[0009] Accordingly, use of adeno-associated virus (AAV) vectors for gene therapy is limited due to the single administration to patients (owing to the patient immune response), the limited range of transgene genetic material suitable for delivery in AAV vectors due to minimal viral packaging capacity (about 4.5kb), and slow AAV-mediated gene expression.

[0010] Closed-ended DNA vectors have been developed that are capable of delivering one or more desired transgenes *in vivo* for therapeutic or other purposes, and which avoid the above-described liabilities of AAV and other virus vector systems. However, methods of producing such ceDNA vectors have relied upon traditional bacterial or insect cell production methods. Such methods can result in contaminants (e.g., nucleic acid contaminants) from the cells used to produce the vectors that are inconvenient or costly to remove and which may have undesirable side effects if included in a ceDNA therapeutic formulation. Accordingly, there is need in the field for a technology that allows for the generation of recombinant vectors to be used in methods of controlling gene expression with minimal off-target effects such as those introduced by such contaminants or other artifacts of the purification method. The methods provided herein lessen or avoid such problems.

SUMMARY

[0011] Conventional methods for production of viral and virally-derived DNA typically use eukaryotic cells, e.g., mammalian or insect cells. One commonly used insect cell line is Sf9. However, not only do these cells both contain enzymes and other proteins which may have a deleterious effect on the DNA to be replicated, but the process of purifying the desired DNA from cell lysates introduces cellular nucleic acids whose presence can make purification of the desired DNA product more difficult. Further, such impurities or contaminants can have a range of deleterious and/or unwanted effects in the subject to which the desired DNA is administered. Additionally, such traditional cell-based production methods can have issues with respect to the quantity of DNA vector product produced, and it is not uncommon for significant engineering of the cell line itself or the production technology to be required to produce desirable yields. The technology described herein relates to a synthetic production method that can readily produce closed circle hairpin loop-containing DNA vectors such as, but not limited to, close-ended DNA vectors (ceDNA vectors) in higher purities and quantities than by conventional means, avoiding the concerns detailed above.

[0012] The disclosure described herein provides synthetic production methods to produce closed-ended DNA vectors using a synthetic production system, which can be a cell-free system. In some examples, the closed-ended DNA vector is a ceDNA vector, which can be used in methods of controlling gene expression in a cell, tissue or system or to introduce new genetic material into a desired cell, tissue or system. In one particular example, the technology described herein relates

to novel cell-free methods of making DNA vectors containing modified AAV inverted terminal repeat sequences (ITRs) and, e.g., one or more expressible transgenes. The methods disclosed herein can be used to produce any closed-ended hairpin loop-containing DNA vector in a cell free system, including but not limited to capsid-free, linear duplex DNA molecules, herein referred to ceDNA vectors, formed from a single strand of DNA with covalently-closed ends (linear, continuous and non-encapsidated structure).

[0013] One exemplary synthetic production method to generate a closed-ended DNA vector, exemplified using the production of a ceDNA vector as disclosed herein, relates to excising the entire molecule that forms the closed-ended DNA vector from a double-stranded DNA construct. In such an example, a double-stranded DNA construct is provided with, in 5' to 3' order: a first restriction endonuclease site; an upstream ITR; an expression cassette; a downstream ITR; and a second restriction endonuclease site. The double-stranded DNA construct is then contacted with one or more restriction endonucleases to generate double-stranded breaks at both of the restriction endonuclease cleavage sites. One endonuclease can target both sites, or each site can be targeted by a different endonuclease as long as the restriction sites are not present within the closed-ended vector template region. This excises the sequence between the restriction endonuclease sites from the rest of the double-stranded DNA construct. This excised molecule will have free 5' and 3' ends, which are then ligated in order to form a ceDNA vector. In some examples, the excised molecule is first annealed to facilitate hairpin formation prior to ligation of the free 5' and 3' ends. In some examples, the unwanted double-stranded DNA construct backbone is cleaved by one or more restriction endonucleases specific for a unique cleavage site in the backbone so that it is degraded and more readily eliminated during purification.

[0014] Another exemplary method of producing a DNA vector, e.g., a ceDNA vector, using the synthetic production method as disclosed herein involves the assembly of various oligonucleotides to form the complete vector. In such an example, a DNA vector, e.g., ceDNA vector is produced by synthesizing a 5' oligonucleotide and a 3' ITR oligonucleotide, which in some examples, are in a hairpin or other three-dimensional configuration (e.g., holliday junction configuration), and ligating the 5' and 3' ITR oligonucleotides to a double-stranded polynucleotide comprising an expression cassette or heterologous nucleic acid sequence. Optionally, a step is added subjecting the oligo(s) to conditions that facilitate the folding of the oligo into a three-dimensional configuration prior to the ligation step. FIG. 11B shows an exemplary method of generating a ceDNA vector comprising ligating a 5' ITR oligonucleotide and a 3' ITR oligonucleotide to a double stranded polynucleotide comprising an expression cassette. In some examples, the 5' and 3' ITR oligonucleotides are 5' and 3' hairpin oligonucleotides or have a hairpin structure or different three-dimensional configuration (e.g., a T- or Y-shaped Holliday junction), and can optionally be provided by *in vitro* DNA synthesis. In some examples, the 5' and 3' ITR oligonucleotides have been cleaved with a

restriction endonuclease to have complementary sticky ends to the double-stranded polynucleotide that has corresponding restriction endonuclease sticky ends. In some examples, the end of the hairpin of the 5' ITR oligonucleotide has a sticky end that is complementary to the 5' sense strand and 3' antisense strand of the double-stranded polynucleotide. In some examples, the end of the hairpin of the 3' ITR oligonucleotide has a sticky end that is complementary to the 3' sense strand and 5' antisense strand of the double-stranded polynucleotide. In some examples, the ends of the hairpin of the 5' ITR oligonucleotide and the 3' ITR oligonucleotide have different restriction endonuclease sticky ends, such that directed ligation to each end of the double-stranded polynucleotide can be achieved. In some examples, the ends of one or both of the ITR oligonucleotides do not have overhangs and such ITR oligo(s) are ligated to the double-stranded polynucleotide by blunt end-joining. In some examples, the unwanted double-stranded DNA polynucleotide backbone is cleaved by one or more restriction endonucleases specific for a unique cleavage site in the backbone so that it is degraded and more readily eliminated during purification.

[0015] Another exemplary method of producing a DNA vector, (e.g., ceDNA vector) involves the formation of a single-stranded linear DNA comprising an expression cassette and subsequently closing the DNA molecule with ligation. In this example, the DNA vector is prepared by synthesizing through any art-known means a single-stranded linear DNA comprising in the 5' to 3' direction a first 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, and then ligating the free ends in order to form a closed-ended ceDNA vector. In one example, using the production of a ceDNA vector as an exemplary DNA vector to be produced, the resulting single-stranded DNA molecule for production of a ceDNA vector comprises, from 5' to 3':

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.

[0016] In this exemplary method, in one example, oligonucleotides may be synthesized that encompass 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. One or more of such oligonucleotides may be ligated in order to form the single-stranded DNA molecule as shown above. Once the single-stranded DNA molecule has been formed, the free 3' and 5' ends of the molecule may be joined by ligation, forming the ceDNA vector.

[0017] Another exemplary method of producing a closed-ended DNA vector is by synthesis of a single-stranded sequence comprising at least one ITR flanking an expression cassette sequence and which also comprises an antisense expression cassette sequence. In one nonlimiting example, ceDNA vector is produced by the method as follows.

[0018] A single-stranded sequence comprising in order from 5' to 3':

a sense first ITR;
a sense expression cassette sequence;
a sense second ITR; and
an antisense expression cassette sequence

is provided. In one example the single-stranded sequence may be synthesized directly through any art-known method. In another example, the single-stranded sequence may be constructed by joining by ligation two or more oligos comprising one or more of the sense first ITR, sense expression cassette sequence, sense second ITR and antisense expression cassette sequence.

[0019] In yet another example, the single-stranded sequence may be obtained by excision of the sequence from a double-stranded DNA construct with subsequent separation of the strands from the excised double-stranded fragment. More specifically, a double-stranded DNA construct comprising a first restriction site, the sense first ITR, the sense expression cassette sequence, the sense second ITR, the antisense expression cassette sequence, and a second restriction site in 5' to 3' order is provided. The region between the two restriction endonuclease cleavage sites is excised by cleavage with at least one restriction endonuclease recognizing such cleavage site(s). The resulting excised double-stranded DNA fragment is treated such that the sense and antisense strands are separated into the desired single-stranded sequence fragments.

[0020] The single-stranded sequence is subjected to an annealing step to facilitate the formation of one or more hairpin loop by the sense first ITR and/or the sense second ITR, and the complementary binding of the sense expression cassette sequence to the antisense expression cassette sequence. The result is a closed-ended structure that did not require ligation to form. Annealing parameters and techniques are well known in the art.

[0021] In all examples of the synthetic production methods to generate DNA vectors as disclosed herein, the ligation step can be a chemical ligation step or an enzymatic ligation step. In some examples, ligation can be conducted using a ligation-competent enzyme, e.g., DNA ligase, e.g. to ligate 5' and 3' sticky overhangs, or blunt ends. In some examples, the ligation enzyme is a ligase enzyme other than a Rep protein. In some examples, the ligation enzyme is an AAV Rep protein.

[0022] In all examples of the synthetic methods to generate DNA vectors as disclosed herein, the method is an *in vitro* method. In a preferred example, the method is a cell-free method, i.e., not performed in, or in the presence of a cell, e.g., an insect cell.

[0023] It will be appreciated by one of ordinary skill in the art that one or more enzymes for the synthetic production method or one or more of the oligonucleotide components can be produced from a cell and used in the methods of the disclosure in purified form. Accordingly, in some examples, the synthetic production method is a cell-free method, however, a restriction enzyme and/or ligase enzyme can be produced from a cell. In one example, a cell, such as a bacterial cell, comprising an expression vector expressing one or more of the restriction endonucleases or the ligase enzymes can be present. Therefore, while the methods disclosed herein are primarily directed to cell-free synthetic methods to generate the DNA vectors disclosed herein, also encompassed in one example are synthetic production methods where a cell, e.g., bacterial cell but not an insect cell is present and can be used to express one or more of the enzymes required in the method.

[0024] One example of the technology described herein is use the synthetic production methods to generate ceDNA vectors. The ceDNA vectors described herein are capsid-free, linear duplex DNA molecules formed from a continuous strand of complementary DNA with covalently-closed ends (linear, continuous and non-encapsidated structure), which comprise a 5' inverted terminal repeat (ITR) sequence and a 3' ITR sequence, where the 5' ITR and the 3' ITR can have the same symmetrical three-dimensional organization with respect to each other, (i.e., symmetrical or substantially symmetrical), or alternatively, the 5' ITR and the 3' ITR can have different three-dimensional organization with respect to each other (i.e., asymmetrical ITRs). In addition, the ITRs can be from the same or different serotypes. In some examples, a ceDNA vector can comprise ITR sequences that have a symmetrical three-dimensional spatial organization such that their structure is the same shape in geometrical space, or have the same A, C-C' and B-B' loops in 3D space (i.e., they are the same or are mirror images with respect to each other). In some examples, one ITR can be from one AAV serotype, and the other ITR can be from a different AAV serotype.

[0025] Accordingly, some examples of the technology described herein relate to synthetic production of a ceDNA vector that comprises ITR sequences selected from any of: (i) at least one WT ITR and at least one modified AAV inverted terminal repeat (ITR) (e.g., asymmetric modified ITRs); (ii) two modified ITRs where the mod-ITR pair have a different three-dimensional spatial organization with respect to each other (e.g., asymmetric modified ITRs), or (iii) symmetrical or substantially symmetrical WT-WT ITR pair, where each WT-ITR has the same three-dimensional spatial organization, or (iv) symmetrical or substantially symmetrical modified ITR pair, where each mod-ITR has the same three-dimensional spatial organization.

[0026] Examples of the disclosure relate to synthetic production methods to produce the ceDNA vectors useful for expression of a desired transgene in a cell, tissue, organ, system, or subject as described herein. In particular, provided herein are methods for producing closed-ended DNA vectors, including but not limited to, ceDNA vectors in a cell-free environment, thereby limiting the amount of impurities and preventing introduction of contaminants during the production process that could impact the efficacy and/or safety of a given vector product. Such methods can be used to synthesize a DNA vector, for example a ceDNA vector, expressing any desired transgene. Transgenes can be selected for treatment of a given disease, promoting optimal health, prevention of disease onset, for diagnostic purposes, or as desired by one of skill in the art for a given application.

[0027] In another example of this example and all other examples provided herein, the transgene encodes a protein of interest, e.g., where a protein of interest is a receptor, a toxin, a hormone, an enzyme, or a cell surface protein. In another example of this example and all other examples provided herein, the protein of interest is a receptor. In another example of this example and all other examples provided herein, the protein of interest is an enzyme. Exemplary genes to be targeted and proteins of interest are described in detail in the methods of use and methods of treatment sections herein.

[0028] In some examples, the present application may be defined in any of the following paragraphs:

1. A method of preparing a closed-ended DNA vector comprising: (i) providing a first single-stranded ITR molecule comprising a first ITR; (ii) providing a second single-stranded ITR molecule comprising a second ITR; (iii) 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 DNA vector.

2. A method of preparing a closed-ended DNA vector comprising:

contacting a double-stranded DNA construct comprising: (i) an expression cassette; (ii) a first ITR on the upstream (5'-end) of the expression cassette; (iii) a second ITR on the downstream (3'-end) of the expression cassette; (iii) 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.

3. 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;

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 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; 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 vector.

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 aspect, 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 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.

In another aspect, 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 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.

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

In another aspect, the present disclosure refers to a genetic medicine comprising an isolated closed-ended DNA vector obtained by the process 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 a process 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 a process 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

positioned between wild-type inverted terminal repeat sequences, wherein optionally the heterologous nucleic acid sequence encodes a transgene, and wherein the vector is not in a viral capsid.

[0030] 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 positioned between asymmetric inverted terminal repeat sequences (asymmetric ITRs), wherein at least one of the asymmetric ITRs comprises a functional terminal resolution site and a Rep binding site, and optionally the heterologous nucleic acid sequence encodes a transgene, and wherein the vector is not in a viral capsid.

[0031] 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 positioned between symmetric mutant inverted terminal repeat sequences, wherein at least one of the ITRs comprises a functional terminal resolution site and a Rep binding site, and optionally the heterologous nucleic acid sequence encodes a transgene, and wherein the vector is not in a viral capsid.

[0032] These and other examples of the disclosure are described in further detail below.

DESCRIPTION OF DRAWINGS

[0033] Embodiments of the present disclosure, briefly summarized above and discussed in greater detail below, can be understood by reference to the illustrative embodiments of the disclosure depicted in the appended drawings. However, the appended drawings illustrate only typical embodiments of the disclosure and are therefore not to be considered limiting of scope, for the disclosure may admit to other equally effective embodiments.

[0034] FIG. 1A illustrates an exemplary structure of a ceDNA vector comprising asymmetric ITRs. In this embodiment, the exemplary ceDNA vector comprises an expression cassette containing CAG promoter, WPRE, and BGHpA. An open reading frame (ORF) encoding a transgene, e.g., a luciferase transgene is inserted into the cloning site (R3/R4) between the CAG promoter and WPRE. The expression cassette is flanked by two inverted terminal repeats (ITRs) – the wild-type AAV2 ITR on the upstream (5'-end) and the modified ITR on the downstream (3'-end) of the expression cassette, therefore the two ITRs flanking the expression cassette are asymmetric with respect to each other.

[0035] FIG. 1B illustrates an exemplary structure of a ceDNA vector comprising asymmetric ITRs with an expression cassette containing CAG promoter, WPRE, and BGHpA. An open reading frame (ORF) encoding a transgene, e.g., a Luciferase transgene is inserted into the cloning site between

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; 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 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 is 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 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.

7. 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.

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

9. The method of claim 1 or claim 2, wherein the second ITR is selected from a wild-type ITR and a modified ITR.

10. 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.

11. 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.

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

13. The method of claim 12, wherein the cis-regulatory element is selected from the group consisting of a promoter, an enhancer, a posttranscriptional regulatory element and a polyadenylation signal.

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

15. The method of claim 14, wherein the transgene sequence encodes a protein.

16. The method of claim 15, wherein the transgene sequence encodes a reporter protein, a therapeutic protein, an antigen, a gene editing protein, or a cytotoxic protein.

17. The method of claim 14, wherein the transgene sequence is a functional nucleotide sequence.

18. The method of any of the preceding claims, wherein the closed-ended DNA vector is a ceDNA vector.

19. A closed-ended DNA vector generated by the method of any of the preceding claims.

20. A pharmaceutical composition comprising the closed-ended DNA vector of claim 19 and optionally, an excipient.

21. 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.

22. The method of claim 21, wherein the double-stranded DNA construct is a bacmid, plasmid, minicircle, or a linear double-stranded DNA molecule.

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

24. The method of claim 23, wherein the ligation is effected by T4 ligase or an AAV Rep protein.

25. The method of any of claims 21-24, wherein at least one of the first ITR and the second ITR comprises at least one RBE site.

26. The method of any of claims 21-25, wherein the expression cassette sequence comprises at least one cis-regulatory element.

27. The method of any of claims 21-26, wherein the cis-regulatory element is selected from the group consisting of a promoter, an enhancer, a posttranscriptional regulatory element and a polyadenylation signal.

28. The method of any of claims 21-27, wherein the expression cassette sequence comprises a transgene sequence.

29. The method of claim 28, wherein the transgene sequence encodes a protein.

30. The method of claim 29, wherein the transgene sequence encodes a reporter protein, a therapeutic protein, an antigen, a gene editing protein, or a cytotoxic protein.

31. The method of claim 28, wherein the transgene sequence is a functional nucleotide sequence.

32. A closed-ended DNA vector generated by the method of any claims 21-31.

33. A pharmaceutical composition comprising the closed-ended DNA vector of claim 32 and optionally, an excipient.

34. 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.

35. The method of claim 34, 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.

36. The method of claim 34 or 35, 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.

37. The method of claim 34 or 35, 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.

38. The method of any of claims 34-37, 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.

39. The method of any of claims 34-38, 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.

40. The method of any of claims 34-39, wherein at least one of the sense first ITR and the sense second ITR is an AAV ITR or an AAV-derived ITR.

41. The method of any of claims 34-40, wherein the sense expression cassette sequence comprises at least one cis-regulatory element.

42. The method of claim 41, wherein the cis-regulatory element is selected from the group consisting of a promoter, an enhancer, a posttranscriptional regulatory element and a polyadenylation signal.

43. The method of any of claims 34-42, wherein the sense expression cassette sequence comprises a transgene sequence.

44. The method of claim 43, wherein the transgene sequence encodes a protein.

45. The method of claim 44, wherein the transgene sequence encodes a reporter protein, a therapeutic protein, an antigen, a gene editing protein, or a cytotoxic protein.

46. The method of claim 43, wherein the transgene sequence is a functional nucleotide sequence.

47. The method of any of claims 34-46, wherein the closed-ended DNA vector is a ceDNA vector.

48. The method of claim 47, wherein the ceDNA vector is purified.

49. A closed-ended DNA vector generated by the method of any of claims 34-48.

50. A pharmaceutical composition comprising the closed-ended DNA vector of claim 49 and optionally, an excipient.

51. 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.

52. The method of claim 51, 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.

53. The method of claim 51, 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.

54. The method of any of claims 51-53, wherein the annealing step results in one or both of the sense first ITR and the sense second ITR forming a hairpin loop.

55. The method of any of claims 51-54, wherein at least one of the sense first ITR and the sense second ITR comprises at least one RBE site.

56. The method of any of claims 51-55, wherein at least one of the sense first ITR and the sense second ITR is an AAV ITR or an AAV-derived ITR.

57. The method of any of claims 51-56, wherein the sense expression cassette sequence comprises at least one cis-regulatory element.

58. The method of claim 57, wherein the cis-regulatory element is selected from the group consisting of a promoter, an enhancer, a posttranscriptional regulatory element and a polyadenylation signal.

59. The method of any of claims 51-58, wherein the sense expression cassette sequence comprises a transgene sequence.

60. The method of claim 59, wherein the transgene sequence encodes a protein.

61. The method of claim 60, wherein the transgene sequence encodes a reporter protein, a therapeutic protein, an antigen, a gene editing protein, or a cytotoxic protein.

62. The method of claim 59, wherein the transgene sequence is a functional nucleotide sequence.

63. The method of any of claims 51-62, wherein the closed-ended DNA vector is a ceDNA vector.

64. The method of claim 63, wherein the ceDNA vector is purified.

65. A closed-ended DNA vector generated by the method of any of claims 51-64.

66. A pharmaceutical composition comprising the closed-ended DNA vector of claim 65 and optionally, an excipient.

67. 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.

68. The method of claim 67, wherein the double-stranded DNA construct is a bacmid, plasmid, minicircle, or a linear double-stranded DNA molecule.

69. The method of claim 67 or 68, wherein the annealing step results in one or both of the sense first ITR and the sense second ITR forming a hairpin loop.

70. The method of any of claims 67-69, wherein the sense first ITR is selected from a wild-type ITR and a modified ITR and/or wherein the sense second ITR is selected from a wild-type ITR and a modified ITR.

71. The method of any of claims 67-70, wherein at least one of the sense first ITR and the sense second ITR comprises at least one RBE site.

72. The method of any of claims 67-71 wherein at least one of the sense first ITR and the sense second ITR is an AAV ITR or an AAV-derived ITR.

73. The method of any of claims 67-72, wherein the sense expression cassette sequence comprises at least one cis-regulatory element.

74. The method of claim 73, wherein the cis-regulatory element is selected from the group consisting of a promoter, an enhancer, a posttranscriptional regulatory element and a polyadenylation signal.

75. The method of any of claims 67-74, wherein the sense expression cassette sequence comprises a transgene sequence.

76. The method of claim 67, wherein the closed-ended DNA vector is a ceDNA vector.

77. A closed-ended DNA vector generated by the method of any of claims 67-76.

78. A pharmaceutical composition comprising the closed-ended DNA vector of claim 77 and optionally, an excipient.

79. An isolated closed-ended DNA vector obtained by or obtainable by a process according to claim 1.

80. A genetic medicine comprising an isolated closed-ended DNA vector obtained by the process according to claim 1.

81. A cell comprising the closed-ended DNA vector of claim 80.

82. A transgenic animal comprising the closed ended DNA vector of claim 80.

83. Use of a closed-ended DNA vector obtained by or obtainable by a process according to claim 1 in the manufacture of a medicament for treating a subject.

84. Use of a composition comprising a closed-ended DNA vector obtained by or obtainable by a process according to claim 1, 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.

85. The use of claim 84, wherein the therapeutic protein is a therapeutic antibody.