nucleotide of the reference sequence identifier in Table 4, is identical to or equivalent to the modification shown in the reference sequence identifier in Table 4.

162. The crRNA of any one of claims 159-161, further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' end of the 5' terminus and three PS bonds linking the last four nucleotides at the 3' end of the 3' terminus.

163. The crRNA of any one of claims 137-162, wherein the crRNA combined with a trRNA forms a ribonucleoprotein complex with an *S. pyogenes* Cas9.

164. A tracr RNA (trRNA) comprising one or more modifications within one or more of the following regions:

a. the first five nucleotides at the 5' end of the 5' terminus;

b. the upper stem region;
c. the bulge region;
d. the lower stem region;
e. the nexus region;
f. the hairpin 1 region;
g. the hairpin 2 region; and
h. the last five nucleotides at the 3' end of the 3' terminus.

165. The trRNA of claim 164, wherein at least one modification comprises a 2'-O-methyl (2'-O-Me) modified nucleotide.

166. The trRNA of claim 164 or claim 165, wherein at least one modification comprises a 2'-fluoro (2'-F) modified nucleotide.

167. The trRNA of any one of claims 164-166, wherein at least one modification comprises a phosphorothioate (PS) bond between nucleotides.

168. The trRNA of any one of claims 164-167, wherein the first four nucleotides at the 5' end of the 5' terminus, and the last four nucleotides at the 3' end of the 3' terminus are linked with phosphorothioate (PS) bonds.

169. The trRNA of any one of claims 164-168, wherein the first three nucleotides at the 5' end of the 5' terminus and the last three nucleotides at the 3' end of the 3' terminus are modified.

170. The trRNA of claim 169, wherein the modification comprises 2'-O-Me.

171. The trRNA of claim 169, wherein the modification comprises 2'-F.

172. The trRNA of any one of claims 164-171, wherein the first four nucleotides at the 5' end of the 5' terminus and the last four nucleotides at the 3' end of the 3' terminus are linked with a PS bond, and wherein the first three nucleotides at the 5' end of the 5' terminus and the last three nucleotides at the 3' end of the 3' terminus comprise 2'-O-Me modifications.

173. The trRNA of any one of claims 164-171, wherein the first four nucleotides at the 5' end of the 5' terminus and the last four nucleotides at the 3' end of the 3'terminus are linked with a PS bond, and wherein the first three nucleotides at the 5' end of the 5' terminus and the last three nucleotides at the 3' end of the 3' terminus comprise 2'-F modifications.

174. The trRNA of any one of claims 164-173, wherein each nucleotide in the upper stem region is modified with 2'-O-Me.

175. The trRNA of any one of claims 164-174, wherein B1 and B2 within the bulge region are modified with 2'-O-Me.

176. The trRNA of any one of claims 164-175, wherein N3, N4, N5, N15, N16, N17, and/or N18 in the nexus region are modified with 2'-O-Me.

177. The trRNA of any one of claims 164-176, wherein each nucleotide in the hairpin 1 region is modified with 2'-O-Me.

178. The trRNA of any one of claims 164-177, wherein each nucleotide in the hairpin 2 region is modified with 2'-O-Me.

179. A tracr RNA (trRNA) comprising 2'-O-Me modified nucleotides at:

a. each nucleotide in the upper stem;
b. B1 and/or B2 within the bulge region;
c. N3, N4, N5, N15, N16, N17, and/or N18 in the nexus region;
d. each nucleotide in the hairpin 1 region; and
e. each nucleotide in the hairpin 2 region.

180. The trRNA of claim 179, further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' end of the 5' terminus and three PS bonds linking the last four nucleotides at the 3' end of the 3' terminus.

181. The trRNA of claim 179 or claim 180, further comprising 2'-O-Me or 2'-F modified nucleotides at the first three nucleotides at the 5' end of the 5' terminus, and 2'-O-Me or 2'-F modified nucleic acids at the last three nucleotides at the 3' end of the 3' terminus.

182. The trRNA of any of claims 164-181, wherein N15, N16, N17, and N18 are modified with 2'-F.

183. The trRNA of any of claims 164-182, wherein LS1, LS3, and LS5 are modified with 2'-F, and LS2, LS4, and LS6 are modified with 2'-O-Me.

184. The trRNA of any one of claims 164-183, further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' end of the 5' terminus and three PS bonds linking the last four nucleotides at the 3' end of the 3' terminus.

185. The trRNA of any one of claims 164-184, further comprising 2'-O-Me or 2'-F modified nucleotides at the first three nucleotides at the 5' end of the 5' terminus, and 2'-O-Me or 2'-F modified nucleotides at the last three nucleotides at the 3' end of the 3' terminus.

186. A trRNA comprising any one of SEQ ID Nos: 188-227, including the modifications of Table 4.

187. A trRNA comprising nucleic acids having at least 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 85, 80, 75, or 70% identity to any one of SEQ ID Nos: 188-227, wherein the modification at each nucleotide of the trRNA that corresponds to a nucleotide of the reference sequence identifier in Table 4, is identical to or equivalent to the modification shown in the reference sequence identifier in Table 4.

188. The trRNA of any one of claims –186-187, further comprising three phosphorothioate (PS) bonds linking the first four nucleotides at the 5' end of the 5' terminus and three PS bonds linking the last four nucleotides at the 3' end of the 3' terminus.

189. The trRNA of any one of claims 164-188, wherein the trRNA combined with a crRNA forms a ribonucleoprotein complex with an *S. pyogenes* Cas9.

190. A dual guide comprising a crRNA and a trRNA, wherein the crRNA comprises any one of SEQ ID Nos: 1-187 as depicted in Table 4, and wherein the trRNA comprises the nucleic acids of any one of SEQ ID Nos: 188-227 as depicted in Table 4.

191. A dual guide comprising a crRNA of any one of claims 137-163 and a trRNA of any one of claims 164-189.

192. A dual guide comprising a crRNA of any one of claims 137-163 and an unmodified trRNA.

193. A dual guide comprising an unmodified crRNA and a trRNA of any one of claims 137-189.

194. A dual guide of any one of claims 190-193, wherein the dual guide forms a ribonucleoprotein complex with an *S. pyogenes* Cas9.

195. An LNP composition comprising an sgRNA of any one of claims 1-78 and 129-136.

196. An LNP composition comprising a gRNA comprising or consisting of the RNA of any one of claims 1-193.

197. A composition comprising an sgRNA of any one of claims 1-78 and 129-136 associated with a lipid nanoparticle (LNP).

198. A composition comprising a crRNA of any of claims 137-163 associated with a lipid nanoparticle (LNP).

199. A composition comprising a trRNA of any of claims 164-189 associated with a lipid nanoparticle (LNP).

200. A composition comprising the sgRNA of any one of claims 1-78 and 129-136, the gRNA of any one of claims 79-128, the crRNA of any one of claims 137-163, the trRNA of any one of claims 164-189, the dgRNA of any one of claims 190-194, or the

composition of any one of claims 195-199, further comprising a nuclease or an mRNA which encodes the nuclease.

201. The composition of claim 200, wherein the nuclease is a Cas protein.

202. The composition of claim 201, wherein the Cas protein is a Cas9.

203. The composition of claim 202, wherein the Cas9 is an *S. pyogenes* Cas9.

204. The composition of any one of claims 200-203, wherein the nuclease is a nickase.

205. The composition of any one of claims 200-204, wherein the nuclease is modified.

206. The composition of claim 205, wherein the modified nuclease comprises a nuclear localization signal (NLS).

207. The composition of any one of claims 200-206, comprising an mRNA which encodes the nuclease.

208. A pharmaceutical formulation comprising the sgRNA of any one of claims 1-78 and 129-136, the gRNA of any one of claims 79-128, the crRNA of any one of claims 137-163, the trRNA of any one of claims 164-189, the dgRNA of any one of claims 190-194, or the composition of any one of claims 195-207 and a pharmaceutically acceptable carrier.

209. A method of modifying a target DNA comprising, delivering a Cas protein or a nucleic acid encoding a Cas protein, and any one or more of the following to a cell:

a. the sgRNA of any one of claims 1-78 and 129-136;
b. the gRNA of any one of claims 79-128;
c. the crRNA of any one of claims 137-163;
d. the trRNA of any one of claims 164-189;
e. the dgRNA of any one of claims 190-194;
f. the composition of any one of claims 195-207; or
g. the pharmaceutical formulation of claim 208.

210. The method of claim 209, wherein the method results in an insertion or deletion in a gene.

211. The method of claim 209 further comprising delivering to the cell a template, wherein at least a part of the template incorporates into a target DNA at or near a double strand break site induced by the Cas protein.

212. The sgRNA of any one of claims 1-78 and 129-136 for use in preparing a medicament for treating a disease or disorder.

213. The crRNA of any one of claims 737-163 for use in preparing a medicament for treating a disease or disorder.

214. The trRNA of any one of claims 164-189 for use in preparing a medicament for treating a disease or disorder.

215. The crRNA of any one of claims 137-163 in combination with the trRNA of any one of claims 164-189 for use in preparing a medicament for treating a disease or disorder.

216. The pharmaceutical formulation of claim 208 for use in preparing a medicament for treating a disease or disorder.