

CLAIMS

What is claimed is:

1. A method of preparing a closed-ended DNA vector comprising:
 - providing a first single-stranded 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; andligating 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 DNA vector.
2. The method of claim 1, wherein at least one of the first ITR and the second ITR are synthesized.
3. The method of claim 1, wherein the double-stranded expression cassette sequence was obtained by excision from a double-stranded DNA construct comprising the expression cassette sequence.
4. The method of claim 3, 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.
5. The method of claim 3 or claim 4, wherein the double-stranded DNA construct is a bacmid, plasmid, minicircle, or a linear double-stranded DNA molecule.
6. The method of claim 4, wherein the first restriction endonuclease and the second restriction endonuclease are the same restriction endonuclease.
7. The method of claim 4, wherein the first restriction endonuclease and the second restriction endonuclease are different restriction endonucleases.
8. 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.
9. The method of any of the preceding claims, 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.
10. The method of any of the preceding claims, wherein the ligation is selected from a chemical ligation and a protein-assisted ligation.
11. The method of claim 10, wherein the ligation is effected by T4 ligase or an AAV Rep protein.
12. The method of any of the preceding claims, wherein the first ITR is selected from a wild-type ITR and a modified ITR.
13. The method of claim 1 or claim 2, wherein the second ITR is selected from a wild-type ITR and a modified ITR.
14. 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.

15. 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.
16. The method of claim 15, 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.
17. The method of claim 15, 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.
18. The method of any of the preceding claims, wherein the expression cassette sequence comprises at least one cis-regulatory element.
19. The method of claim 18, wherein the cis-regulatory element is selected from the group consisting of a promoter, an enhancer, a posttranscriptional regulatory element and a polyadenylation signal.
20. The method of claim 19, wherein the posttranscriptional regulatory element comprises a WHP posttranscriptional regulatory element (WPRE).
21. The method of claim 19, wherein the promoter is selected from the group consisting of a CAG promoter, an AAT promoter, an LP1 promoter, and an EF1a promoter.
22. The method of any of the preceding claims, wherein the expression cassette sequence comprises a transgene sequence.
23. The method of claim 22, wherein the transgene sequence is at least 2000 nucleotides in length.
24. The method of claim 22, wherein the transgene sequence encodes a protein.
25. The method of claim 24, wherein the transgene sequence encodes a reporter protein, a therapeutic protein, an antigen, a gene editing protein, or a cytotoxic protein.
26. The method of claim 22, wherein the transgene sequence is a functional nucleotide sequence.
27. The method of any of the preceding claims, wherein the closed-ended DNA vector is a ceDNA vector.
28. The method of claim 27, wherein the ceDNA vector is purified.
29. A closed-ended DNA vector generated by the method of any of the preceding claims.
30. A pharmaceutical composition comprising the closed-ended DNA vector of claim 29 and optionally, an excipient.
31. 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 cassettewith 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.

32. The method of claim 31, wherein the double-stranded DNA construct is a bacmid, plasmid, minicircle, or a linear double-stranded DNA molecule.

33. The method of claim 31 or 32, wherein a single restriction endonuclease is used to effect the excision.

34. The method of claim 31 or 32, wherein two different restriction endonucleases are used to effect the excision.

35. The method of any of the preceding claims, wherein the ligation is selected from a chemical ligation and a protein-assisted ligation.

36. The method of claim 35, wherein the ligation is effected by T4 ligase or an AAV Rep protein.

37. The method of any of claims 31-36, wherein the first ITR is selected from a wild-type ITR and a modified ITR.

38. The method of any of claims 31-37, wherein the second ITR is selected from a wild-type ITR and a modified ITR.

39. The method of any claims 31-38, wherein at least one of the first ITR and the second ITR comprises at least one RBE site.

40. The method of any of claims 31-39 wherein at least one of the first ITR and the second ITR is an AAV ITR or an AAV-derived ITR.

41. The method of any of claims 31-40, 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.

42. The method of any of claims 31-41, 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.

43. The method of any of claims 31-42, wherein the expression cassette sequence comprises at least one cis-regulatory element.

44. The method of any of claims 31-43, wherein the cis-regulatory element is selected from the group consisting of a promoter, an enhancer, a posttranscriptional regulatory element and a polyadenylation signal.

45. The method of claim 44, wherein the posttranscriptional regulatory element comprises a WHP posttranscriptional regulatory element (WPRE).

46. The method of claim 44, wherein the promoter is selected from the group consisting of a CAG promoter, an AAT promoter, an LP1 promoter, and an EF1a promoter.

47. The method of any of claims 31-46, wherein the expression cassette sequence comprises a transgene sequence.

48. The method of claim 47, wherein the transgene sequence is at least 2000 nucleotides in length.

49. The method of claim 47, wherein the transgene sequence encodes a protein.

50. The method of claim 49, wherein the transgene sequence encodes a reporter protein, a therapeutic protein, an antigen, a gene editing protein, or a cytotoxic protein.
51. The method of claim 47, wherein the transgene sequence is a functional nucleotide sequence.
52. The method of any of claims 31-51, wherein the closed-ended DNA vector is a ceDNA vector.
53. The method of claim 52, wherein the ceDNA vector is purified.
54. A closed-ended DNA vector generated by the method of any claims 31-54.
55. A pharmaceutical composition comprising the closed-ended DNA vector of claim 54 and optionally, an excipient.
56. A method of preparing a 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;
an antisense second ITR;
an antisense expression cassette sequence; and
an antisense first ITR;
forming a hairpin-comprising polynucleotide from the single-stranded molecule; and ligating the 5' and 3' ends to form the closed-ended DNA vector.
57. The method of claim 56, wherein at least one of the sense first ITR, the sense expression cassette sequence, the sense second ITR, the antisense second ITR, the antisense expression cassette sequence, and the antisense first ITR are synthesized.
58. The method of claim 56 or 57, wherein the single-stranded DNA molecule is constructed by synthesizing one or more of the sense first ITR, the sense expression cassette sequence, the sense second ITR, the antisense second ITR, the antisense expression cassette sequence, and the antisense first ITR as oligonucleotides and ligating such oligonucleotides to form the single-stranded DNA molecule.
59. The method of claim 56 or 57, 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.
60. The method of any of claims 56-59, wherein the step of forming a hairpin-comprising polynucleotide from the single-stranded molecule is effected by annealing the single-stranded molecule under conditions whereby one or more of the ITRs forms a hairpin loop.
61. The method of any of claims 56-60, wherein the ligation is selected from a chemical ligation and a protein-assisted ligation.
62. The method of claim 61, wherein the ligation is effected by T4 ligase or an AAV Rep protein.

63. The method of any of claims 56-62, wherein the sense first ITR is selected from a wild-type ITR and a modified ITR.
64. The method of any of claims 56-63, wherein the sense second ITR is selected from a wild-type ITR and a modified ITR.
65. The method of any of claims 56-64, wherein at least one of the sense first ITR, the antisense first ITR, the sense first ITR and the sense second ITR comprises at least one RBE site.
66. The method of any of claims 56-65 wherein at least one of the sense first ITR and the sense second ITR is an AAV ITR or an AAV-derived ITR.
67. The method of claim 66, wherein the sequence of the sense 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.
68. The method of claim 66, 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.
69. The method of any of claims 56-68, wherein the sense expression cassette sequence comprises at least one cis-regulatory element.
70. The method of claim 69, wherein the cis-regulatory element is selected from the group consisting of a promoter, an enhancer, a posttranscriptional regulatory element and a polyadenylation signal.
71. The method of claim 70, wherein the posttranscriptional regulatory element comprises a WHP posttranscriptional regulatory element (WPRE).
72. The method of claim 70, wherein the promoter is selected from the group consisting of a CAG promoter, an AAT promoter, an LP1 promoter, and an EF1a promoter.
73. The method of any of claims 56-72, wherein the sense expression cassette sequence comprises a transgene sequence.
74. The method of claim 73, wherein the transgene sequence is at least 2000 nucleotides in length.
75. The method of claim 73, wherein the transgene sequence encodes a protein.
76. The method of claim 75, wherein the transgene sequence encodes a reporter protein, a therapeutic protein, an antigen, a gene editing protein, or a cytotoxic protein.
77. The method of claim 73, wherein the transgene sequence is a functional nucleotide sequence.
78. The method of any of claims 56-77, wherein the closed-ended DNA vector is a ceDNA vector.
79. The method of claim 78, wherein the ceDNA vector is purified.
80. A closed-ended DNA vector generated by the method of any of claims 56-79.
81. A pharmaceutical composition comprising the closed-ended DNA vector of claim 80 and optionally, an excipient.
82. 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.
83. The method of claim 82, wherein at least one of the sense first ITR, the sense expression cassette sequence, the sense second ITR and the antisense expression cassette sequence are synthesized.
84. The method of claim 82 or 83, wherein the single-stranded DNA molecule is constructed by synthesizing one or more of the sense first ITR, the sense expression cassette sequence, the sense second ITR and the antisense expression cassette sequence and ligating such oligonucleotides to form the single-stranded DNA molecule.
85. The method of claim 82 or 83, 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.
86. The method of any of claims 82-85, wherein the annealing step results in one or both of the sense first ITR and the sense second ITR forming a hairpin loop.
87. The method of any of claims 82-86, wherein the sense first ITR is selected from a wild-type ITR and a modified ITR.
88. The method of any of claims 82-87, wherein the sense second ITR is selected from a wild-type ITR and a modified ITR.
89. The method of any of claims 82-88, wherein at least one of the sense first ITR and the sense second ITR comprises at least one RBE site.
90. The method of any of claims 82-89 wherein at least one of the sense first ITR and the sense second ITR is an AAV ITR or an AAV-derived ITR.
91. The method of claim 90, wherein the sequence of the sense 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.
92. The method of claim 90, 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.
93. The method of any of claims 82-92, wherein the sense expression cassette sequence comprises at least one cis-regulatory element.
94. The method of claim 93, wherein the cis-regulatory element is selected from the group consisting of a promoter, an enhancer, a posttranscriptional regulatory element and a polyadenylation signal.
95. The method of claim 94, wherein the posttranscriptional regulatory element comprises a WHP posttranscriptional regulatory element (WPRE).
96. The method of claim 94, wherein the promoter is selected from the group consisting of a CAG promoter, an AAT promoter, an LP1 promoter, and an EF1a promoter.
97. The method of any of claims 82-96, wherein the sense expression cassette sequence comprises a transgene sequence.

98. The method of claim 97, wherein the transgene sequence is at least 2000 nucleotides in length.
99. The method of claim 97, wherein the transgene sequence encodes a protein.
100. The method of claim 99, wherein the transgene sequence encodes a reporter protein, a therapeutic protein, an antigen, a gene editing protein, or a cytotoxic protein.
101. The method of claim 97, wherein the transgene sequence is a functional nucleotide sequence.
102. The method of any of claims 82-101, wherein the closed-ended DNA vector is a ceDNA vector.
103. The method of claim 102, wherein the ceDNA vector is purified.
104. A closed-ended DNA vector generated by the method of any of claims 82-103.
105. A pharmaceutical composition comprising the closed-ended DNA vector of claim 104 and optionally, an excipient.
106. A method of preparing a closed-ended DNA vector comprising:
providing 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;
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.
107. The method of claim 106, wherein the double-stranded DNA construct is a bacmid, plasmid, minicircle, or a linear double-stranded DNA molecule.
108. The method of claim 106 or 107, wherein a single restriction endonuclease is used to effect the excision.
109. The method of claim 106 or 107, wherein two different restriction endonucleases are used to effect the excision.
110. The method of any of claims 106-109, wherein the annealing step results in one or both of the sense first ITR and the sense second ITR forming a hairpin loop.
111. The method of any of claims 106-110, wherein the sense first ITR is selected from a wild-type ITR and a modified ITR.

112. The method of any of claims 106-111, wherein the sense second ITR is selected from a wild-type ITR and a modified ITR.
113. The method of any of claims 106-112, wherein at least one of the sense first ITR and the sense second ITR comprises at least one RBE site.
114. The method of any of claims 106-113 wherein at least one of the sense first ITR and the sense second ITR is an AAV ITR or an AAV-derived ITR.
115. The method of claim 114, wherein the sequence of the sense 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.
116. The method of claim 114, 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.
117. The method of any of claims 106-116, wherein the sense expression cassette sequence comprises at least one cis-regulatory element.
118. The method of claim 117, wherein the cis-regulatory element is selected from the group consisting of a promoter, an enhancer, a posttranscriptional regulatory element and a polyadenylation signal.
119. The method of claim 118, wherein the posttranscriptional regulatory element comprises a WHP posttranscriptional regulatory element (WPRE).
120. The method of claim 118, wherein the promoter is selected from the group consisting of a CAG promoter, an AAT promoter, an LP1 promoter, and an EF1a promoter.
121. The method of any of claims 106-120, wherein the sense expression cassette sequence comprises a transgene sequence.
122. The method of claim 121, wherein the transgene sequence is at least 2000 nucleotides in length.
123. The method of claim 121, wherein the transgene sequence encodes a protein.
124. The method of claim 123, wherein the transgene sequence encodes a reporter protein, a therapeutic protein, an antigen, a gene editing protein, or a cytotoxic protein.
125. The method of claim 121, wherein the transgene sequence is a functional nucleotide sequence.
126. The method of any of claims 106-125, wherein the closed-ended DNA vector is a ceDNA vector.
127. The method of claim 126, wherein the ceDNA vector is purified.
128. A closed-ended DNA vector generated by the method of any of claims 106-127.
129. A pharmaceutical composition comprising the closed-ended DNA vector of claim 128 and optionally, an excipient.
130. An isolated closed-ended DNA vector obtained by or obtainable by a process according to any of claims 1-28, 31-53, 56-79, 82-103, and 106-127.
131. A genetic medicine comprising an isolated closed-ended DNA vector obtained by the process according to any of claims 1-28, 31-53, 56-79, 82-103, and 106-127.
132. A cell comprising a the closed-ended DNA vector of claim 130.

133. A transgenic animal comprising the closed ended DNA vector of claim 130.
134. A method of treating a subject by administering a closed-ended DNA vector obtained by or obtainable by a process according to any of claims 1-28, 31-53, 56-79, 82-103, and 106-127.
135. A method for delivering a therapeutic protein to a subject, the method comprising:
administering to a subject a composition comprising a closed-ended DNA vector obtained by or obtainable by a process according to any of claims 1-28, 31-53, 56-79, 82-103, and 106-127, wherein at least one heterologous nucleotide sequence encodes a therapeutic protein.
136. The method of claim 135, wherein the therapeutic protein is a therapeutic antibody.
137. A kit comprising a closed-ended DNA vector obtained by or obtainable by a process according to any of claims 1-28, 31-53, 56-79, 82-103, and 106-127, and a nanocarrier, packaged in a container with a packet insert.
138. A kit for producing a closed-ended DNA vector obtained by or obtainable by a process according to any of claims 1-28, 31-53, 56-79, 82-103, and 106-127
139. A kit for producing a closed-ended DNA vector obtained by or obtainable by a process according to any of claims 1-28, comprising a first-single stranded ITR molecule comprising a first ITR, a second single-stranded ITR molecule comprising a second ITR and at least one reagent for ligation of the first-single stranded ITR molecule and second single-stranded ITR molecule to a double stranded polynucleotide molecule.
140. A kit for producing a closed-ended DNA vector obtained by or obtainable by a process according to any of claims 31-53, comprising:
- a. 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, wherein the expression cassette has a restriction endonuclease site for insertion of a transgene, and
 - b. at least one ligation reagent for ligation
141. A kit for producing a closed-ended DNA vector obtained by or obtainable by a process according to any of claims 56-79, comprising:
- 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;
 - 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

b. at least one ligation reagent for ligation

142. A kit for producing a closed-ended DNA vector obtained by or obtainable by a process according to any of claims 82-103, comprising:

a. 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

b. at least one ligation reagent for ligation.

143. A kit for producing a closed-ended DNA vector obtained by or obtainable by a process according to any of claims 106-127, comprising:

a. 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

b. at least one ligation reagent for ligation.

144. The kit of any of claims 138-143, wherein the at least one reagent for ligation is a reagent for chemical ligation.

145. The kit of claim 144, wherein the at least one reagent for ligation is a reagent for protein-assisted ligation.
146. The kit of claim 146, wherein the ligation is effected by T4 ligation or an AAV Rep protein.
147. The kit of any of claims 138-146, wherein the first-single stranded ITR molecule and second single-stranded ITR molecule comprise a restriction endonuclease cleave site at their ends.
148. The kit of any of claims 138-147, wherein the kit further comprises at least one restriction endonuclease enzyme.