

CLAIMS

1. A method comprising, contacting a first RNA with a second RNA,

wherein the first RNA and the second RNA comprise at least five RNA nucleotides that are complementary, and wherein the contacting forms a stem structure or a stem loop
5 structure, and

ligating the first RNA and the second RNA with a ligating enzyme
 - (i) within the stem structure, or
 - (ii) at an end of the stem structure, thereby forming a loop at the end of the stem structure.
- 10 2. The method of claim 1, wherein the contacting forms a stem structure and the ligating enzyme ligates the first RNA and the second RNA at an end of the stem structure, thereby forming a loop at the end of the stem structure.
3. The method of claim 1, wherein the contacting forms a stem loop structure and the ligating enzyme ligates the first RNA and the second RNA within a stem of the stem loop
15 structure.
4. The method of any one of the preceding claims, wherein the ligating enzyme is selected from the group consisting of T4 RNA ligase 1, T4 RNA Ligase 2, RtcB Ligase, Thermo-stable 5' App DNA/RNA Ligase, ElectroLigase, T4 DNA Ligase, T3 DNA Ligase, T7 DNA Ligase, Taq DNA Ligase, SplintR Ligase E. coli DNA Ligase, 9°N DNA Ligase, CircLigase,
20 CircLigase II, DNA Ligase I, DNA Ligase III, and DNA Ligase IV.
5. The method of claim 2, wherein the ligating enzyme is T4 RNA ligase 1.
6. The method of claim 3, wherein the ligating enzyme is T4 RNA ligase 2.
7. The method of any one of the preceding claims, wherein the first and/or second RNA is chemically synthesized.
- 25 8. The method of any one of the preceding claims, wherein the first RNA is a clustered regularly interspersed short palindromic repeats (CRISPR) RNA (crRNA) and the second RNA is a trans-activating RNA (tracrRNA).

9. The method of any one of the preceding claims wherein a guide RNA (gRNA) is produced.
10. The method of any one of the preceding claims, wherein the first RNA and/or the second RNA is chemically synthesized.
- 5 11. The method of any one of claims 1-9, wherein the first and/or the second RNA is enzymatically synthesized.
12. The method of any one of the preceding claims, wherein ligating the first RNA and the second RNA with a ligating enzyme creates phosphodiester linkages between the first and the second RNA.
- 10 13. The method of any one of the preceding claims, wherein the first RNA and/or second RNA nucleotide is engineered to allow for non-covalent assembly.
14. The method of any one of the preceding claims, wherein the stem loop has a length of between about 2-50 nucleotides.
15. The method of any one of the preceding claims, wherein the first RNA and the second RNA comprise at least two RNA nucleotides that have perfect complementarity.
- 15 16. The method of claim 14, wherein the first RNA and the second RNA comprise at least three, four, five, six or seven consecutive RNA nucleotides that have perfect complementarity.
17. The method of claim 15 or 16, wherein the RNA nucleotides that have perfect complementarity are present in a top stem and/or in a bottom stem.
- 20 18. The method of any one of the preceding claims, wherein the first RNA and the second RNA comprise at least five, six, or seven consecutive RNA nucleotides that are complementary at a lower stem formed by the first RNA and the second RNA.
19. The method of any one of the preceding claims, wherein the first RNA and the second RNA comprise at least four to fourteen consecutive RNA nucleotides that are complementary at an upper stem.
- 25 20. The method of claim 19, wherein the first RNA and the second RNA comprise four consecutive RNA nucleotides that are complementary at an upper stem.

21. The method of claim 19, wherein the first and the second RNA comprise five consecutive RNA nucleotides that are complementary at an upper stem.
22. The method of claim 19, wherein the first and the second RNA comprise seven consecutive RNA nucleotides that are complementary at an upper stem.
- 5 23. The method of claim 19, wherein the first and the second RNA comprises 14 consecutive RNA nucleotides that are complementary at an upper stem.
24. The method of any one of the preceding claims, wherein the first and the second RNA comprise 7 consecutive RNA nucleotides that are complementary at a lower stem.
25. The method of any one of the preceding claims, wherein the first RNA and/or the second
10 RNA is engineered to create a ligation site for a ligation enzyme.
26. The method of any of the preceding claims, wherein the stem loop comprises a loop of 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 nucleotides.
27. The method of any one of the preceding claims, wherein the stem loop comprises a tetraloop.
- 15 28. The method of claim 16, wherein the loop comprises 7 nucleotides.
29. The method of any one of claims 26-28, wherein ligating the first RNA and the second RNA occurs at a ligation site that is at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 base pairs from the loop.
30. The method of claim 29, wherein the ligation site is 2 or 3 base pairs from the loop.
- 20 31. The method of any one of the preceding claims, wherein ligating the first RNA and the second RNA occurs at a ligation site that is at least 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 base pairs from a bulge.
32. The method of claim 31, wherein ligating the first RNA and the second RNA occurs at a ligation site that is 3, 4, 5, or 11 base pairs from the bulge.
- 25 33. The method of any one of the preceding claims, wherein the first RNA and/or second RNA is enzymatically produced.

34. The method of any one the preceding claims, wherein the first RNA comprises a 3' sequence that is capable of base pairing with a portion of the second RNA.
35. The method of any one of the preceding claims, wherein the first RNA comprises a phosphate at the 5' terminus.
- 5 36. The method of claim 35, wherein the first RNA is a donor RNA.
37. The method of any one the preceding claims, wherein the second RNA comprises a variable protospacer region.
38. The method of claim 37, wherein the second RNA is an acceptor RNA.
39. The method of claim 35, wherein the first RNA comprises an adenosine triphosphate at
10 the 5' terminus.
40. The method of any one of the preceding claims, wherein about 8-50 nucleotides are complementary and allow for base pairing between the first and the second RNA.
41. The method of claim 40, wherein the 8-50 nucleotides are partially complementary.
42. The method of claim 41, wherein the 8-50 nucleotides are from about 50% to 99%
15 complementary.
43. The method of claim 41, wherein the 8-50 nucleotides are perfectly complementary.
44. The method of any one of the preceding claims, wherein the first and the second RNA have different nucleotide lengths.
45. The method of claim 44, wherein the first RNA has from about 20-100 nucleotides.
- 20 46. The method of claim any one of the preceding claims, wherein the second RNA has from about 20-70 nucleotides.
47. The method of any one of the preceding claims, wherein base pairing occurs in a lower stem.
48. The method of claim 47, wherein 7 nucleotides are complementary in the lower stem
25 and allow for base pairing between the first RNA and the second RNA.

49. The method of any claim 47 or 48, wherein the base pairing occurs in an upper stem.
50. The method of claim 49, wherein 2 nucleotides are complementary in the upper stem and allow for base pairing between the first RNA and the second RNA.
51. The method of any one of claims 9-50, wherein the gRNA has a length of about 100
5 nucleotides, about 125 nucleotides, about 150 nucleotides, about 175 nucleotides, about 200 nucleotides, or greater than about 200 nucleotides.
52. The method of any one of the preceding claims, wherein the gRNA is an extended guide RNA, prime editor guide RNA (pegRNA), or a Cas12 guide RNA such as Cas12a guide RNA, Cas12b guide RNA, Cas12c guide RNA, Cas12d, guide RNA, Cas12e guide RNA,
10 Cas12f guide RNA, Cas12g guide RNA, Cas12h guide RNA, Cas12i guide RNA, Cas12j guide RNA, or Cas12k guide RNA.
53. The method of any one of the preceding claims, wherein the gRNA comprises one or more of the following: a spacer, a lower stem, a bulge, an upper stem, a nexus and a hairpin.
54. The method of any one of the preceding claims, wherein the first RNA and the second
15 RNA are present at a ratio of about 0.5:1, 0.6:1, 0.7:1, 0.8:1, 0.9:1, 1:1, 1:0.9, 1:0.8, 1:0.7, 1:0.6, or 1:0.5.
55. The method of any one of the preceding claims, wherein the gRNA is produced at a yield of about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99% or more.
56. The method of any one of the preceding claims, wherein the gRNA is produced at 50%,
20 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99% or more improvement in yield as compared to conventional synthetic methods.
57. A method of producing a synthetic guide RNA (gRNA) comprising:
providing a first RNA comprising a 5' -monophosphate;
providing a second RNA';
25 providing an oligonucleotide that has partial complementarity to the first RNA and the second RNA, wherein the complementarity of the oligonucleotide allows for base pairing with the first and the second RNA; and

providing a ligase to catalyze ligation between the first and the second RNA, thus producing the synthetic gRNA.

58. A method of producing a synthetic guide RNA (gRNA) comprising:

- providing a first RNA comprising a 5' –monophosphate;
- 5 providing a second RNA comprising a blocked 3' end; and
- providing a ligase to catalyze ligation between the first and the second RNA, thus producing the synthetic gRNA.

59. The method of any one of claims 57-58, wherein the first RNA is a trans-activating RNA (tracrRNA), and the second RNA is a clustered regularly interspersed short palindromic repeats (CRISPR) RNA (crRNA).

60. The method of claim 57, wherein the oligonucleotide is about 100 nucleotides long.

61. A method of producing a synthetic guide RNA (gRNA) comprising:

- providing two or more RNA fragments;
- providing an oligonucleotide that has partial complementarity to the two or more
- 15 RNA fragments, wherein the complementarity of the oligonucleotide allows for base pairing with the two or more RNA fragments; and
- providing a ligase to catalyze ligation between the two or more RNA fragments, thus producing the synthetic guide RNA.

62. The method of claim 61, wherein the two or more RNA fragments are ligated at an overhang, blunt end, or at a bulge.

63. A guide RNA (gRNA) or prime editing guide RNA (pegRNA) synthesized by the method of any one of claim 1-62.

64. A method for targeted transcription activation, targeted transcription repression, targeted epigenome modification, or targeted genome modification, the method comprising

25 introducing into a eukaryotic cell:

- (a) a synthetic guide RNA (gRNA) as defined in any one of the preceding claims;
- (b) at least one CRISPR/Cas protein or a nucleic acid encoding the at least one CRISPR/Cas protein;

wherein interactions between (a) and (b) and a target sequence in chromosomal DNA leads to targeted transcription activation, targeted transcription repression, targeted epigenome modification, or targeted genome modification.

65. A method for targeted RNA modification, the method comprising introducing into a
5 eukaryotic cell:

(a) a synthetic guide RNA (gRNA) as defined in any one of the preceding claims;

(b) at least one CRISPR/Cas protein or a nucleic acid encoding the at least one CRISPR/Cas protein;

wherein interactions between (a) and (b) and an RNA expressed by chromosomal
10 DNA leads to a modification of the RNA expressed by the chromosomal DNA.

66. The method of claim 65, wherein the RNA expressed by the chromosomal DNA is a messenger RNA (mRNA).

67. The method of any one of claims 64-66, wherein the CRISPR/Cas protein is selected from Cas9, Cpf1, SaCas, Cas12, Cas13, or modified versions thereof.

15 68. A method for producing synthetic guide RNA (gRNA) according to any one of claims 1-67.

69. The method of claim 68, wherein the second RNA comprises a 3' sequence that is capable of base pairing with a portion of the first RNA.

70. The method of claim 68 or 69, wherein the second RNA comprises a variable
20 protospacer region.

71. The method of any one of claims 68-70, wherein the first RNA comprises a phosphate at the 5' terminus.

72. The method of any one of claims 68-71, wherein the contacting forms a stem loop structure and the ligating enzyme ligates the first RNA and the second RNA within a stem of
25 the stem loop structure.

73. The method of claim 72, wherein the ligating enzyme is T4 RNA ligase 2.

74. The method of claim 73, wherein the stem loop comprises GC base pairs in the upper stem.
75. The method of claim 74, wherein the upper stem comprises a nucleotide sequence at least about 80% identical to CGAUACGACAGAAC.
- 5 76. The method of claim 74, wherein the upper stem comprises a nucleotide sequence at least about 80% identical to CGCCG.
77. The method of claim 74, wherein the upper stem comprises a nucleotide sequence at least about 80% identical to CGGCCGC.
78. The method of claim 74, wherein the upper stem comprises a nucleotide sequence at least
10 about 80% identical to CGCGC.
79. The method of claim 74, wherein the upper stem comprises a nucleotide sequence at least about 80% identical to CGAU.
80. The method of claim 72, wherein the stem loop comprises GC base pairs in the lower stem.
- 15 81. The method of any one of claims 68-79, wherein the lower stem does not comprise GC base pairs.
82. The method of any one of claims 68-81, wherein the upper stem does not comprise a GC base pair.
83. The method of any one of claims 68-81, wherein the upper the stem comprises at least 1,
20 2, 3, 4, 5, or 6, 7, 8, 9, 10, 11, or 12 GC base pairs.
84. The method of claim 83, wherein the upper portion of the stem comprises 2 GC nucleotides.
85. The method of any one of claims 68-84, wherein ligating the first and the second RNA results in a yield of at least 60%, 70%, 80%, 90%, or more than 95% of full-length product.
- 25 86. The method of claim 85, wherein ligating the first and the second RNA results in a yield of at least about 60%.

87. The method of any one of the claims 1-63 or 68-86, wherein the gRNA is produced at a quantity of at least 1 gram.
88. The method of claim 87, wherein large scale comprises at least 5 grams, 10 grams, 20 grams, 30 grams, 40 grams, 50 grams, 60 grams, 70 grams, 80 grams, 90 grams, or 100 grams.
89. The method of any one of claims 1-63 or 68-88, wherein the gRNA is produced at a quantity of less than 1 gram.
90. The method of claim 89, wherein the gRNA is produced at a quantity of about 0.05 grams, 0.1 grams, 0.2 grams, 0.3 grams, 0.4 grams, 0.5 grams, 0.6 grams, 0.7 grams, 0.8 grams, or 0.9g.
91. The method of any one of claims 68-90, wherein the method produces gRNA at a purity of about 50%, 60%, 70%, 80%, 90%, or more than 90%.
92. The method of any one of claims 68-91, wherein the first RNA is synthesized in a 3' to 5' direction.
93. The method of any one of claims 68-92, wherein the second RNA is synthesized in a 3' to 5' direction.
94. The method of any one of claims 68-93, wherein the gRNA has a length of about 100 nucleotides, about 125 nucleotides, about 150 nucleotides, about 175 nucleotides, about 200 nucleotides, or greater than about 200 nucleotides.
95. The method of any one of claims 68-94, wherein the loop comprises 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 nucleotides.
96. The method of claim 95, wherein the loop is a tetraloop.
97. The method of claim 95, wherein the loop comprises 7 nucleotides.
98. The method of any one of claims 68-97, wherein ligating the first RNA and the second RNA occurs at a ligation site that is at least about 3 base pairs from the loop.
99. The method of claim 98, wherein the ligation site is 1, 2, 3, 4, 5, 6, or 10 base pairs from the loop.

100. The method of any one of claims 1-63 or 68-99, wherein the first and/or second RNA comprises one or more backbone modifications.
101. The method of claim 100, wherein the one or more backbone modifications comprises a 2' O-methyl or a phosphorothioate modification.
- 5 102. The method of claim 100, wherein the one or more backbone modifications is selected from 2'-O-methyl 3'-phosphorothioate, 2'-O-methyl, 2'-ribo 3'-phosphorothioate, deoxy, or 5' phosphate modification.
103. The method of claim 101 or 102, wherein the one or more modifications are present at the site of ligation.
- 10 104. The method of claim 103, wherein the one or more modifications are present in the donor RNA and/or the acceptor RNA.
105. The method of claim 104, wherein the 3' and/or the 5' end of the donor RNA has one or more backbone modifications.
106. The method of claim 104, wherein the 3' and/or the 5' end of the acceptor RNA has one
15 or more backbone modifications.
107. The method of any one of claims 1-63 or 68-106, wherein the concentration of the first and/or second RNA is between about 1 g/L and 5 g/L.
108. The method of claim 107, wherein the concentration of the first and/or second RNA is about 1 g/L.
- 20 109. The method of claim 107, wherein the concentration of the first and/or second RNA is about 3 g/L.
110. A composition produced by the method of any one of the preceding claims comprising a first RNA comprising a phosphate at a 5' terminus and a second RNA comprising a variable protospacer region, wherein the first and the second RNA are non-covalently bound.
- 25 111. A composition produced by the method of any one of claims 1-103 comprising a first RNA comprising a phosphate at a 5' terminus and a second RNA comprising a variable protospacer region, and wherein the first and the second RNA are bound to a ligase.

112. The composition of claim 111, wherein the ligase is a T4 RNA ligase 2.
113. A composition comprising an RNA comprising a nucleotide sequence at least about 80% identical to CGAUACGACAGAAC.
114. The composition of claim 113, wherein the nucleotide sequence is identical to
5 CGAUACGACAGAAC.
115. A composition comprising an RNA comprising a nucleotide sequence at least about 80% identical to CGCCG.
116. The composition of claim 115, wherein the nucleotide sequence is identical to CGCCG.
117. A composition comprising an RNA comprising a nucleotide sequence at least about 80%
10 identical to CGGCCGC.
118. The composition of claim 117, wherein the nucleotide sequence is identical to CGGCCGC.
119. A composition comprising an RNA comprising a nucleotide sequence at least about 80% identical to CGCGC.
- 15 120. The composition of claim 119, wherein the nucleotide sequence is identical to CGCGC.
121. A kit comprising the composition of any one of claims 110-120.
122. A kit comprising a first RNA comprising trans-activating RNA (tracrRNA) sequence, a second RNA comprising a variable protospacer region, and a ligase.
123. The kit of claim 122, wherein the ligase is a T4 RNA Ligase 2.