where two or more synthetic RNAs are ligated using an enzyme. It was surprisingly found that a ligation-based approach increases the purity, yield and the integrity of the gRNA produced. The resultant purity, yield and integrity of the gRNA allows for increased editing efficiencies and a reduction of off-target editing as compared to previous methods of gRNA synthesis. The ligation-based method for the synthesis of gRNA includes methods comprising using two or more partially complementary synthetic RNAs that are subsequently ligated ("template approach"), and methods that do not require complementarity between two or more synthetic RNAs ("non-templated approach").

In one aspect, the present disclosure refers to 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 loop 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, wherein the ligation produces a linear guide RNA.

In one aspect, the present disclosure refers to 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 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, wherein the ligation produces a circular guide RNA.

In one example, a method is provided 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 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.

In some examples, 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.

In some examples, 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 structure.

In some examples, 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, CircLigase II, DNA Ligase I, DNA Ligase III, and DNA Ligase IV. Accordingly, in some examples, the ligating enzyme is T4 RNA ligase 1. In some examples, the ligating enzyme is T4 RNA ligase 2. In some examples, the ligating enzyme is RtcB Ligase. In some examples, the ligating enzyme is Thermo-stable 5′ App DNA/RNA Ligase. In some examples, the ligating enzyme is ElectroLigase. In some examples, the ligating enzyme is T4 DNA Ligase. In some examples, the ligating enzyme is T3 DNA Ligase. In some examples, the ligating enzyme is T7 DNA Ligase. In some examples, the ligating enzyme is Taq DNA Ligase. In some examples, the ligating enzyme is SplintR Ligase *E. coli* DNA Ligase. In some examples, the ligating enzyme is 9°N DNA Ligase. In some

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 loop 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,

wherein the ligation produces a linear guide RNA.

2. The method of claim 1, wherein the first RNA and/or second RNA is at least 100 nucleotides long.

3. The method of claim 1 or 2, 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, CircLigase II, DNA Ligase I, DNA Ligase III, and DNA Ligase IV.

4. The method of claim 1 or 2, wherein the first RNA and/or the second RNA is (i) chemically or enzymatically synthesized; (ii) creates phosphodiester linkages between the first and the second RNA when ligated; (iii) is engineered to allow for non-covalent assembly; and/or (iv) is engineered to create a ligation site for a ligation enzyme.

5. The method of any one of claims 1-4, 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).

6. The method of claim 1, wherein the guide RNA (gRNA):

(a) has a length of between 100 nucleotidesand 200 nucleotides; and/or

(b) 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, Cas12f guide RNA, Cas12g guide RNA, Cas12h guide RNA, Cas12i guide RNA, Cas12j guide RNA, or Cas12k guide RNA; and/or

(c) comprises one or more of the following: a spacer, a lower stem, a bulge, an upper stem, a nexus and a hairpin; and/or

(d) is produced at a yield of 50%or more.

7. The method of any one of claims 1-6, wherein the stem loop has a length of between 2-50 nucleotides.

8. The method of any one of claims 1-7, wherein the first and the second RNA comprise an upper stem, a lower stem, and a bulge formed by the first RNA and the second RNA.

9. The method of claim 8, wherein the first RNA and the second RNA comprise:

(a) at least two RNA nucleotides that have perfect complementarity; and/or

(b) at least five consecutive RNA nucleotides that are complementary at a lower stem formed by the first RNA and the second RNA; and/or

(c) at least four consecutive RNA nucleotides that are complementary at an upper stem; and/or

(d) seven consecutive RNA nucleotides that are complementary at a lower stem.

10. The method of any one of claims 1-9, wherein the stem loop comprises a loop of 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 nucleotides.

11. The method of claim 8 or 9, 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.

12. The method of claim 8 or 9, 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 the bulge.

13. The method of any one of claims 1-12, wherein the first RNA comprises

(a) a 3′ sequence that is capable of base pairing with a portion of the second RNA; and/or

(b) a phosphate at the 5′ terminus; and/or(c) an adenosine triphosphate at the 5′ terminus; and/or (d) is a donor DNA.

14. The method of any one of claims 1-13, wherein the second RNA comprises a variable protospacer region and/or is an acceptor RNA.

15. The method of any one of claims 1-14, wherein between 8-50 nucleotides are at least 50% complementary and allow for base pairing between the first and the second RNA.

16. The method of any one of claims 1-15, wherein:

the first and the second RNA have at least 20 nucleotides.

17. The method of any one of claims 1-16, wherein the first RNA and the second RNA are present at a ratio of 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.

18. The method of claim 1, wherein:

(a) the second RNA comprises a 3′ sequence that is capable of base pairing with a portion of the first RNA; and/or

(b) the second RNA comprises a variable protospacer region; and/or

(c) the first RNA comprises a phosphate at the 5′ terminus; and/or

(d) 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 structure.

19. The method of claim 18, wherein the ligating enzyme is T4 RNA ligase 2.

20. The method of claim 19, wherein the stem loop comprises at least 1 GC base pair in the upper and/or lower stem.

21. The method of claim 20, wherein the upper stem comprises a nucleotide sequence (i) at least 80% identical to CGAUACGACAGAAC; (ii) at least 80% identical to CGCCG; (iii) at least 80% identical to CGCCG; (iv) at least 80% identical to CGGCCGC; (v) at least 80% identical to CGCGC; or (vi) at least 80% identical to CGAU.

22. The method of any one of claims 18-23, wherein ligating the first and the second RNA results in a yield of at least 60% of full-length product.

23. The method of any one of the claims 18-22, wherein the gRNA is produced:

(a) at a quantity of at least 1 gram; (b) between 5 grams and 100 grams; or (c) between 0.05 grams and 0.9 grams; and/or

at a purity of at least 50%.

24. The method of any one of claims 18-23, wherein:

(a) the first RNA is synthesized in a 3′ to 5′ direction; and/or

(b) the second RNA is synthesized in a 3′ to 5′ direction; and/or

(c) the gRNA has a length of 100 nucleotides, 125 nucleotides, 150 nucleotides, 175 nucleotides, 200 nucleotides, or greater than 200 nucleotides; and/or

(d) the loop comprises 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 nucleotides ; and/or

(e) ligating the first RNA and the second RNA occurs at a ligation site that is at least 3 base pairs from the loop.

25. The method of any one of claims 18-24, wherein the first and/or second RNA comprises one or more backbone modifications.

26. The method of any one of claims 18-25, wherein the concentration of the first and/or second RNA is between 1g/L and 5 g/L.

27. 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 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,

wherein the ligation produces a circular guide RNA.