wherein the expression cassette has a restriction endonuclease site for insertion of a transgene, and (ii) at least one ligation reagent for ligation

53. A kit for producing a closed-ended DNA vector obtained by or obtainable by a process according to any of paragraphs 3-39, comprising: (i) single-stranded DNA molecule comprising in order in the 5' to 3' direction: a sense first ITR; a sense expression cassette sequence; a sense second ITR; an antisense second ITR; an antisense expression cassette sequence; and an antisense first ITR; wherein the sense expression cassette sequence and the antisense expression cassette sequence have a restriction endonuclease site for insertion of a transgene, and (i) at least one ligation reagent for ligation.
54. A kit for producing a closed-ended DNA vector obtained by or obtainable by a process according to any of paragraphs 4-39, comprising: (i) a single stranded-DNA molecule comprising in order of 5' to 3' direction: a sense first ITR; a sense expression cassette sequence; a sense second ITR; and an antisense expression cassette sequence; wherein the sense expression cassette sequence and the antisense expression cassette sequence have a restriction endonuclease site for insertion of a transgene, and (ii) at least one ligation reagent for ligation.
55. A kit for producing a closed-ended DNA vector obtained by or obtainable by a process according to any of paragraphs 5-39, comprising: (i) a double-stranded DNA construct comprising in order in the 5' to 3' direction: a first restriction endonuclease cleavage site; a sense first ITR; a sense expression cassette sequence; a sense second ITR; an antisense expression cassette sequence; and a second restriction endonuclease cleavage site; wherein the sense expression cassette sequence and the antisense expression cassette sequence have a restriction endonuclease site for insertion of a transgene, and (ii) at least one ligation reagent for ligation.
56. The kit of any of paragraphs 49-55, wherein the at least one reagent for ligation is a reagent for chemical ligation.
57. The kit of any of paragraphs 49-56, wherein the at least one reagent for ligation is a reagent for protein-assisted ligation.
58. The kit of any of paragraphs 49-57, wherein the ligation is effected by T4 ligation or an AAV Rep protein.
59. The kit of any of paragraphs 49-58, wherein the first-single stranded ITR molecule and second single-stranded ITR molecule comprise a restriction endonuclease cleave site at their ends.
60. The kit of any of paragraphs 49-59, wherein the kit further comprises at least one restriction endonuclease enzyme.

In one aspect, the present disclosure refers to a cell-free method of preparing a non-viral, linear and closed-ended DNA (ceDNA) vector comprising:

providing a first single-stranded inverted terminal repeat (ITR) molecule comprising a first ITR;

providing a second single-stranded ITR molecule comprising a second ITR;

providing a double-stranded polynucleotide comprising an expression cassette sequence; and

ligating the 5' and 3' ends of the first ITR molecule to a first end of the double-stranded molecule and ligating the 5' and 3' ends of the second ITR molecule to the second end of the double stranded molecule to form the closed-ended DNA (ceDNA) vector, wherein at least one of the first ITR and the second ITR are synthesized, and wherein at least one of the first ITR and the second ITR comprises an overhang region complementary to the first end of the expression cassette sequence or the second end of the expression cassette sequence, respectively.

In another aspect, the present disclosure refers to a closed-ended DNA vector generated by the method as disclosed herein.

In another aspect, the present disclosure refers to a pharmaceutical composition comprising the closed-ended DNA vector as disclosed herein and optionally, an excipient.

In another example, the present disclosure refers to a method of preparing a closed-ended DNA vector comprising:

contacting a double-stranded DNA construct comprising:

an expression cassette;

a first ITR on the upstream (5'-end) of the expression cassette;

a second ITR on the downstream (3'-end) of the expression cassette;

and at least two restriction endonuclease cleavage sites flanking the ITRs such that the restriction endonucleases are distal to the expression cassette

with one or more restriction endonucleases that can cleave the double-stranded DNA construct at the restriction endonuclease cleavage sites to excise the sequences between the restriction endonuclease cleavage sites from the double-stranded DNA construct; and ligating the 5' and 3' ends of the excised sequence to form a closed-ended DNA vector.

In another aspect, the present disclosure refers to a cell-free method of preparing a non-viral, linear and closed-ended DNA (ceDNA) vector comprising:

synthesizing a single-stranded DNA molecule comprising in order in the 5' to 3' direction:

a first ITR;

a sense expression cassette sequence;

a second ITR; and

an antisense expression cassette sequence;

and annealing the molecule.

In another example, the present disclosure refers to a method of preparing a closed-ended DNA vector comprising:

synthesizing a single-stranded DNA molecule comprising in order in the 5' to 3' direction:

a sense first ITR;

a sense expression cassette sequence;

a sense second ITR; and

an antisense expression cassette sequence;

and annealing the molecule.

In another aspect, the present disclosure refers to cell-free method of preparing a non-viral, linear and closed-ended DNA (ceDNA) vector comprising:

providing a double-stranded DNA construct comprising in order in the 5' to 3' direction:

a first restriction endonuclease cleavage site;

a first ITR;

a sense expression cassette sequence;

a second ITR;

an antisense expression cassette sequence; and

a second restriction endonuclease cleavage site;

contacting the double-stranded DNA construct with one or more restriction endonucleases that can cleave the double-stranded DNA construct at the first restriction endonuclease cleavage site and the second restriction endonuclease cleavage site to excise the double-stranded sequence between the restriction endonuclease cleavage sites from the double-stranded polynucleotide;

separating the excised double-stranded sequence into a sense strand and an antisense strand; and

performing an annealing step wherein each of the sense strand and the antisense strand forms a closed-ended DNA vector, wherein the annealing step results in one or both of the first ITR and the second ITR forming a hairpin loop.

In another aspect, the present disclosure refers to an isolated closed-ended DNA vector obtained by or obtainable by the method as disclosed herein.

In another aspect, the present disclosure refers to a genetic medicine comprising an isolated closed-ended DNA vector obtained by the method as disclosed herein.

In another aspect, the present disclosure refers to a cell comprising the closed-ended DNA vector as disclosed herein.

In another aspect, the present disclosure refers to a transgenic animal comprising the closed ended DNA vector as disclosed herein.

In another aspect, the present disclosure refers to use of a closed-ended DNA vector obtained by or obtainable by the method as disclosed herein in the manufacture of a medicament for treating a subject.

In another aspect, the present disclosure refers to use of a composition comprising a closed-ended DNA vector obtained by or obtainable by the method as disclosed herein, in the manufacture of a medicament for delivering a therapeutic protein to a subject, wherein at least one heterologous nucleotide sequence encodes a therapeutic protein.

[0029] In some examples, one example of the technology described herein relates to a synthetically produced non-viral capsid-free DNA vector with covalently-closed ends (ceDNA vector), wherein the ceDNA vector comprises at least one heterologous nucleotide sequence, operably

CLAIMS

What is claimed is:

1. A cell-free method of preparing a non-viral, linear and closed-ended DNA (ceDNA) vector comprising:

providing a first single-stranded inverted terminal repeat (ITR) molecule comprising a first ITR;

providing a second single-stranded ITR molecule comprising a second ITR;

providing a double-stranded polynucleotide comprising an expression cassette sequence; and

ligating the 5' and 3' ends of the first ITR molecule to a first end of the double-stranded molecule and ligating the 5' and 3' ends of the second ITR molecule to the second end of the double stranded molecule to form the closed-ended DNA (ceDNA) vector, wherein at least one of the first ITR and the second ITR are synthesized, and wherein at least one of the first ITR and the second ITR comprises an overhang region complementary to the first end of the expression cassette sequence or the second end of the expression cassette sequence, respectively.

2. The method of claim 1, wherein each of the first and the second single-stranded ITR molecules comprises an overhang region in an A-stem of the ITR between hairpin loop sequences B and C and a Rep binding element (an RBE element), wherein the overhang is complementary to an overhang created by endonuclease cleavage at a restriction site engineered into the expression cassette.

3. The method of claim 1, wherein the RBE elements and the ITR D regions are present in the double-stranded polynucleotide.

4. The method of claim 1, wherein the double-stranded expression cassette sequence is obtained by excision from a double-stranded DNA construct comprising the expression cassette sequence.

5. The method of claim 1, wherein the double-stranded polynucleotide comprising the expression cassette is provided via DNA synthesis or PCR chain assembly.

46. The method of claim 4, wherein within the double-stranded DNA construct the expression cassette sequence is flanked at the 5' end by a first restriction endonuclease cleavage site and at the 3' end by a second restriction endonuclease cleavage site.

7. The method of claim 6, wherein the first restriction endonuclease and the second restriction nuclease are the same restriction endonuclease or different restriction endonucleases.

8. The method of claim 4 or claim 6, wherein the double-stranded DNA construct is a bacmid, plasmid, minicircle, or a linear double-stranded DNA molecule.

9. The method of any of the preceding claims, wherein at least one of the first ITR and the second ITR are annealed prior to ligation to the expression cassette sequence.

10. The method of any of the preceding claims, wherein the ligation is effected by T4 ligase or an AAV Rep protein.

11. The method of any of the preceding claims, wherein the first ITR is selected from a wild-type ITR and a modified ITR.

12. The method of any one of the preceding claims, wherein the second ITR is selected from a wild-type ITR and a modified ITR.

13. The method of any of the preceding claims, wherein at least one of the first ITR and the second ITR comprises at least one RBE site.

14. The method of any of the preceding claims wherein at least one of the first ITR and the second ITR is an AAV ITR or an AAV-derived ITR.

15. The method of claim 14, wherein the sequence of the first ITR is selected from any of the left ITR sequences set forth in Table 4B or Table 5 or SEQ ID NO: 2, 5-9, 32-48, or wherein the sequence of the second ITR is selected from any of the right ITR sequences set forth in Table 4A or Table 5 or SEQ ID NO: 1, 3, 10-14, 15-31.

16. The method of any of the preceding claims, wherein the expression cassette sequence comprises at least one cis-regulatory element.

17. The method of claim 16, wherein the cis-regulatory element is selected from the group consisting of a promoter, an enhancer, a posttranscriptional regulatory element and a polyadenylation signal.

18. The method of claim 16, wherein the cis-regulatory element is a promoter selected from the group consisting of a CAG promoter, an AAT promoter, an LP1 promoter, a cytomegalovirus (CMV) promoter, and an EF1a promoter.

19. The method of claim 16, wherein the cis-regulatory element is a posttranscriptional regulatory element comprising a WHP posttranscriptional regulatory element (WPRE).

20. The method of any of the preceding claims, wherein the expression cassette sequence comprises a transgene sequence.

21. The method of claim 20, wherein the transgene sequence encodes a protein.

22. The method of claim 21, wherein the transgene sequence encodes a reporter protein, a therapeutic protein, an antigen, a gene editing protein, or a cytotoxic protein.

23. The method of claim 20, wherein the transgene sequence is a functional nucleotide sequence.

24. The method of any one of claims 20-23, wherein the transgene sequence is at least 2000 nucleotides in length.

25. A cell-free method of preparing a non-viral, linear and closed-ended DNA (ceDNA) vector comprising: synthesizing a single-stranded DNA molecule comprising in order in the 5' to 3' direction:

a first ITR;

a sense expression cassette sequence;

a second ITR; and

an antisense expression cassette sequence;

and annealing the molecule.

26. The method of claim 25, wherein the single-stranded DNA molecule is constructed by synthesizing one or more of the first ITR, the sense expression cassette sequence, the second ITR and the antisense expression cassette sequence and ligating such oligonucleotides to form the single-stranded DNA molecule.

27. The method of claim 25, wherein the single-stranded DNA molecule is provided by excision of the molecule from a double-stranded DNA polynucleotide, followed by denaturation of the excised double-stranded fragment to produce the single-stranded DNA molecule.

28. The method of any of claims 25-27, wherein the annealing step results in one or both of the first ITR and the second ITR forming a hairpin loop.

29. The method of any of claims 25-28, wherein at least one of the first ITR and the second ITR comprises at least one RBE site.

30. The method of any of claims 25-29, wherein at least one of the first ITR and the second ITR is an AAV ITR or an AAV-derived ITR.

31. The method of any of claims 25-30, wherein the sense expression cassette sequence comprises at least one cis-regulatory element.

32. The method of claim 31, wherein the cis-regulatory element is selected from the group consisting of a promoter, an enhancer, a posttranscriptional regulatory element and a polyadenylation signal.

33. The method of claim 32, wherein the cis-regulatory element is a promoter selected from the group consisting of a CAG promoter, an AAT promoter, an LP1 promoter, a cytomegalovirus (CMV) promoter, and an EF1a promoter.

34. The method of claim 32, wherein the cis-regulatory element is a posttranscriptional regulatory element comprising a WHP posttranscriptional regulatory element (WPRE).

35. The method of any of claims 25-34, wherein the sense expression cassette sequence comprises a transgene sequence.

36. The method of claim 35, wherein the transgene sequence encodes a protein.

37. The method of claim 36, wherein the transgene sequence encodes a reporter protein, a therapeutic protein, an antigen, a gene editing protein, or a cytotoxic protein.

38. The method of claim 35, wherein the transgene sequence is a functional nucleotide sequence.

39. The method of any one of clams 25-38, wherein the ceDNA vector is purified.

40. A cell-free method of preparing a non-viral, linear and closed-ended DNA (ceDNA) vector comprising:

providing a double-stranded DNA construct comprising in order in the 5' to 3' direction:

a first restriction endonuclease cleavage site;

a first ITR;

a sense expression cassette sequence;

a second ITR;

an antisense expression cassette sequence; and

a second restriction endonuclease cleavage site;

contacting the double-stranded DNA construct with one or more restriction endonucleases that can cleave the double-stranded DNA construct at the first restriction endonuclease cleavage site and the second restriction endonuclease cleavage site to excise the double-stranded sequence between the restriction endonuclease cleavage sites from the double-stranded polynucleotide;

separating the excised double-stranded sequence into a sense strand and an antisense strand; and

performing an annealing step wherein each of the sense strand and the antisense strand forms a closed-ended DNA vector, wherein the annealing step results in one or both of the first ITR and the second ITR forming a hairpin loop.

41. The method of claim 40, wherein the double-stranded DNA construct is a bacmid, plasmid, minicircle, or a linear double-stranded DNA molecule.

42. The method of any of claims 40-41, wherein the first ITR is selected from a wild-type ITR and a modified ITR and/or wherein the second ITR is selected from a wild-type ITR and a modified ITR.

43. The method of any of claims 40-42, wherein at least one of the first ITR and the second ITR comprises at least one RBE site.

44. The method of any of claims 40-43 wherein at least one of the first ITR and the second ITR is an AAV ITR or an AAV-derived ITR.

45. The method of any of claims 40-44, wherein the sense expression cassette sequence comprises at least one cis-regulatory element.

46. The method of claim 45, wherein the cis-regulatory element is selected from the group consisting of a promoter, an enhancer, a posttranscriptional regulatory element and a polyadenylation signal.

47. The method of claim 46, wherein the cis-regulatory element is a promoter selected from the group consisting of a CAG promoter, an AAT promoter, an LP1 promoter, a cytomegalovirus (CMV) promoter, and an EF1a promoter.

48. The method of claim 46, wherein the cis-regulatory element is a posttranscriptional regulatory element comprising a WHP posttranscriptional regulatory element (WPRE).

49. The method of any of claims 40-46, wherein the sense expression cassette sequence comprises a transgene sequence.

50. A closed-ended DNA vector generated by the method of any of claims 1-49.

51. A pharmaceutical composition comprising the closed-ended DNA vector of claim 50 and optionally, an excipient.

52. An isolated closed-ended DNA vector obtained by or obtainable by the method of any of claims 1-49.

53. A genetic medicine comprising an isolated closed-ended DNA vector obtained by the method of any of claims 1-49.

54. A cell comprising the closed-ended DNA vector of claim 53.

55. A transgenic animal comprising the closed ended DNA vector of claim 53.

56. Use of a closed-ended DNA vector obtained by or obtainable by the method of any of claims 1-49 in the manufacture of a medicament for treating a subject.

57. Use of a composition comprising a closed-ended DNA vector obtained by or obtainable by the method of any of claims 1-49, in the manufacture of a medicament for delivering a therapeutic protein to a subject, wherein at least one heterologous nucleotide sequence encodes a therapeutic protein.

58. The use of claim 57, wherein the therapeutic protein is a therapeutic antibody.