CLAIMS

1. A method of producing a therapeutic circular DNA vector, the method comprising:

(a) providing a sample comprising a template DNA vector comprising a therapeutic sequence and a backbone sequence;

(b) amplifying the template DNA vector using a polymerase-mediated rolling-circle amplification to generate a linear concatemer;

(c) digesting the linear concatemer with a type IIs restriction enzyme that cuts a first site and a second site per unit of the linear concatemer, wherein the first and second sites flank the therapeutic sequence and form self-complementary overhangs, thereby producing a linear therapeutic fragment and a linear backbone fragment, wherein the linear therapeutic fragment comprises the therapeutic sequence and the linear backbone fragment comprises the backbone sequence, or a portion thereof; and

(d) contacting the linear backbone fragment and the linear therapeutic fragment with a ligase to produce a circular backbone and a therapeutic circular DNA vector lacking a type IIs restriction site.

2. The method of claim 1, wherein the linear backbone fragment of (c) comprises a type IIs restriction site, the circular backbone of (d) comprises the type IIs restriction site, and the type IIs restriction enzyme cuts the circular backbone and does not cut the therapeutic circular DNA vector.

3. A method of producing a therapeutic circular DNA vector, the method comprising:

(a) providing a sample comprising a template DNA vector comprising a therapeutic sequence and a backbone sequence;

(b) amplifying the template DNA vector using a polymerase-mediated rolling-circle amplification to generate a linear concatemer;

(c) digesting the linear concatemer with one or more restriction enzymes that cut at least a first site, a second site, and a third site per unit of the linear concatemer, wherein: (i) the first and second sites flank the therapeutic sequence and form self-complementary overhangs, and (ii) the third site is within the backbone sequence and forms an overhang that is non-complementary to the first or second site, thereby producing a linear therapeutic fragment comprising the therapeutic sequence and at least two linear backbone fragments each comprising a portion of the backbone sequence; and

(d) contacting the linear therapeutic fragment with a ligase to produce a therapeutic circular DNA vector in solution.

4. The method of any one of claims 1-3, wherein the method further comprises diluting the DNA between steps (c) and step (d).

5. The method of any one of claims 1-4, wherein the DNA concentration at the beginning of step (d) is greater than or equal to 20 μg/mL but less than 160 μg/mL.

6. The method of any one of claims 1-5, wherein the ligase is a T4 ligase.

7. The method of any one of claims 3-6, wherein the linear concatemer is digested with a single restriction enzyme that cuts the first site, the second site, and the third site.

8. The method of claim 7, wherein the one or more restriction enzymes cut a fourth site of the linear concatemer per unit, wherein the fourth site is within the backbone sequence and forms an overhang that is non-complementary to the first or second site, and wherein the digestion produces at least three linear backbone fragments each comprising a portion of the backbone sequence.

9. The method of claim 8, wherein the single restriction enzyme cuts a fourth site of the linear concatemer per unit, wherein the fourth site is within the backbone sequence and forms an overhang that is non-complementary to the first or second site, and wherein the digestion produces at least three linear backbone fragments each comprising a portion of the backbone sequence.

10. The method of any one of claims 1, 2, 4-7, and 9, wherein the restriction enzyme is a type IIs restriction enzyme that is BsaI.

11. The method of any one of claims 1-10, wherein no restriction enzyme inactivation step precedes step (d), and/or wherein no temperature increase is performed between steps (c) and (d), and/or wherein no temperature increase is performed immediately after step (d), and/or wherein steps (c) and (d) occur simultaneously.

12. The method of any one of claims 1-11, further comprising:

(e) contacting the therapeutic circular DNA vector with a topoisomerase or a helicase.

13. The method of any one of claims 1-12, further comprising:
(f) contacting the linear backbone fragments with an exonuclease.

14. The method of any one of claims 1-13, wherein the therapeutic sequence comprises two or more transcription units, and/or wherein the therapeutic sequence encodes one or more therapeutic proteins.

15. The method of any one of claims 1-14, wherein the therapeutic sequence encodes a therapeutic nucleic acid.

16. The method of claim 15, wherein the therapeutic nucleic acid is an RNA molecule; optionally, wherein the RNA molecule is a self-replicating RNA molecule, a short hairpin RNA, or a microRNA.

17. The method of any one of claims 1-16, further comprising formulating the therapeutic circular DNA vector in a pharmaceutically acceptable carrier to produce a pharmaceutical composition; optionally wherein the pharmaceutical composition comprises at least 1.0 mg of the therapeutic circular DNA vector in a pharmaceutically acceptable carrier; optionally wherein the therapeutic circular DNA vector in the pharmaceutical composition is at least 70% supercoiled monomer; and/or wherein the pharmaceutical composition comprises no more than 1.0% of residual protein or backbone sequence.

18. The method of claim 17, wherein the pharmaceutical composition comprises <1.0% protein content by mass, less than <1.0% RNA content by mass, and less than <5 EU/mg endotoxin.

19. A pharmaceutical composition produced by the method of claim 17 or 18.

20. The pharmaceutical composition of claim 19, for use in expressing a therapeutic sequence in an individual.