CLAIMS

What is claimed is:

1. A method of producing a closed-ended DNA (ceDNA) vector, the method comprising:

(a) contacting a double-stranded DNA construct having a sense strand and an antisense strand with at least a first restriction endonuclease and at least a second restriction endonuclease, wherein the double-stranded DNA construct comprises:

a transgene expression cassette,

a first non-palindromic restriction endonuclease recognition site and a corresponding first cleavage site upstream of the transgene expression cassette, and

a second non-palindromic restriction endonuclease recognition site and a corresponding second cleavage site downstream of the transgene expression cassette;

wherein the first restriction endonuclease is capable of cleaving the double-stranded DNA construct at the first cleavage site, and wherein the second restriction endonuclease is capable of cleaving the double-stranded DNA construct at the second cleavage site,

and wherein contacting the double-stranded DNA construct with the first restriction endonuclease and the second restriction endonuclease releases an insert having a first end comprising a first single-stranded overhang and a second end comprising a second single-stranded overhang;

(b) ligating the first end comprising the first single-stranded overhang to a first oligonucleotide comprising one or more hairpin structures; and

(c) ligating the second end comprising the second single-stranded overhang to a second oligonucleotide comprising one or more hairpin structures;

thereby producing the ceDNA vector.

2. The method of claim 1, wherein the first oligonucleotide comprises an inverted terminal repeat (ITR) and/or wherein the second oligonucleotide comprises an ITR.

3. The method of any one of claims 1-2, wherein

the first oligonucleotide and the second oligonucleotide are different;

the first oligonucleotide and the second oligonucleotide are the same;

the first restriction endonuclease and the second restriction endonuclease are different restriction endonucleases;

the first restriction endonuclease and the second restriction endonuclease are the same restriction endonucleases;

each of the oligonucleotides independently includes 1, 2, 3, 4, or more stem-loop regions; and/or

each of the oligonucleotides independently includes 2 or 3 stem-loop regions.

4. The method of any one of claims 1-3, wherein:

the first non-palindromic restriction endonuclease recognition site and the corresponding first cleavage site are separate and distinct sites from each other, and wherein both sites are located upstream of the transgene expression cassette;

the first cleavage site is about 1 to about 22 nucleotides away from the first non-palindromic restriction endonuclease recognition site in at least one of the sense strand and the antisense strand of the construct;

the second non-palindromic restriction endonuclease recognition site and the corresponding second cleavage site are separate and distinct sites from each other, and wherein both sites are located downstream of the expression cassette;

the second cleavage site is about 1 to about 22 nucleotides away of the second non-palindromic restriction endonuclease recognition site in at least one of the sense strand and the antisense strand of the construct; and/or

the first non-palindromic restriction endonuclease recognition site and the second non-palindromic restriction endonuclease recognition site are each a double-stranded polynucleotide having different 5' to 3' nucleotide sequences in each of the sense strand and the antisense strand.

5. The method of claim 4, wherein the first cleavage site is about 1 to about 8 nucleotides away from the first non-palindromic restriction endonuclease recognition site in at least one of the sense strand and the antisense strand of the construct; and/or wherein the second cleavage site is about 1 to about 8 nucleotides away from the second non-palindromic restriction endonuclease recognition site in at least one of the sense and the antisense strand of the construct.

6. The method of any one of claims 1-5, wherein:

one or both of the single-stranded overhangs at the ends of the insert are 5' overhangs;

one or both of the single-stranded overhangs at the ends of the insert are 3' overhangs; and/or

the 5' end of each oligonucleotide is phosphorylated.

7. The method of any one of claims 1-6, wherein the first oligonucleotide comprising one or more hairpin structures and the second oligonucleotide comprising one or more hairpin structures are

each a single-stranded oligonucleotide that self-anneals to form a three-dimensional configuration; and/or

wherein the first oligonucleotide comprising one or more hairpin structures and the second oligonucleotide comprising one or more hairpin structures each self-anneal to further form a single-stranded overhang at either the 5’ end or the 3’ end of each oligonucleotide.

8. The method of claim 7, wherein the three-dimensional configuration is a T- or Y-shaped stem-loop structure.

9. The method of claim 8, wherein the first oligonucleotide and the second oligonucleotide each self-anneal to further form a single-stranded overhang at the 5’ end of each oligonucleotide and/or wherein the first oligonucleotide and the second oligonucleotide each self-anneal to further form a single-stranded overhang at the 3’ end of each oligonucleotide.

10. The method of any one of claims 7-9, wherein:

the single-stranded overhang of the first oligonucleotide and the single-stranded overhang of the second oligonucleotide are each about 1 to about 12 nucleotides in length;

the single-stranded overhang of the first oligonucleotide and the single-stranded overhang of the second oligonucleotide are each about 1 to about 8 nucleotides in length;

the single-stranded overhang of the first oligonucleotide and the single-stranded overhang of the second oligonucleotide are each about 2 to about 6 nucleotides in length;

the single-stranded overhang of the first oligonucleotide and the single-stranded overhang of the second oligonucleotide are each about 3, about 4, about 5, or about 6 nucleotides in length; and/or

the single-stranded overhang of the first oligonucleotide and the single-stranded overhang of the second oligonucleotide are each 3 or 4 nucleotides in length.

11. The method of claim 10, wherein:

the 5’ to 3’ nucleotide sequences of the single-stranded overhang of the first oligonucleotide and the single-stranded overhang of the second oligonucleotide are non-complementary to each other;

the 5’ to 3’ nucleotide sequences of the single-stranded overhang of the first oligonucleotide and the single-stranded overhang of the second oligonucleotide are the same;

the first oligonucleotide and the second oligonucleotide have the same nucleotide sequence;

the single-stranded overhangs at each end of the insert comprise the same 5’ to 3’ nucleotide sequence;

the single-stranded overhang of the first oligonucleotide and the single-stranded overhang of the second oligonucleotide are each complementary to both of the single-stranded overhangs at the ends of the insert; and/or

the 5’ to 3’ nucleotide sequences of the single-stranded overhang of the first oligonucleotide and the single-stranded overhang of the second oligonucleotide are different.

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

the first oligonucleotide and the second oligonucleotide comprise different nucleotide sequences

the 5’ to 3’ nucleotide sequences of the single-stranded overhangs at each end of the insert are different;

the single-stranded overhang of the first oligonucleotide and the single-stranded overhang of the second oligonucleotide are each complementary to only one of the single-stranded overhangs at the ends of the insert.

13. The method of any one of claims 9-12, wherein the single-stranded overhang of the first oligonucleotide and/or the single-stranded overhang of the second oligonucleotide comprise a 5’ to 3’ nucleotide sequence selected from the group consisting of CTCT, CTCA, CACT, CTC, and GCT.

14. The method of any one of claims 1-13, wherein:

one or both of the first oligonucleotide and the second oligonucleotide is synthetic;

the first oligonucleotide and the second oligonucleotide are each about 40 nucleotides to about 75 nucleotides in length;

the first oligonucleotide and the second oligonucleotide are each about 45 nucleotides to about 65 nucleotides in length; and/or

the first oligonucleotide and the second oligonucleotide each independently comprise a nucleotide sequence selected from the group consisting of SEQ ID NO: 1; SEQ ID NO: 2; SEQ ID NO: 3; SEQ ID NO: 4; SEQ ID NO: 5; SEQ ID NO: 6; SEQ ID NO: 7; and SEQ ID NO: 8.

15. The method of any one of claims 1-14, wherein:

each hairpin structure and/or each T- or Y-shaped stem-loop structure of the first oligonucleotide and each hairpin structure and/or each T- or Y-shaped stem-loop structure of the second oligonucleotide comprises a stem region that is at least about 4 base pairs in length;

each hairpin structure and/or each T- or Y-shaped stem-loop structure of the first oligonucleotide and each hairpin structure and/or each T- or Y-shaped stem-loop structure of the second oligonucleotide comprises a stem region that is about 4 base pairs to about 20 base pairs in length;

each hairpin structure and/or each T- or Y-shaped stem-loop structure of the first oligonucleotide and each hairpin structure and/or each T- or Y-shaped stem-loop structure of the

second oligonucleotide comprises a stem region that is about 4 base pairs to about 15 base pairs in length;

each hairpin structure and/or each T- or Y-shaped stem-loop structure of the first oligonucleotide and each hairpin structure and/or each T- or Y-shaped stem-loop structure of the second oligonucleotide comprises a stem region that is about 4 base pairs to about 6 base pairs in length;

each hairpin structure and/or each T- or Y-shaped stem-loop structure of the first oligonucleotide and each hairpin structure and/or each T- or Y-shaped stem-loop structure of the second oligonucleotide comprises a stem region that is about 6 base pairs to about 8 base pairs in length; and/or

the stem region length does not include any single-stranded overhang.

16. The method of any one of claims 1-15, wherein at least one of the restriction endonucleases is a Type IIS restriction endonuclease;

the first restriction endonuclease and the second restriction endonuclease are the same restriction endonuclease;

the first restriction endonuclease and the second restriction endonuclease are different restriction endonucleases;

each of the first and second restriction endonucleases is a Type IIS restriction endonuclease;

each of the first and second restriction endonucleases is a Type IIS restriction endonuclease independently selected from the group consisting of *Acu*I, *Alw*I, *Alw*26I, *Bas*I, *Bbs*I, *Bbv*I, *Bce*AI, *Bcg*I, *BCi*VI, *Bco*DI, *Bru*AI, *Bmr*I, *Bpi*I, *Bpu*EI, *Bsa*I, *Bsa*XI, *Bse*GI, *Bse*RI, *Bsg*I, *Bsm*AI, *Bsm*BI, *Bsm*FI, *Bsm*I, *Bsp*CNI, *Bsp*MI, *Bsp*QI, *Bsr*DI, *Bsr*I, *Btg*ZI, *Bts*CI, *Bts*I, *Mu*tI, *Csp*CI, *Ear*I, *Eci*I, *Eco*31I, *Esp*3I, *Fau*I, *Fok*I, *Hga*I, *Hph*I, *Hpy*AV, *Lgu*I, *Mbo*II, *Mly*I, *Mme*I, *Mnl*I, *Mva*1269I, *Nme*AIII, *Paq*CI, *Ple*I, *Sap*I, *Sfa*NI, and an isoschizomer thereof;

the at least one Type IIS restriction endonuclease is selected from group consisting of *Bbs*I, *Bsa*I, *Esp*3I, and *Sap*I, and an isoschizomer thereof;

the at least one Type IIS restriction endonuclease is *Bsa*I or an isoschizomer thereof; and/or

the at least one Type IIS restriction endonuclease is *Esp*3I or an isoschizomer thereof.

17. The method of any one of claims 1-16, wherein after the ligating, the first non-palindromic restriction endonuclease recognition site and the second non-palindromic restriction endonuclease recognition site are not regenerated in the resulting ceDNA vector.

18. The method of any one of claims 1-17, wherein the double-stranded DNA construct further comprises a least a first partial ITR and a second partial ITR each flanking the transgene expression cassette.

19. The method of claim 18, wherein:

the first partial ITR is upstream of the transgene expression cassette and downstream of the first non-palindromic restriction endonuclease recognition site and the corresponding first cleavage site;

the second partial ITR is downstream of the transgene expression cassette and upstream of the second non-palindromic restriction endonuclease recognition site and the corresponding second cleavage site;

the first cleavage site is adjacent to the first partial ITR and the second cleavage site is adjacent to the second partial ITR;

the double-stranded DNA construct further comprises a first spacer between the first partial ITR and the transgene expression cassette; and/or

the double-stranded DNA construct further comprises a second spacer between the second partial ITR and the transgene expression cassette.

20. The method of any one of claims 1-19, wherein the double-stranded DNA construct is selected from the group consisting of a bacmid, a plasmid, a minicircle, and a linear double-stranded DNA molecule.

21. The method of any one of claims 1-20, wherein the resulting ceDNA vector comprises the transgene expression cassette and at least a first ITR and a second ITR each flanking the transgene expression cassette.

22. The method of claim 21, wherein:

the first ITR is upstream of the transgene expression cassette;

the second ITR is downstream of the transgene expression cassette;

the first ITR comprises nucleotide sequences from the first oligonucleotide and the first partial ITR;

the second ITR comprises nucleotide sequences from the second oligonucleotide and the second partial ITR.

the first ITR is devoid of the first non-palindromic restriction endonuclease recognition site;

the second ITR is devoid of the second non-palindromic restriction endonuclease recognition site;

the first ITR and the second ITR each comprise a hairpin structure and/or a T- or Y-shaped stem-loop structure; and/or

the T- or Y-shaped stem-loop structure comprises a stem comprising A-A’ and D-D’ stem regions and two B-B’ and C-C’ loops.

23. The method of claim 21 or 22, wherein:

one or both of the first ITR and the second ITR is selected from the group consisting of: an adeno-associated virus (AAV) ITR and an AAV-derived ITR;

one of the first ITR or the second ITR is a wild-type ITR;

both of the first ITR and the second ITR are wild-type ITRs;

one or both of the first ITR and the second ITR are modified ITRs;

the first ITR and the second ITR are symmetrical or substantially symmetrical to each other; the first ITR and the second ITR are asymmetrical ITRs; and/or

one or both of the first ITR and the second ITR comprise one or more modifications selected from the group consisting of an addition, a deletion, a truncation, and a point mutation.

24. The method of claim 23, wherein:

the one or more modifications are located in an A-A' stem region, a B-B' loop, a C-C' loop, and/or a D-D' stem region of one or both of the first ITR and the second ITR;

the one or more modifications are located in the B-B' loop and/or the C-C' loop of one or both of the first ITR and the second ITR; and/or

the B-B' loop and the C-C' loop of one of the first ITR and the second ITR are truncated.

25. The method of any one of claims 21-24, wherein:

the transgene expression cassette further comprises a first spacer between the first ITR and the transgene expression cassette;

the transgene expression cassette further comprises a first spacer between the second ITR and the transgene expression cassette; and/or

the transgene expression cassette further comprises a first spacer between the first ITR and the transgene expression cassette, and a second spacer between the second ITR and the transgene expression cassette.

26. The method of any one of claims 1-25, wherein the transgene expression cassette comprises a transgene.

27. The method of claim 26, wherein the transgene encodes a therapeutic protein.

28. The method of claim 27, wherein the therapeutic protein is selected from the group consisting of an enzyme, a coagulation factor or co-factor, an antibody or an antigen-binding fragment thereof, an antigen, a gene-editing protein, and a cytotoxic protein.

29. The method of any one of claims 1-28, wherein the transgene expression cassette further comprises a genetic element selected from the group consisting of a promoter, an enhancer, an intron, a posttranscriptional regulatory element, and a polyadenylation signal.

30. The method of any one of claims 1-29, wherein the ligating is effected by a ligase or an AAV Rep protein.

31. The method of claim 30, wherein the ligase is T4 ligase.

32. The method of any one of claims 1-31, wherein:
the method further comprises isolating or purifying the resulting ceDNA vector;
the method further comprises isolating or purifying the insert prior to the ligating;
the method does not comprise isolating or purifying the insert prior to the ligating; and/or
steps (a), (b), and (c) are performed in a single reaction vessel.

33. The method of any one of claims 1-32, wherein the resulting ceDNA vector comprises at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% of a monomeric species of the ceDNA vector.

34. An inverted terminal repeat (ITR) nucleotide sequence selected from the group consisting of:

SEQ ID NO:	Left or Right	Sequence (5’ to 3’)
18	Left	CTCACTGAGGCCGCCCGGGCAAAGCCCGGGCGT CGGGCGACCTTTGGTCGCCCGGCCTCAG
19	Left	CTCACTGAGGCCGCCCGGGCAAAGCCCGGGCGT CGGGCGACCTTTGGTCGCCCGGCCTCAGTGAGC GAGCGAGCGCGCAGAGAGGGAGTGGCCAACTC CATCACTAGGGGTTCCT
20	Left	CTCTCTGAGGCCGCCCGGGCAAAGCCCGGGCGT CGGGCGACCTTTGGTCGCCCGGCCTCAG

21	Left	CTCTCTGAGGCCGCCCGGGCAAAGCCCGGGCGT CGGGCGACCTTTGGTCGCCCGGCCTCAGTGAGC GAGCGAGCGCGCAGAGAGGGAGTGGCCAACTC CATCACTAGGGGTTCCT
22	Left	CACTGAGGCCGCCCGGGCAAAGCCCGGGCGTC GGGCGACCTTTGGTCGCCCGGCCTCAG
23	Left	CACTGAGGCCGCCCGGGCAAAGCCCGGGCGTC GGGCGACCTTTGGTCGCCCGGCCTCAGTGAGC GAGCGAGCGCGCAGAGAGGGAGTGGCCAACT CCATCACTAGGGGTTCCT
24	Left	CTCACTGAGGCCGCCCGGGCAAAGCCCGGGCG TCGGGCGACCTTTGGTCGCCCGGCCTCAG
25	Left	CTCACTGAGGCCGCCCGGGCAAAGCCCGGGCG TCGGGCGACCTTTGGTCGCCCGGCCTCAGTGAG CGAGCGAGCGCGCAGAGAGGGAGTGGCCAACT CCATCACTAGGGGTTCCT
26	Right	CTGAGGCCGGGCGACCAAAGGTCGCCCGACGCC CGGGCTTTGCCCGGGCGGCCTCAGTGAG
27	Right	AGGAACCCCTAGTGATGGAGTTGGCCACTCCCTC TCTGCGCGCTCGCTCGCTCACTGAGGCCGGGCGA CCAAAGGTCGCCCGACGCCCGGGCTTTGCCCGGG CGGCCTCAGTGAG
28	Right	CTGAGGCCGGGCGACCAAAGGTCGCCCGACGCCC GGGCGGCCTC
29	Right	AGGAACCCCTAGTGATGGAGTTGGCCACTCCCTCT CTGCGCGCTCGCTCGCTCACTGAGGCCGGGCGACC AAAGGTCGCCCGACGCCCGGGCGGCCTC
30	Right	CTGAGGCCGGGCGACCAAAGGTCGCCCGACGCCC GGGCTTTGCCCGGGCGGCCTCAGTGAG; and
31	Right	AGGAACCCCTAGTGATGGAGTTGGCCACTCCCTCTC TGCGCGCTCGCTCGCTCACTGAGGCCGGGCGACCAA AGGTCGCCCGACGCCCGGGCTTTGCCCGGGCGGCCT CAGTGAG

35. The ITR nucleotide sequence of claim 34, wherein the nucleotide sequence further includes a spacer selected from the group consisting of:

SEQ ID NO:	Left or Right	Sequence (5’ to 3’)
32	Left	TGTAGTTAATGATTAACCCGCCATGCTACTTAT CGCGGCCGC
33	Left	TGTAGTTAATGATTAACCCGCCATGCTACTTAT CTACGTAGCCATGCATATG
34	Left	TGTAGTTAATGATTAACCCGCCATGCTACTTATC
35	Left	TGTAGTTAATGATTAACCCACCATGCTACTTATG GCCTGCAGG
36	Left	TGTAGTTAATGATTAACCCACCATGCTACTTATC ACCAATTG
37	Right	TCTAGAGCATGGCTACGTAGATAAGTAGCATGG CGGGTTAATCATTAACTACACCTGCAGG
38	Right	GCTAGCCCACAATCTGCCTCCCAGTAGTACATG ACATTAGTTTATTAATAGCCT
39	Left	TCCACAATCTGCCTCCCAGTAGTACATGACATT AGTTTATTAATAGCCT; and
40	Right	CTTAAGCATGGCTACGTAGATAAGTAGCATGGC GGGTTAATCATTAACTACACCTGCAGG

36. A closed-ended DNA (ceDNA) vector comprising a transgene expression cassette and at least a first inverted terminal repeat (ITR) and a second ITR flanking the transgene expression cassette; wherein the first ITR and the second ITR each comprise a nucleotide sequence selected from the group consisting of the ITR nucleotide sequences of any one of claims 34-35.

37. The ceDNA vector of claim 36, wherein the vector comprises double-stranded DNA.

38. A pharmaceutical composition comprising the ceDNA vector of any one of claims 36-37 and at least one pharmaceutically acceptable excipient.

39. A lipid nanoparticle composition comprising the ceDNA vector of any one of claims 36-37.

40. An isolated host cell comprising the ceDNA vector of any one of claims 36-37.

41. A transgenic animal comprising the ceDNA vector of any one of claims 36-37.

42. The ceDNA vector of any one of claims 36-37, or the pharmaceutical composition of claim 38, or the lipid nanoparticle composition of claim 39 for use in the treatment of a disorder, disease, or condition in a subject or delivery of a therapeutic protein to a subject.

43. A DNA vector for use in synthetic production of a closed-ended DNA vector (ceDNA), comprising:

a multiple cloning site capable of receiving a transgene;

a first non-palindromic restriction endonuclease recognition site and a corresponding first cleavage site upstream of the multiple cloning site;

a second non-palindromic restriction endonuclease recognition site and a corresponding second cleavage site downstream of the multiple cloning site; and

a first partial ITR and a second partial ITR each flanking the multiple cloning site.

44. The DNA vector of claim 43, wherein:

the first partial ITR is upstream of the multiple cloning site and downstream of the first non-palindromic restriction endonuclease recognition site and the corresponding first cleavage site;

the second partial ITR is downstream of the multiple cloning site and upstream of the second non-palindromic restriction endonuclease recognition site and the corresponding second cleavage site;

the multiple cloning site is capable of receiving a transgene and one or more additional genetic elements selected from the group consisting of a promoter, an enhancer, an intron, a posttranscriptional regulatory element and a polyadenylation signal;

the first non-palindromic restriction endonuclease recognition site is specific for a first restriction endonuclease, and the second non-palindromic restriction endonuclease recognition site is specific for at least a second restriction endonuclease.

45. The DNA vector of claim 43 or 44, further comprising:

one or more spacers; and/or

an origin of replication and a selectable marker gene.

46. The DNA vector of claim 44, wherein the first restriction endonuclease and the second restriction endonuclease are the same restriction endonuclease;

the first restriction endonuclease and the second restriction endonuclease are different restriction endonucleases;

at least one of the restriction endonucleases is a Type IIS restriction endonuclease; each of the first and second restriction endonucleases is a Type IIS restriction endonuclease; and/or

the DNA vector comprises a nucleotide sequence selected from the group consisting of SEQ ID NO: 9; SEQ ID NO: 10; SEQ ID NO: 11; SEQ ID NO: 12; SEQ ID NO: 13; SEQ ID NO: 14; SEQ ID NO: 15; SEQ ID NO: 16; and SEQ ID NO: 17.

47. The DNA vector of claim 46, wherein:

each Type IIS restriction endonuclease is independently selected from the group consisting of *Acu*I, *Alw*I, *Alw*26I, *Bas*I, *Bbs*I, *Bbv*I, *Bce*AI, *Bcg*I, *BCi*VI, *Bco*DI, *Bru*AI, *Bmr*I, *Bpi*I, *Bpu*EI, *Bsa*I, *Bsa*XI, *Bse*GI, *Bse*RI, *Bsg*I, *Bsm*AI, *Bsm*BI, *Bsm*FI, *Bsm*I, *Bsp*CNI, *Bsp*MI, *Bsp*QI, *Bsr*DI, *Bsr*I, *Btg*ZI, *Bts*CI, *Bts*I, *Mut*I, *Csp*CI, *Ear*I, *Eci*I, *Eco3*1I, *Esp*3I, *Fau*I, *Fok*I, *Hga*I, *Hph*I, *Hpy*AV, *Lgu*I, *Mbo*II, *Mly*I, *Mme*I, *Mnl*I, *Mva*1269I, *Nme*AIII, *Paq*CI, *Ple*I, *Sap*I, *Sfa*NI, and an isoschizomer thereof; and/or

each Type IIS restriction endonuclease is selected from the group consisting of BbsI, BsaI, Esp3I, and SapI, and an isoschizomer thereof.

48. A kit for preparing a closed-ended DNA (ceDNA) vector comprising a transgene, the kit comprising:

the DNA vector of any one of claims 43-47;

at least one restriction endonuclease capable of cleaving the DNA vector at the multiple cloning site to allow the multiple cloning site to receive a transgene;

at least one restriction endonuclease capable of cleaving at the first and second cleavage sites;

a ligase; and

instructions for use.

49. A double-stranded circular DNA construct engineered to facilitate preparation of a closed-ended DNA (ceDNA) vector comprising a transgene expression cassette, the double-stranded circular DNA construct comprising:

a transgene expression cassette;

a first non-palindromic restriction endonuclease recognition site and a corresponding first cleavage site upstream of the transgene expression cassette;

a second non-palindromic restriction endonuclease recognition site and a corresponding second cleavage site downstream of the transgene expression cassette; and

a first partial ITR and a second partial ITR each flanking the transgene expression cassette.

50. The double-stranded circular DNA construct of claim 49, wherein:

the first partial ITR is upstream of the transgene expression cassette and downstream of the first non-palindromic restriction endonuclease recognition site and the corresponding first cleavage site;

the second partial ITR is downstream of the transgene expression cassette and upstream of the second non-palindromic restriction endonuclease recognition site and the corresponding second cleavage site; and/or

the first non-palindromic restriction endonuclease recognition site is specific for a first restriction endonuclease and the second non-palindromic restriction endonuclease recognition site are specific for at least a second restriction endonuclease.

51. The double-stranded circular DNA construct of claim 50, wherein the first restriction endonuclease and the second restriction endonuclease are the same restriction endonuclease; or the first restriction endonuclease and the second restriction endonuclease are different restriction endonucleases.

52. The double-stranded circular DNA construct of any one of claims 49-51, wherein at least one of the restriction endonucleases is a Type IIS restriction endonuclease or wherein each of the first and second restriction endonucleases is a Type IIS restriction endonuclease.

53. A kit for preparing a closed-ended DNA (ceDNA) vector comprising a transgene expression cassette, the kit comprising:

the double-stranded DNA construct of any one of claims 49-52;

at least one restriction endonuclease capable of cleaving the double-stranded DNA construct at the first and second cleavage sites;

a ligase; and

instructions for use.

54. A method of producing a double-stranded DNA construct from a plasmid template via rolling-circle amplification, comprising the steps of:

(a) contacting the plasmid template with a thermostable polymerase having strand-displacement activity, wherein the ratio of plasmid template concentration (in ng/µl) to polymerase concentration (in U/µl) is greater than about 1;

(b) contacting the plasmid template with an oligonucleotide primer and dNTPs;

(c) incubating the plasmid template, the polymerase, the oligonucleotide primer, and the dNTPs at a temperature of about 40°C or less, for a time period of at least about 5 hours;

thereby producing a double-stranded DNA construct.

55. The method of claim 54, wherein:

the ratio of plasmid template concentration (in ng/µl) to polymerase concentration (in U/µl) is greater than about 1, about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, or about 20;

the plasmid template concentration is about 0.01 ng/µl, about 0.05 ng/µl, about 0.1 ng/µl, about 0.15 ng/µl, about 0.2 ng/µl, about 0.21 ng/µl, about 0.22 ng/µl, about 0.23 ng/µl, about 0.24 ng/µl, about 0.2 ng/µl 5, about 0.26 ng/µl, about 0.27 ng/µl, about 0.28 ng/µl, about 0.29 ng/µl, about 0.3 ng/µl, about 0.35 ng/µl, about 0.4 ng/µl, about 0.45 ng/µl, about 0.5 ng/µl, about 0.6 ng/µl, about 0.7 ng/µl, about 0.8 ng/µl, about 0.9 ng/µl, or about 1.0 ng/µl;

the polymerase concentration is about 0.01 U/µl, about 0.02 U/µl, about 0.03 U/µl, about 0.04 U/µl, about 0.05 U/µl, about 0.06 U/µl, about 0.07 U/µl, about 0.08 U/µl, about 0.09 U/µl, about 0.1 U/µl, about 0.15 U/µl, about 0.2 U/µl, about 0.25 U/µl, about 0.3 U/µl, about 0.35 U/µl, about 0.4 U/µl, or about 0.45 U/µl;

the temperature in step (c) is less than about 40°C, about 39°C, about 38°C, about 37°C, about 36°C, about 35°C, about 34°C, about 33°C, about 32°C, about 31°C, about 30°C, about 29°C, about 28°C, about 27°C, about 26°C, about 25°C, about 24°C, about 23°C, about 22°C, or about 21°C;

the time period is at least about 5 hours, about 6 hours, about 7 hours, about 8 hours, about 9 hours, about 10 hours, about 11 hours, about 12 hours, about 13 hours, about 14 hours, about 15 hours, about 16 hours, about 17 hours, about 18 hours, about 19 hours, about 20 hours, about 21 hours, about 22 hours, about 23 hours, about 24 hours, about 25 hours, about 26 hours, about 27 hours, about 28 hours, about 29 hours, about 30 hours, about 31 hours, about 21 hours, about 33 hours, about 34 hours, about 35 hours, about 36 hours, about 37 hours, about 38 hours, about 39 hours, or about 40 hours;

the time period is less than about 6 hours, about 6 hours, about 7 hours, about 8 hours, about 9 hours, about 10 hours, about 11 hours, about 12 hours, about 13 hours, about 14 hours, about 15 hours, about 16 hours, about 17 hours, about 18 hours, about 19 hours, about 20 hours, about 21 hours, about 22 hours, about 23 hours, about 24 hours, about 25 hours, about 26 hours, about 27 hours, about 28 hours, about 29 hours, about 30 hours, about 31 hours, about 21 hours, about 33 hours, about 34 hours, about 35 hours, about 36 hours, about 37 hours, about 38 hours, about 39 hours, or about 40 hours;

the oligonucleotide primer concentration is less than about 50 µM;

the oligonucleotide primer concentration is at least about 10 µM;

the thermostable polymerase is Phi29 DNA polymerase or a derivative or variant thereof.

the method is performed in a total reaction volume of at least about 100 µl;

the method is performed in a total reaction volume of at least about 100 µl, about 200 µl, about 300 µl, about 400 µl, about 500 µl, about 600 µl, about 700 µl, about 800 µl, about 900 µl, about 1 ml, about 2 ml, about 3 ml, about 4 ml, about 5 ml, about 6 ml, about 7 ml, about 8 ml, about

9 ml, about 10 ml, about 15 ml, about 20 ml, about 25 ml, about 30 ml, about 35 ml, about 40 ml, about 45 ml, about 50 ml, about 55 ml, about 60 ml, about 65 ml, about 70 ml, about 75 ml, about 80 ml, about 85 ml, about 90 ml, about 95 ml, about 100 ml, about 200 ml, about 300 ml, about 400 ml, about 500 ml, about 600 ml, about 700 ml, about 800 ml, about 900 ml, about 1 L, about 2 L, about 3 L, 4 L, about 5 L, about 6 L, about 7 L, about 8 L, about 9 L, about 10 L, about 20 L, about 30 L, about 40 L, about 50 L, about 60 L, about 70 L, about 80 L, about 90 L, about 100 L, about 200 L, about 300 L, about 400 L, about 500 L, about 600 L, about 700 L, about 800 L, about 900 L, or about 1000 L; and/or

wherein the dNTP concentration is about 4 mM.

56. The method of claim 54 or 55, wherein the oligonucleotide primer hybridizes to a backbone sequence in the plasmid template and/or wherein the oligonucleotide primer is a universal primer.

57. A method of producing a closed-ended DNA (ceDNA) vector, the method comprising:

(a) producing a double-stranded DNA construct using the method of any one of claims 54-56;

(b) performing the method of any one of claims 1-33 to produce a ceDNA vector from the double-stranded DNA construct.

58. A method of preparing a closed-ended DNA (ceDNA) vector, the method comprising:

(a) contacting a double-stranded DNA construct with at least one restriction endonuclease, wherein the restriction endonuclease is a Type IIs restriction endonuclease, wherein the construct comprises:

a transgene expression cassette;

a first non-palindromic restriction endonuclease recognition site and a corresponding first cleavage site upstream of the transgene expression cassette, and

a second non-palindromic restriction endonuclease recognition site and a corresponding second cleavage site downstream of the transgene expression cassette;

the Type IIs one restriction endonuclease is capable of cleaving the construct at the first and second cleavage sites to release an insert having single-stranded overhangs at the 5' and 3' ends of the insert that are compatible with the overhangs of a first inverted terminal repeat (ITR) oligonucleotide and a second inverted terminal repeat (ITR) oligonucleotide; and

(b) ligating the 5' and 3' ends of the insert to the first inverted terminal repeat (ITR) oligonucleotide and the second ITR oligonucleotide to form the ceDNA vector.