

WHAT IS CLAIMED IS:

1. A double-stranded DNA molecule comprising in 5' to 3' direction of the top strand:
 - a. a first inverted repeat, wherein a first and a second restriction site for nicking endonuclease are arranged on opposite strands in proximity of the first inverted repeat such that nicking results in:
 - i. a top strand 5' single strand DNA overhang comprising the first inverted repeat or a fragment thereof upon separation of the top from the bottom strand of the first inverted repeat; or
 - ii. a bottom strand 3' single strand DNA overhang comprising the first inverted repeat upon separation of the top from the bottom strand of the first inverted repeat; and
 - b. a second inverted repeat, wherein a third and a fourth restriction site for nicking endonuclease are arranged on opposite strands in proximity of the second inverted repeat such that nicking results in:
 - i. a top strand 3' single strand DNA overhang comprising the second inverted repeat or a fragment thereof upon separation of the top from the bottom strand of the second inverted repeat; or
 - ii. a bottom strand 5' single strand DNA overhang comprising the second inverted repeat upon separation of the top from the bottom strand of the second inverted repeat.
2. A double-stranded DNA molecule comprising in 5' to 3' direction of the top strand:
 - a. a first hairpinned inverted repeat;
 - b. a nick of the bottom strand or a nick of the top strand;
 - c. a nick of the bottom strand or a nick of the top strand; and
 - d. a second hairpinned inverted repeat.
3. A method for preparing a hairpin-ended DNA molecule, wherein the method comprises:
 - a. providing a double-stranded DNA molecule, wherein the double-stranded DNA molecule comprises in 5' to 3' direction of the top strand:

- i. a first inverted repeat, wherein a first and a second restriction site for nicking endonuclease are arranged on opposite strands in proximity of the first inverted repeat such that nicking results in:
 - (1) a top strand 5' single strand DNA overhang comprising the first inverted repeat or a fragment thereof upon separation of the top from the bottom strand of the first inverted repeat; or
 - (2) a bottom strand 3' single strand DNA overhang comprising the first inverted repeat upon separation of the top from the bottom strand of the first inverted repeat;
- ii. a second inverted repeat, wherein a third and a fourth restriction site for nicking endonuclease are arranged on opposite strands in proximity of the second inverted repeat such that nicking results in:
 - (1) a top strand 3' single strand DNA overhang comprising the second inverted repeat or a fragment thereof upon separation of the top from the bottom strand of the second inverted repeat; or
 - (2) a bottom strand 5' single strand DNA overhang comprising the second inverted repeat upon separation of the top from the bottom strand of the second inverted repeat.
- b. incubating the double-stranded DNA molecule with one or more nicking endonucleases recognizing the four restriction sites, thereby creating the two single strand DNA overhangs as specified in step a upon separation of the top from the bottom strand;
- c. denaturing and thereby creating a DNA fragment that comprises the two single strand DNA overhangs; and
- d. annealing the single strand DNA overhangs and thereby creating a hairpinned inverted repeat on both ends of the DNA fragment resulting from step c.

4. The double-stranded DNA molecule of claim 1 or claim 2, or the method of claim 3, wherein the double-stranded DNA molecule is an isolated DNA molecule.

5. The double-stranded DNA molecule of claim 1, or the method of claim 3, wherein the first, second, third, and fourth restriction sites for nicking endonuclease are all restriction sites for the same nicking endonuclease.

6. The double-stranded DNA molecule of claim 1 or claim 2, or the method of claim 3, wherein:

- a. the first and the second inverted repeats of the double-stranded DNA molecule are the same; or
- b. the first and/or the second inverted repeat of the double-stranded DNA molecule is an ITR of a parvovirus; or
- c. the first and/or the second inverted repeat of the double-stranded DNA molecule is a modified ITR of a parvovirus; or
- d. the first and/or the second inverted repeat of the double-stranded DNA molecule has a nucleotide sequence that is at least 50%, 60%, 70%, 80%, 90%, 95%, 98%, or at least 99% identical to the ITR of a parvovirus.

7. The double-stranded DNA molecule or method of claim 6, wherein the parvovirus is a Dependoparvovirus, a Bocaparvovirus, an Erythroparvovirus, a Protoparvovirus, or a Tetraparvovirus.

8. The double-stranded DNA molecule of claim 1, or the method of claim 3, wherein the double-stranded DNA molecule is characterized in that:

- a. the first nick is within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 nucleotides from the 5' nucleotide of the ITR closing base pair of the first inverted repeat; and
- b. the second nick is within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 nucleotides from the 3' nucleotide of the ITR closing base pair of the first inverted repeat; and
- c. the third nick is within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 nucleotides from the 5' nucleotide of the ITR closing base pair of the second inverted repeat, and the fourth nick is within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 nucleotides from the 3' nucleotide of the ITR closing base pair of the second inverted repeat; or

d. the third nick is within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 nucleotides from the 3' nucleotide of the ITR closing base pair of the second inverted repeat; and the fourth nick is within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 nucleotides from the 5' nucleotide of the ITR closing base pair of the second inverted repeat;

wherein the first nick and the fourth nick are two resulting nicks on the top strand, with the first nick being the one closer to the 5' end of the top strand and the fourth nick being the one closer to the 3' end of the top strand, and wherein the second nick and the third nick are two resulting nicks on the bottom strand, with the third nick being the one closer to the 5' end of the bottom strand and the second nick being the one closer to the 3' end of the bottom strand.

9. The double-stranded DNA molecule of claim 1, or the method of claim 3, wherein the double-stranded DNA molecule is characterized in that:

a. the first nick is within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 nucleotides from the 3' nucleotide of the ITR closing base pair of the first inverted repeat; and

b. the second nick is within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 nucleotides from the 5' nucleotide of the ITR closing base pair of the first inverted repeat; and

c. the third nick is within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 nucleotides from the 3' nucleotide of the ITR closing base pair of the second inverted repeat, and the fourth nick is within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 nucleotides from the 5' nucleotide of the ITR closing base pair of the second inverted repeat; or

d. the third nick is within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 nucleotides from the 5' nucleotide of the ITR closing

base pair of the second inverted repeat, and the fourth nick is within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 nucleotides from the 3' nucleotide of the ITR closing base pair of the second inverted repeat;

wherein the first nick and the fourth nick are two resulting nicks on the top strand, with the first nick being the one closer to the 5' end of the top strand and the fourth nick being the one closer to the 3' end of the top strand, and wherein the second nick and the third nick are two resulting nicks on the bottom strand, with the third nick being the one closer to the 5' end of the bottom strand and the second nick being the one closer to the 3' end of the bottom strand.

10. The double-stranded DNA molecule of claim 1, or the method of claim 3, wherein the double-stranded DNA molecule is a plasmid, wherein the plasmid is circular or linear.

11. The double-stranded DNA molecule or method of claim 10, wherein the plasmid further comprises a restriction enzyme site in the region 5' to the first inverted repeat and 3' to the second inverted repeat wherein the restriction enzyme site is not present in any of the first inverted repeat, second inverted repeat, and the region between the first and second inverted repeats, wherein cleavage with the restriction enzyme results in single strand DNA overhangs that do not anneal at detectable levels under conditions that favor annealing of the first and/or second inverted repeat.

12. The double-stranded DNA molecule or method of claim 10, wherein the plasmid further comprises a fifth and a sixth restriction site for nicking endonuclease in the region 5' to the first inverted repeat and 3' to the second inverted repeat, wherein the fifth and sixth restriction sites for nicking endonuclease are:

- a. on opposite strands; and
- b. create a break in the double stranded DNA molecule such that the single strand DNA overhangs of the break do not anneal at detectable levels inter- or intramolecularly under conditions that favor annealing of the first and/or second inverted repeat.

13. The double-stranded DNA molecule or method of claim 12, wherein the fifth and the sixth nick are 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides apart.

14. The double-stranded DNA molecule or method of claim 12, wherein the first, second, third, fourth, fifth, and sixth restriction sites for nicking endonuclease are all target sequences for the same nicking endonuclease.

15. The double-stranded DNA molecule of claim 1, or the method of claim 3, wherein one or more of the nicking endonuclease site of the double-stranded DNA molecule is a target sequence of an endogenous nicking endonuclease.

16. The double-stranded DNA molecule or method of claim 12, wherein the plasmid further comprises an open reading frame encoding a nicking endonuclease that recognizes the first, second, third, fourth, fifth, and/or sixth restriction site for nicking endonuclease.

17. The double-stranded DNA molecule of claim 1, or the method of claim 3, wherein the nicking endonuclease that recognizes the first, second, third, and/or fourth restriction site for nicking endonuclease is Nt. BsmAI; Nt. BtsCI; N. ALwI; N. BstNBI; N. BspD6I; Nb. MvaI269I; Nb. BsrDI; Nt. BtsI; Nt. BsaI; Nt. Bpu10I; Nt. BsmBI; Nb. BbvCI; Nt. BbvCI; or Nt. BspQI.

18. The double-stranded DNA molecule or method of claim 12 wherein the nicking endonuclease that recognizes the fifth and sixth restriction site for nicking endonuclease Nt. BsmAI; Nt. BtsCI; N. ALwI; N. BstNBI; N. BspD6I; Nb. MvaI269I; Nb. BsrDI; Nt. BtsI; Nt. BsaI; Nt. Bpu10I; Nt. BsmBI; Nb. BbvCI; Nt. BbvCI; or Nt. BspQI.

19. The double-stranded DNA molecule of claim 1 or claim 2, or the method of claim 3, wherein the double-stranded DNA molecule further comprises a transcription unit located between the first and the second inverted repeats, and wherein the size of the transcription unit is at least 4 kb, at least 4.5 kb, at least 5 kb, at least 5.5 kb, at least 6 kb, at least 6.5 kb, at least 7 kb, at least 7.5 kb, at least 8 kb, at least 8.5 kb, at least 9 kb, at least 9.5 kb, or at least 10 kb.

20. The double-stranded DNA molecule of claim 2, wherein the double-stranded DNA molecule is protected from an exonuclease, optionally wherein the exonuclease is RecBCD exonuclease.

21. The double-stranded DNA molecule of claim 1 or claim 2, or the method of claim 3, wherein the double-stranded DNA molecule lacks at least one replication-associated protein binding sequence ("RABS").

22. The double-stranded DNA molecule of claim 1 or claim 2, or the method of claim 3, wherein the double-stranded DNA molecule lacks a viral capsid protein encoding sequence.

23. The double-stranded DNA molecule of claim 1 or claim 2, or the method of claim 3, wherein the first or the second inverted repeat of the double-stranded DNA molecule lacks a functional RABS.

24. The double-stranded DNA molecule of claim 1 or claim 2, or the method of claim 3, wherein the first and the second inverted repeat of the double-stranded DNA molecule each lacks a functional RABS.

25. The double-stranded DNA molecule of claim 1 or claim 2, or the method of claim 3, wherein the double-stranded DNA molecule lacks a terminal resolution site (TRS).

26. The double-stranded DNA molecule of claim 1 or claim 2, or the method of claim 3, wherein the first or the second inverted repeat of the double-stranded DNA molecule lacks a functional TRS.

27. The double-stranded DNA molecule of claim 1 or claim 2, or the method of claim 3, wherein the first and the second inverted repeat of the double-stranded DNA molecule each lacks a functional TRS.

28. The double-stranded DNA molecule of claim 1 or claim 2, or the method of claim 3, wherein the first or the second inverted repeat of the double-stranded DNA molecule lacks a functional RABS and a functional TRS.

29. The double-stranded DNA molecule of claim 1 or claim 2, or the method of claim 3, wherein the first and the second inverted repeat of the double-stranded DNA molecule each lacks a functional RABS and a functional TRS.

30. The double-stranded DNA molecule of claim 29, wherein the mobilization risk of the double-stranded DNA molecule when administered to a host is lower than control DNA molecules with the at least one functional RABS and/or with at least one functional TRS by 100%, 95%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, or 50%.

31. Isolated double-stranded DNA molecules of claim 4, wherein:

- a. the isolated double-stranded DNA molecules are free of fragments of the double-stranded DNA molecule; and/or
- b. fragments of the double-stranded DNA molecules are no more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, or 10% of the isolated double-stranded DNA molecules; and/or
- c. the isolated double-stranded DNA molecules are free of nucleic acid contaminants that are not fragments of the double-stranded DNA molecules; and/or
- d. nucleic acid contaminants that are not fragments of the double-stranded DNA molecules are less than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, or 10% of the isolated double-stranded DNA molecules; and/or
- e. the isolated double-stranded DNA molecules are free of baculoviral DNA; and/or
- f. baculoviral DNA is less than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, or 10% of the isolated double-stranded DNA molecules.

32. A delivery vehicle comprising the double-stranded DNA molecule of claim 2, optionally wherein the delivery vehicle comprises a hybridosome, a liposome, a lipid nanoparticle, or a transfection reagent.

33. The method of claim 3, wherein the double-stranded DNA molecule of step a is provided by:

- a. culturing a host cell comprising the double-stranded DNA molecule under conditions resulting in amplification of the double-stranded DNA molecule; and
- b. releasing the double-stranded DNA molecule from the host cell.

34. The method of claim 3, wherein the double-stranded DNA molecule of step a is provided by *in vitro* replication, isothermal DNA amplification, or chemical synthesis.

35. The method of claim 33, wherein the host cell is a bacterial host cell.

36. The double-stranded DNA molecule of claim 1 or claim 2, or the method of claim 3, wherein the double-stranded DNA molecule:

- a. lacks any functional RABS; and/or
- b. lacks any functional TRS; and/or
- c. lacks or has reduced (by at least 50%, 60%, 70%, 80%, 90%, 95%, 98%, 99%, or at least 99.9%) P5 AAV promoter activity; and/or
- d. lacks any other elements required for viral replication.

37. The double-stranded DNA molecule or method of claim 36, wherein

- a. any RABS in the double-stranded DNA molecule is deleted or mutated to be functionally inactive; and/or
- b. any TRS in the double-stranded DNA molecule is deleted or mutated to be functionally inactive; and/or
- c. any P5 AAV promoter is deleted or mutated to be functionally inactive; and/or
- d. any other element required for viral replication in the double-stranded DNA molecule is deleted or mutated to be functionally inactive.

38. The double stranded DNA molecule or method of claim 37, wherein the deletion is only of the RABS and/or TRS and/or any P5 AAV promoter and/or any other element required for replication but does not delete an entire stem/loop structure.

39. The double-stranded DNA molecule of claim 36, wherein the double-stranded DNA molecule is not detectably replicated in a cell when the cell is also infected with the wildtype virus from which the ITRs of the double-stranded DNA molecule were derived and a helper virus, or wherein the ability of the double-stranded DNA molecule is reduced by at least 50%, 60%, 70%, 80%, 90%, 95%, 98%, 99%, or at least 99.9% in such an assay.

40. The double-stranded DNA molecule of claim 39, wherein the double-stranded DNA molecule is derived from an adeno-associated virus and the wild type virus is the same adeno-associated virus.

41. The double-stranded DNA molecule or method of claim 19, wherein:

a. the 5' end of the top strand of the transcription unit of the double-stranded DNA molecule is 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, about 20, about 21, about 22, about 23, about 24, about 25, about 26, about 27, about 28, about 29, about 30, about 31, about 32, about 33, about 34, about 35, about 36, about 37, about 38, about 39, about 40, about 41, about 42, about 43, about 44, about 45, about 46, about 47, about 48, about 49, about 50, about 51, about 52, about 53, about 54, about 55, about 56, about 57, about 58, about 59, about 60, about 61, about 62, about 63, about 64, about 65, about 66, about 67, about 68, about 69, about 70, about 71, about 72, about 73, about 74, about 75, about 76, about 77, about 78, about 79, about 80, about 81, about 82, about 83, about 84, about 85, about 86, about 87, about 88, about 89, about 90, about 91, about 92, about 93, about 94, about 95, about 96, about 97, about 98, about 99, about 100 nucleotides, greater than 100 nucleotides, at least 150 nucleotides, at least 200 nucleotides, at least 300 nucleotides, or at least 400 nucleotides apart from the 3' top strand end of the inverted repeat; or

b. the 3' end of the top strand of the transcription unit of the double-stranded DNA molecule is 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, about 20, about 21, about 22, about 23, about 24, about 25, about 26, about 27, about 28, about 29, about 30, about 31, about 32, about 33, about 34, about 35, about 36, about 37, about 38, about 39, about 40, about 41, about 42, about 43, about 44, about 45, about 46, about 47, about 48, about 49, about 50, about 51, about 52, about 53, about 54, about 55, about 56, about 57, about 58, about 59, about 60, about 61, about 62, about 63, about 64, about 65, about 66, about 67, about 68, about 69, about 70, about 71, about 72, about 73, about 74, about 75, about 76, about 77, about 78, about 79, about 80, about 81, about 82, about 83, about 84, about 85, about 86, about 87, about 88, about 89, about 90, about 91, about 92, about 93, about 94, about 95, about 96, about 97, about 98, about 99, about 100 nucleotides, greater than 100 nucleotides, at least 150 nucleotides, at

least 200 nucleotides, at least 300 nucleotides, or at least 400 nucleotides apart from the 5' top strand end of the inverted repeat.

42. The double-stranded DNA molecule of claim 2, wherein one, two, three, or all nicks (such as nicks resulting from the nicking endonuclease digestion) are ligated.

43. The double-stranded DNA molecule or method of claim 19, wherein:

- a. the bottom strand of the double-stranded DNA molecule is in anti-sense orientation with respect to an open reading frame in the transcription unit; or
- b. the top strand of the double-stranded DNA molecule is in anti-sense orientation with respect to an open reading frame in the transcription unit.

44. A DNA molecule resulting from the method of claim 3.

45. The double-stranded DNA molecule of claim 1 or claim 2, or the method of claim 3, wherein the double-stranded DNA molecule:

- a. comprises a first inverted repeat that lacks any transcriptional activity; and/or
- b. comprises a second inverted repeat that lacks any transcriptional activity; and/or
- c. lacks or has reduced (by at least 50%, 60%, 70%, 80%, 90%, 95%, 98%, 99%, or at least 99.9%) P5 AAV promoter activity; and/or
- d. lacks any other elements in the inverted repeats that are transcriptionally active.

46. The double-stranded DNA molecule of claim 1 or claim 2, or the method of claim 3, wherein the first and the second inverted repeat of the double-stranded DNA molecule each further comprises a modification of at least one nucleotide to replace or delete occurrence of CpG motifs.

47. The double-stranded DNA molecule of claim 1 or claim 2, or the method of claim 3, wherein the double-stranded DNA molecule is characterized in that the first inverted repeat and the second inverted repeat each comprise a palindromic sequence that is 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%,

94%, 95%, 96%, 97%, 98%, or 99% identical when read forwards as when read backwards on the complementary strand, and wherein

a. the first and the second restriction site for nicking endonuclease are at least 19, at least 20, at least 25, at least 30, at least 35, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 105, at least 110, at least 115, at least 120, at least 125, at least 130, at least 135, at least 140, at least 145, at least 150, at least 155, at least 160, at least 165, at least 170, at least 175, at least 180, at least 185, at least 190, at least 195, or at least 200 nucleotides apart; and/or

b. the third and the fourth restriction sites for nicking endonuclease at least 19, at least 20, at least 25, at least 30, at least 35, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 105, at least 110, at least 115, at least 120, at least 125, at least 130, at least 135, at least 140, at least 145, at least 150, at least 155, at least 160, at least 165, at least 170, at least 175, at least 180, at least 185, at least 190, at least 195, or at least 200 nucleotides apart.

48. The double-stranded DNA molecule of claim 1 or claim 2, or the method of claim 3, wherein the first and the second inverted repeat of the double-stranded DNA molecule are each characterized in that the sequence stretching from one nucleotide of the ITR closing base pair to the other nucleotide of the ITR closing base pair has a Gibbs free energy of unfolding under physiological conditions of no more than -10 kcal/mol.

49. The double-stranded DNA molecule of claim 1, or the method of claim 3, wherein the double-stranded DNA molecule is characterized in that the length of each single strand DNA overhang is independently at least 20, at least 21, at least 22, at least 23, at least 24, at least 25, at least 26, at least 27, at least 28, at least 29, at least 30, at least 31, at least 32, at least 33, at least 34, at least 35, at least 36, at least 37, at least 38, at least 39, at least 40, at least 41, at least 42, at least 43, at least 44, at least 45, at least 46, at least 47, at least 48, at least 49, at least 50, at least 51, at least 52, at least 53, at least 54, at least 55, at least 56, at least 57, at least 58, at least 59, at least 60, at least 61, at least 62, at least 63, at least 64, at least 65, at least 66, at least 67, at least 68, at least 69, at least 70, at least 71, at least 72, at least 73, at least 74, at least 75, at least 76, at least 77, at least 78, at least 79, at least 80, at least 81, at least 82, at least 83, at least 84, at least 85, at least 86, at least 87, at least 88, at least 89, at least 90, at least 91, at least 92, at least 93, at least 94, at least 95, at least 96, at least 97, at least 98, at least 99, or at least 100 nucleotides in length.

50. The double-stranded DNA molecule or method of claim 19, wherein the transcription unit of the double-stranded DNA molecule comprises a transgene in the range of from about 500 to about 50,000 nucleotides in length, from about 500 to about 75,000 nucleotides in length, from about 500 to about 10,000 nucleotides in length, from about 1000 to about 10,000 nucleotides in length, or from about 500 to about 5,000 nucleotides in length.

51. The method of claim 11 further comprising,
e. incubating the double-stranded DNA molecule of step a or step b, or the fragments resulting from step c, with one or more restriction enzymes resulting in the break in the double stranded DNA molecule; and
f. incubating the fragments of the double-stranded DNA molecule with an exonuclease thereby digesting the fragments of the double-stranded DNA molecule except the fragment resulting from step d.

52. The method of claim 12 further comprising,
e. incubating the double-stranded DNA molecule of step a or step b, or the fragments resulting from step c, with one or more nicking endonuclease recognizing the fifth and sixth restriction sites resulting in the break in the double stranded DNA molecule; and
f. incubating the fragments of the double-stranded DNA molecule with an exonuclease thereby digesting the fragments of the double-stranded DNA molecule except the fragment resulting from step d.

53. The method of claim 3 further comprising ligating the nicks of the fragment resulting from step d with a ligase to form a circular DNA.

54. The method of claim 51 further comprising after step f, repairing the nicks of the fragment resulting from step d with a ligase to form a circular DNA.

55. The method of claim 52 further comprising after step f, repairing the nicks of the fragment resulting from step d with a ligase to form a circular DNA.

56. The double-stranded DNA molecule of method of any one of claims 19, 41, 43, or 50, wherein the transcription unit is an expression cassette.