

We claim:

1. A guide RNA (gRNA) comprising a guide region and a conserved region, the conserved region comprising one or more of:
 - (a) a shortened repeat/anti-repeat region, wherein the shortened repeat/anti-repeat region lacks 2-24 nucleotides relative to SEQ ID NO: 500, wherein
 - (i) one or more of nucleotides 37-48 and 53-64 is deleted; and
 - (ii) nucleotide 36 is linked to nucleotide 65 by at least 2 nucleotides; or
 - (b) a shortened hairpin 1 region, wherein the shortened hairpin 1 region lacks 2-10, nucleotides relative to SEQ ID NO: 500, wherein
 - (i) one or more of nucleotides 82-86 and 91-95 is deleted relative to SEQ ID NO: 500; and
 - (ii) nucleotide 81 is linked to nucleotide 96 by at least 4 nucleotides; or
 - (c) a shortened hairpin 2 region, wherein the shortened hairpin 2 region lacks 2-18, nucleotides relative to SEQ ID NO: 500, wherein
 - (i) one or more of nucleotides 113-121 and 126-134 is deleted relative to SEQ ID NO: 500; and
 - (ii) nucleotide 112 is linked to nucleotide 135 by at least 4 nucleotides;

wherein at least 10 nucleotides are modified nucleotides.
2. The gRNA of claim 1, wherein one or more of nucleotides 37-64 is substituted relative to SEQ ID NO: 500.
3. The gRNA of claim 1, wherein one or more of nucleotides 82-96 is substituted relative to SEQ ID NO: 500.
4. The gRNA of claim 1, wherein the gRNA is a single-guide RNA (sgRNA) and wherein the gRNA comprises (a) a shortened repeat/anti-repeat region, wherein the shortened repeat/anti-repeat region lacks 2-24 nucleotides relative to SEQ ID NO: 500, wherein
 - (i) one or more of nucleotides 37-48 and 53-64 is deleted relative to SEQ ID NO: 500; and
 - (ii) nucleotide 36 is linked to nucleotide 65 by at least 2 nucleotides.
5. The gRNA of claim 1, wherein the guide region has
 - a. an insertion of one nucleotide or a deletion of 1-4 nucleotides within positions 1-24 relative to SEQ ID NO: 500, or

- b. a length of 25, 24, 23, 22, 21, or 20 nucleotides, optionally wherein (i) the guide region has a length of 25, 24, 23, or 22 nucleotides, optionally a length of 23-24 nucleotides, further optionally a length of 24 nucleotides.
6. The gRNA of any one of claims 1-3, wherein the 3' nucleotide of the gRNA is a uridine or a modified uridine.
7. The gRNA of claim 1, wherein the gRNA further comprises a 3' tail, optionally (i) wherein the 3' tail comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides, further optionally wherein the 3' tail comprises 1, 2, 3, 4, or 5 nucleotides, further optionally wherein the 3' tail is 1 nucleotide in length, and/or (ii) wherein the 3' tail terminates with a uridine or a modified uridine, further optionally wherein the 3' tail consists of a uridine or a modified uridine.
8. The gRNA of claim 5, wherein the 3' tail comprises a modification of any one or more of the nucleotides present in the 3' tail, further optionally wherein the modification of the 3' tail is one or more of 2'-O-methyl (2'-OMe) modified nucleotide and a phosphorothioate (PS) linkage between nucleotides, and further optionally wherein the 3' tail is fully modified.
9. The gRNA of claim 1, wherein one or both of nucleotides 144 and 145 are deleted relative to SEQ ID NO: 500.
10. The gRNA of claim 1, wherein the gRNA does not comprise a 3' tail.
11. The gRNA of claim 1, wherein (1) the shortened repeat/anti-repeat region lacks 2-24 nucleotides, optionally 12-24, 18-24, or 20-22 nucleotides; and/or (2) the shortened repeat/anti-repeat region has a length of 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 nucleotides, optionally a length of 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 nucleotides, further optionally a length of 28, 29, 30, 31, 32, 33, or 34 nucleotides, or 30, 31, or 32 nucleotides.
12. The gRNA of claim 1, wherein nucleotides 37-64 of SEQ ID NO: 500 form the upper stem, and one or more base pairs of the upper stem of the shortened repeat/anti-repeat region are deleted, optionally wherein
 - a. all of positions 39-48 and all of positions 53-62 of the upper stem of the shortened repeat/anti-repeat region are deleted, optionally wherein nucleotide 38 or 63 is substituted;
 - b. all of positions 38-48 and all of positions 53-63 of the upper stem of the shortened repeat/anti-repeat region are deleted, optionally wherein nucleotide 37 or 64 is substituted;
 - c. all of nucleotides 37-48 and 53-64 of the upper stem of the shortened repeat/anti-repeat region are deleted, optionally wherein nucleotides 36 or 65 is substituted;

optionally wherein the upper stem of the shortened repeat/anti-repeat region comprises no more than one, two, three, or four base pairs.

13. The gRNA of claim 1, wherein the shortened repeat/anti-repeat region has a duplex portion 11 base paired nucleotides in length, optionally wherein the shortened repeat/anti-repeat region has a single duplex portion.
14. The gRNA of claim 1, wherein the shortened repeat/anti-repeat region has a first duplex portion and a second duplex portion, optionally wherein the second duplex portion is 2-3 base paired nucleotides in length, further optionally wherein the first duplex portion is 11 base paired nucleotides in length and the second duplex portion is 3 base paired nucleotides in length.
15. The gRNA of claim 1, wherein the upper stem of the shortened repeat/anti-repeat region includes one or more substitutions relative to SEQ ID NO: 500, optionally wherein one or more of nucleotides 49-52 is substituted relative to SEQ ID NO: 500; and/or wherein the shortened repeat/anti-repeat region is unsubstituted.
16. The gRNA of claim 1, wherein the shortened repeat/anti-repeat region has 12-22 modified nucleotides, optionally wherein the shortened repeat/anti-repeat region does not comprise a phosphorothioate (PS) modification at nucleotide 76, further optionally wherein the shortened repeat/anti-repeat region does not comprise a modification at nucleotide 76.
17. The gRNA of claim 1, wherein
 - a. the shortened hairpin 1 region lacks 2-10 nucleotides, optionally 2-8 or 2-4 nucleotides; and/or
 - b. the shortened hairpin 1 region has a length of 13, 14, 15, 16, 17, 18, 19, 20, or 21 nucleotides.
18. The gRNA of claim 1, wherein the shortened hairpin 1 region has a duplex portion 4-8, optionally 7-8 base paired nucleotides in length, optionally wherein the shortened hairpin 1 region has a single duplex portion.
19. The gRNA of claim 1, wherein
 - a. one or two base pairs of the shortened hairpin 1 region are deleted;
 - b. the stem of the shortened hairpin 1 region is seven or eight base paired nucleotides in length; and/or
 - c. one or more of positions 85-86 and one or more of nucleotides 91-92 of the shortened hairpin 1 region are deleted, optionally wherein nucleotides 86 and 91 or nucleotides 85 and 92 of the shortened hairpin 1 region are deleted.

20. The gRNA of claim 1, wherein one or more of nucleotides 82-95 of the shortened hairpin 1 region is substituted relative to SEQ ID NO: 500, optionally wherein one or more of nucleotides 87-90 is substituted relative to SEQ ID NO: 500; and/or wherein the shortened hairpin 1 region is unsubstituted.
21. The gRNA of claim 1, wherein the shortened hairpin 1 region has 6-15 modified nucleotides.
22. The gRNA of claim 1, wherein
 - a. the shortened hairpin 2 region lacks 2-18, optionally 2-16 nucleotides; and/or
 - b. the shortened hairpin 2 region has a length of 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 nucleotides, optionally a length of 28, 29, 30, 31, 32, 33, or 34 nucleotides.
23. The gRNA of claim 1, wherein one or more of nucleotides 113-121 and one or more of nucleotides 126-134 of the shortened hairpin 2 region are deleted.
24. The gRNA of claim 1, wherein the shortened hairpin 2 region comprises an unpaired region and/or has two duplex portions.
25. The gRNA of claim 1, wherein the shortened hairpin 2 region has a duplex portion of 4 base paired nucleotides in length, optionally 4-6 base paired nucleotides in length, optionally 4 base paired nucleotides in length.
26. The gRNA of claim 1, wherein nucleotides 109-138 of SEQ ID NO: 500 form the upper stem, and the upper stem of the shortened hairpin 2 region comprises one, two, three, or four base pairs, optionally wherein
 - a. at least one pair of nucleotides 113 and 134, nucleotides 114 and 133, nucleotides 115 and 132, nucleotides 116 and 131, nucleotides 117 and 130, nucleotides 118 and 129, nucleotides 119 and 128, nucleotides 120 and 127, and nucleotides 121 and 126 are deleted; or
 - b. all of positions 113-121 and 126-134 of the shortened hairpin 2 region are deleted.
27. The gRNA of claim 1, wherein one or more of nucleotides 113-134 of the shortened hairpin 2 region is substituted relative to SEQ ID NO: 500, or one or more of nucleotides 122-125 is substituted relative to SEQ ID NO: 500; and/or wherein the shortened hairpin 2 region is unsubstituted.
28. The gRNA of claim 1, wherein the shortened hairpin 2 region comprises at least two modified nucleotides, optionally at least four modified nucleotides, optionally 6-15 modified nucleotides.

29. The gRNA of claim 1, wherein the guide region of the gRNA comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 modified nucleotides, optionally 1, 2, or 3 modified nucleotides; 4, 5, 6, 7, 8, 9, 10, 11, or 12 modified nucleotides; or 6, 7, 8, 9, 10, 11, or 12 modified nucleotides.
30. The gRNA of claim 1, wherein (1) the guide region does not comprise a modified nucleotide 3' of the first three nucleotides of the guide region; (2) wherein the guide region does not comprise a modified nucleotide; or (3) wherein nucleotides 1-3 of the guide region are modified and nucleotides in the guide region other than nucleotides 1-3 are not modified.
31. The gRNA of claim 1, comprising (1) a 3' end modification; (2) a 5' end modification; (3) a modification in the upper stem region of the repeat/anti-repeat region; (4) a modification in the hairpin 1 region; and/or (5) a modification in the hairpin 2 region.
32. The gRNA of claim 29, wherein:
 - a. the modification in the hairpin 2 region comprises a modification at 1, 2, 3, or 4 nucleotides of nucleotides 106-109, optionally wherein the modification comprises a 2'-O-methyl (2'-O-Me) modification;
 - b. the modification in the repeat/anti-repeat region does not comprise a modification at nucleotide 76; and/or
 - c. the modification in the repeat/anti-repeat region does not comprise a phosphorothioate (PS) modification at nucleotide 76.
33. The gRNA of claim 29, wherein the 5' end modification comprises a modification of any one or more of the first 1, 2, 3, or 4 nucleotides, optionally comprising one, two, three or four modified nucleotides; and/or wherein the 3' end modification comprises a modification of any one or more of the last 1, 2, 3, or 4 nucleotides, optionally comprising one, two, three or four modified nucleotides, optionally wherein the 5' end modification and/or 3' end modification comprises one or more of a phosphorothioate (PS) linkage between nucleotides, a 2'-OMe modified nucleotide, a 2'-O-moe modified nucleotide, a 2'-F modified nucleotide; and an inverted abasic modified nucleotide.
34. The gRNA of claim 29, wherein the 5' end modification comprises
 - a. at least one PS linkage, and wherein one or more of:
 - i. there is one PS linkage, and the linkage is between the first and second nucleotides;
 - ii. there are two PS linkages between the first three nucleotides;
 - iii. there are PS linkages between any one or more of the first four nucleotides; and

- iv. there are PS linkages between any one or more of the first five nucleotides optionally further comprising at least one 2'-O-Me, 2'-O-moe, inverted abasic, or 2'-F modified nucleotide; and/or

b. a modification to:

- i. one or more of the first 1-4 nucleotides, wherein the modification is a PS linkage, inverted abasic nucleotide, 2'-OMe, 2'-O-moe, or 2'-F;
- ii. the first nucleotide with 2'-Ome, 2'-O-moe, or 2'-F, and an optional one or two PS linkages to the next nucleotide or the first nucleotide of the 3' tail;
- iii. the first or second nucleotide with 2'-Ome, 2'-O-moe, or 2'-F, and optionally one or more PS linkages;
- iv. the first, second, or third nucleotides with 2'-Ome, 2'-O-moe, or 2'-F, and optionally one or more PS linkages; and/or
- v. the first, second, third, or forth nucleotides with 2'-Ome, 2'-O-moe, or 2'-F, and optionally one or more PS linkages.

35. The gRNA of claim 1, wherein the 3' end modification comprises

a. at least one PS linkage, and wherein one or more of:

- i. there is one PS linkage, and the linkage is between the last and second to last nucleotides;
- ii. there are two PS linkages between the last three nucleotides; and

b. there are PS linkages between any one or more of the last four nucleotides, optionally further comprising at least one 2'-O-Me, 2'-O-moe, inverted abasic, or 2'-F modified nucleotide; and/or

c. a modification to:

- i. one or more of the last 1-4 nucleotides, wherein the modification is a PS linkage, inverted abasic nucleotide, 2'-OMe, 2'-O-moe, or 2'-F;
- ii. the last nucleotide with 2'-OMe, 2'-O-moe, or 2'-F, and an optional one or two PS linkages to the next nucleotide or the first nucleotide of the 3' tail;
- iii. the last or second to last nucleotide with 2'-OMe, 2'-O-moe, or 2'-F, and optionally one or more PS linkages;
- iv. the last, second to last, or third to last nucleotides with 2'-OMe, 2'-O-moe, or 2'-F, and optionally one or more PS linkages; and/or
- v. the last, second to last, third to last, or fourth to last nucleotides with 2'-OMe, 2'-O-moe, or 2'-F, and optionally one or more PS linkages.

36. The gRNA of claim 1, wherein the modification in the repeat/anti-repeat region, the hairpin 1 region, or the hairpin 2 region comprises a modified nucleotide selected from 2'-O-methyl (2'-OMe) modified nucleotide, 2'-O-(2-methoxyethyl) (2'-O-moe) modified nucleotide, a 2'-fluoro (2'-F) modified nucleotide, or a phosphorothioate (PS) linkage between nucleotides, optionally a modified nucleotide selected from 2'-O-methyl (2'-OMe) modified nucleotide or a phosphorothioate (PS) linkage between nucleotides.
37. The gRNA of claim 1, wherein at least 20%, 30%, 40%, or 50% of the nucleotides are modified nucleotides.
38. The gRNA of claim 1, wherein a 3' tail of nucleotide 144 is present in the gRNA, and comprises 2'-O-Me modified nucleotides at nucleotides 141-144 and two PS linkages between nucleotides 141-142 and 142-143 respectively.
39. The gRNA of claim 1, wherein one or more positions of 49-52, 87-90, or 122-125 is substituted.
40. A single guide RNA (sgRNA) comprising
 - a. any one of SEQ ID NOs: 17, 9, 25, 32, 1-8, 10-16, 18-19, 21- 24, 26-31, and 33- 42;
 - b. a nucleotide sequence having at least 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 85, 80, 75, or 70% identity to the nucleotide sequence of any one of SEQ ID Nos: 17, 9, 25, 32, 1-8, 10-16, 18, 19, 21-24, 26-31, and 33-42
 - c. a nucleotide sequence having at least 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 85, 80, 75, or 70% identity to the nucleotide sequence of any one of SEQ ID Nos: 17, 9, 25, 32, 1-8, 10-16, 18, 19 21-24, 26-31, and 33-42, wherein the modification at each nucleotide of the gRNA that corresponds to a nucleotide of the reference sequence identifier in Table 1 is identical to or equivalent to the modification shown in the reference sequence identifier in Table 2; or
 - d. comprising a nucleotide sequence having at least 99, 98, 97, 96, 95, 94, 93, 92, 91, or 90% identity to the sequence from X to the 3' end of the nucleotide sequence of any one of SEQ ID Nos: 16, 8, 24, 31, 1-5, 7, 11, 12, 13, 15, 18, 19, 21, 23, 26, 27, 28, 30, 33, 34, 35, 37, 39, 41, 101-291, 301-494, 931-946, 951, and 952, where X is the first nucleotide of the conserved region;
 - e. a nucleotide sequence having at least 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 85, 80, 75, or 70% identity to the nucleotide sequence of any one of SEQ ID NOs: 17, 9, 25, or 32;

- f. a nucleotide sequence having at least 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 85, 80, 75, or 70% identity to the nucleotide sequence of any one of SEQ ID NOs: 16, 8, 24, or 31, wherein (N)₂₀₋₂₅ form the guide region; and/or
 - g. a nucleotide sequence having at least 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 85, 80, 75, or 70% identity to the nucleotide sequence of any one of SEQ ID Nos: 15, 7, 23 or 30, wherein the totality of N's form the guide region.
41. A guide RNA (gRNA) comprising a guide region and a conserved region, the gRNA comprising:
- (a) SEQ ID NO: 17;
 - (b) a nucleotide sequence having at least 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 85, 80, 75, or 70% identity to the nucleotide sequence of SEQ ID NO: 17;
 - (c) a nucleotide sequence having at least 99, 98, 97, 96, 95, 94, 93, 92, 91, or 90% identity to the sequence from X to the 3' end of the nucleotide sequence of SEQ ID NO: 16, where X is the first nucleotide of a conserved region of the gRNA;
 - (d) a nucleotide sequence having at least 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 85, 80, 75, or 70% identity to the nucleotide sequence of SEQ ID NO: 16, wherein (N)₂₀₋₂₅ form a guide region of the gRNA; or
 - (e) a nucleotide sequence having at least 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 85, 80, 75, or 70% identity to the nucleotide sequence of SEQ ID NO: 15, wherein the totality of N's form a guide region of the gRNA.
42. The gRNA of claim 1, comprising
- (a) a nucleotide sequence having at least 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 85, 80, 75, or 70% identity to the nucleotide sequence of any one of SEQ ID Nos: 17, 9, 25, 32;
 - (b) a nucleotide sequence having at least 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 85, 80, 75, or 70% identity to the nucleotide sequence of any one of SEQ ID NOs: 16, 8, 24, or 31, wherein (N)₂₀₋₂₅ form the guide region; or
 - (c) a nucleotide sequence having at least 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 85, 80, 75, or 70% identity to the nucleotide sequence of any one of SEQ ID Nos: 15, 7, 23 or 30, wherein the totality of N's form the guide region.
43. The gRNA of claim 40, further comprising a 3' tail comprising a 2'-O-Me modified nucleotide.
44. The gRNA of claim 1, 40 or 41, wherein the gRNA directs a nuclease to a target sequence for binding, for inducing a single-strand break within the target sequence, or for inducing a double-strand break within the target sequence.

45. The gRNA of claim 1, 40, or 41, wherein the gRNA comprises a conservative substitution, optionally wherein the conservative substitution maintains at least one base pair.
46. A composition comprising a gRNA of claim 1, 40 or 41, associated with a lipid nanoparticle (LNP).
47. A lipid nanoparticle (LNP) composition comprising a gRNA of claim 1, 40, or 41.
48. A composition comprising the gRNA of claim 1, 40 or 41, or an LNP composition comprising of the gRNA of claim 1, 40, or 41, further comprising a nuclease or an mRNA which encodes the nuclease, optionally wherein the nuclease is a Cas protein, optionally a NmeCas9, further optionally an Nme1Cas9, an Nme2Cas9, or an Nme3Cas9, and further optionally wherein the mRNA comprises the sequence of any one of SEQ ID NOs: 636-638.
49. The composition of claim 48, wherein the nuclease has a dCas DNA binding domain, nickase activity, or double strand cleaving activity.
50. The composition of claim 48, wherein the nuclease is modified, optionally wherein the modified nuclease comprises (i) a heterologous functional domain, optionally a deaminase, optionally a cytidine deaminase, and/or (ii) a nuclear localization signal (NLS).
51. The composition of claim 50, wherein the modified nuclease comprises a deaminase and the composition further comprises a UGI or a mRNA encoding a UGI.
52. The composition of claim 50, wherein (i) the nuclease or modified nuclease comprises an amino acid sequence with at least 90%, 93%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to any one of SEQ ID NOs: 600-603, 605, 607-620, or 707-712; (ii) the mRNA encoding the nuclease or modified nuclease comprises a nucleotide sequence with at least 90%, 93%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to any one of SEQ ID NOs: 621-623, 626-643, 645, 647-668, 701-706, and 713-718; (iii) the mRNA encoding the nuclease or modified nuclease comprises an open reading frame (ORF) comprising a sequence with at least 90%, 93%, 95%, 96%, 97%, 98%, or 99%, or with 100% identity to any one of SEQ ID NOs: 621-623, 626-639, and 713-718.
53. A pharmaceutical formulation comprising the gRNA of claim 1, 40 or 41, a composition comprising the gRNA of claim 1, 40, or 41 and a nuclease or a nucleic acid encoding a Cas protein, or an LNP composition comprising the gRNA of claim 1, 40, or 41 and a pharmaceutically acceptable carrier.
54. 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:
 - i. the gRNA of any one of claim 1, 40, or 41;

- ii. an LNP composition comprising the gRNA of claim 1, 40 or 41; and
- iii. a pharmaceutical formulation comprising the gRNA of (i) or the composition of (ii) and a pharmaceutically acceptable carrier; and

optionally, 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, and further optionally wherein the method results in (i) an insertion or deletion in a gene or (ii) at least one base edit

- 55. The gRNA of claim 1, 40, or 41, an LNP composition comprising the gRNA of claim 1, 40, or 41, or a pharmaceutical formulation comprising the gRNA of claim 1, 40, for use in therapy.
- 56. The gRNA of claim 1, 40, or 41, an LNP composition comprising the gRNA of claim 1, 40, or 41, or a pharmaceutical formulation comprising the gRNA of claim 1, 40, for use as a medicament.
- 57. Use of the gRNA of claim 1, 40, or 41, an LNP composition comprising the gRNA of claim 1, 40 or 41, or a pharmaceutical formulation comprising the gRNA of claim 1, 40 or 41 in the manufacture of a medicament for treating a disease or disorder.