METHOD FOR GENERATING CIRCULAR DNA USING HETERODUPLEX THERMOSTABLE LIGATION ASSEMBLY OF PRECURSORS PRODUCED BY ROLLING CIRCLE AMPLIFICATION

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0001] This invention was made with United States Government support from the National Institutes of Health under Grant No. R41GM154562. The Government has certain rights in the invention.

CROSS REFERENCE TO RELATED APPLICATIONS

[0002] This application claims priority to U.S. Provisional Patent Application No. 63/554,752 filed on February 16, 2024 in the name of Xiang LI and Charles J. BIEBERICH entitled "METHOD FOR GENERATING CIRCULAR DNA USING HETERODUPLEX THERMOSTABLE LIGATION ASSEMBLY OF PRECURSORS CREATED BY ROLLING CIRCLE AMPLIFICATION," which is hereby incorporated by reference herein in its entirety.

FIELD

[0003] The present invention relates to methods of generating supercoiled plasmid-vector less circular DNAs from two or more precursor DNA fragments, e.g., generated using rolling circle amplification (RCA). The methods described herein increase the yield of synthetic circular DNA produced by cyclic heteroduplex thermostable ligase assembly (CHTLA).

BACKGROUND

[0004] Gene therapy is experiencing a renaissance after many setbacks and challenges. This resurgence began in the early 2010s, following significant improvements in technology and safety measures. The U.S. Food and Drug Administration (FDA) has approved gene therapies for various conditions, including retinal blindness, spinal muscular atrophy, and hemophilia. As of 2024, there are 36 FDA-approved gene and cell therapies, and hundreds more in the pipeline. Many gene therapies use viral vectors for gene delivery, including lentivirus (LV), adenovirus, and adeno-associated virus (AAV). Viral vectors are known in the art to be produced by transient packaging

cell transfection [Bulcha, 2021], which requires simultaneously transfecting the helper plasmid encoding genes for replication, the capsid/replication plasmid encoding the structural proteins, and the transgene (or transfer) plasmid containing the therapeutic sequences. Therefore, the development of gene therapies requires a large quantity of plasmid DNAs. Disadvantageously, current plasmid manufacturing relies on E. coli fermentation, which requires extensive optimization and suffers from batch-to-batch variations [Reinikainen, 1989]. Consequently, there is a substantial backlog for plasmid production. Companies are investing in new facilities to increase production capacity but disadvantageously, gene expression from transient plasmid transfection is often weak and short-lived [Al-Dosari, 2009; Chong, 2021]. A major contributing factor to this is that the bacterial sequence in the plasmid backbone stimulates the innate immune response that causes rapid plasmid degradation or silencing [Liu, 2007; Williams, 2009]. Thus, there is also an unmet need in the gene and cell therapy field for novel technologies that improve the performance of plasmid DNA.

[0005] It is known that smaller DNA molecules are generally easier to transfect than larger ones. Minicircle DNAs lacking a replication origin and bacterial selection marker have shown much higher expression levels both *in vitro* and *in vivo* [Darquet, 1999]. Minicircle plasmid is derived from a parental plasmid containing a replication origin and antibiotic selection marker flanked with two recombination sites. Expression of a recombinase upon induction releases the origin and selection marker, which forms a mini plasmid, and the remaining sequences form the minicircle plasmid. Disadvantageously, isolating the desired minicircle from mini plasmid and the unprocessed residual parental plasmid is a daunting technical challenge. The application of minicircle DNA in clinical trials has not been reported to date, most likely due to the high production costs. A second mini-DNA technology termed Nanoplasmid was introduced in 2009 [Luke, 2009], wherein the antibiotic selection marker gene was removed in the Nanoplasmid, and a 150 bp siRNA encoding sequence was used as a reverse selection marker. An ~300 bp S6K origin replaces pUC or ColE1; both origins are ~550 bp long. Therefore, the Nanoplasmid backbone is substantially shorter than in a typical plasmid. Superior performance for Nanoplasmids in gene expression, DNA vaccine production, and lentivirus vector preparation has been reported [Williams, 2023], and multiple clinical trials using Nanoplasmids have been completed or are ongoing. Minivec™, produced by VectorBuilder, is another type of mini plasmid produced following the same concept as Nanoplasmids. Despite their promise, large-scale

production of minicircles, Nanoplasmids, and Minivec™ plasmids still relies on E. coli fermentation, and turnaround time remains a substantial hurdle.

[0006] DNA vectors that can be produced without *E. coli* fermentation are also being developed, and a prominent example is termed Doggybone DNA (dbDNA) produced by Touchlight Genomics [Karbowniczek, 2017; Allen, 2018]. dbDNA production can be divided into three steps. The first step is to create circular template DNA with two protelomerase sites flanking the gene of interest. The second step is to amplify the template using Rolling Circle Amplification (RCA) [Dean, 2001]. The RCA product is a double-stranded concatemer of the template DNA ~70 kb in length. The concatemer is then cleaved using protelomerase and sealed to adopt a linear conformation in the doggybone shape. Production of GMP-grade dbDNA can be accomplished in a few weeks, which is significantly shorter than the 3-6 months, or longer, needed to manufacture GMP-grade plasmid DNA. dbDNA showed similar performance for transfection, DNA vaccine production, and lentivirus vector production in comparison with plasmid DNA. Disadvantageously though, dbDNAs are not supercoiled, raising concerns about transfection efficiency, particularly for long sequences of interest.

[0007] To supplant existing approaches, new technologies are still needed that match or outperform plasmid DNA in transfection, virus vector preparation, DNA vaccine production, and other applications in gene and cell therapies. The manufacturing process should be readily scalable and have a short turnaround time from design to production as well as having production costs that are lower than current methods.

SUMMARY

[0008] In some aspects, a method of producing synthetic circular supercoiled DNA (^{SCS}DNA) is described, the method comprising:

(a) generating precursor DNA fragments using rolling circle amplification (RCA);

(b) introducing two or more precursor DNA fragments into a buffer medium comprising a thermostable DNA ligase enzyme, wherein the precursor DNA fragments will correctly assemble to generate a defined DNA sequence;

(c) applying heat to a first temperature to cause the at least two precursor DNA fragments to denature; and

(d) lowering the temperature to a second temperature for: (i) annealing in the presence of the thermostable DNA ligase enzyme, thereby generating double-stranded DNA heteroduplexes formed by base pairing of complementary regions, a portion of the heteroduplexes having single-stranded 5' overhangs and a portion of the heteroduplexes having 3' overhangs, wherein when the 5' intramolecular overhangs on a heteroduplex molecule are complementary and ligation on both DNA strands occurs, ^{SCS}DNA is produced, and when the 3' intramolecular overhangs on a heteroduplex molecule are complementary and ligation on both DNA strands occurs, ^{SCS}DNA is produced; and (ii) substantial supercoiling of the ^{SCS}DNA in the presence of the thermostable type II topoisomerase.

[0009] In some other aspects, a method of producing synthetic circular supercoiled DNA (^{SCS}DNA), the method comprising:

(a) generating precursor DNA fragments using rolling circle amplification (RCA), said method comprising:

priming a circular RCA DNA template using oligonucleotides to form a primed circular RCA DNA template;

amplifying the primed circular RCA DNA template to form a double-stranded DNA multimer by preparing a mixture comprisingthe primed circular RCA DNA template, an effective amount of deoxynucleotide triphosphates (dNTP), a buffer, and an effective amount of strand-displacing DNA polymerase and incubating same;

isolating the double-stranded DNA multimer from the mixture;

cleaving the double-stranded DNA multimer to produce the precursor DNA fragments; and

separating the precursor DNA fragments from a milieu comprising same

(b) introducing two or more precursor DNA fragments into a buffer medium comprising a thermostable DNA ligase enzyme, wherein the precursor DNA fragments will correctly assemble to generate a defined DNA sequence;

(c) applying heat to a first temperature to cause the at least two precursor DNA fragments to denature; and

(d) lowering the temperature to a second temperature for: (i) annealing in the presence of the thermostable DNA ligase enzyme, thereby generating double-stranded DNA heteroduplexes formed by base pairing of complementary regions, a portion of the heteroduplexes having single-

stranded 5' overhangs and a portion of the heteroduplexes having 3' overhangs, wherein when the 5' intramolecular overhangs on a heteroduplex molecule are complementary and ligation on both DNA strands occurs, ^{SCS}DNA is produced, and when the 3' intramolecular overhangs on a heteroduplex molecule are complementary and ligation on both DNA strands occurs, ^{SCS}DNA is produced; and (ii) substantial supercoiling of the ^{SCS}DNA in the presence of the thermostable type II topoisomerase.

[0010] Other aspects, features and embodiments of the invention will be more fully apparent from the ensuing disclosure and appended claims.

BRIEF DESCRIPTION OF THE FIGURES

[0011] FIGURE 1. Two-fragment CHTLA generates synthetic circular supercoiled DNA (^{SCS}DNA). Linear precursor DNA fragments that share overlapping and offset homologies are heat-denatured and annealed. Heteroduplexes (blue + brown strands) form and ligation produces circular DNAs. Two (A and C) of the four (A, B, C, and D) possible heteroduplexes are shown in Figure 1. Subsequent cycles deplete linear precursors and increase circular product yield.

[0012] FIGURE 2. Denaturation and reannealing of two precursor DNA fragments yields four possible heteroduplexes (A, B, C, and D). Two linear precursor DNA fragments with identical overall DNA sequence but different starting and ending nucleotide positions are generated. Denaturation and reannealing permits formation of four possible heteroduplexes comprised of one strand from one precursor and a complementary strand from the second precursor. Two of the heteroduplexes have 5' overhangs and two have 3' overhangs. The overhangs are complementary and can be ligated to form a closed circular DNA, e.g., as also illustrated in Figure 1.

[0013] FIGURE 3A. An embodiment of the cyclic heteroduplex thermostable ligation assembly (CHTLA) workflow to produce ^{SCS}DNA from RCA-generated precursors. RE1 and RE2, restriction enzymes 1 and 2, respectively. T5, digestion with T5 exonuclease.

[0014] FIGURE 3B. DNA precursors and CHTLA products (with or without T5 exonuclease digestion) to generate an approximately 3.4 kb ^{SCS}DNA were analyzed using agarose gel electrophoresis. The gel was stained with ethidium bromide after electrophoresis was complete. The white arrow designates the concatemers and products from non-specific ligation generated during the CHTLA reaction. The green arrowhead designates the supercoiled form of ^{SCS}DNA. **L,** 1 kb DNA ladder; **P**, precursor DNA fragments for CHTLA, which is a mixture of RE-digested

double-stranded DNA multimers (i.e., RE-digested RCA products) with two restriction enzymes; **2, 4, 8, and 16**, designates the number of restriction enzymes used to digest the double-stranded DNA multimers to generate the precursor DNA fragments used in the subsequent CHTLA reaction.

[0015] FIGURE 4A. An illustration of the effect of precursor diversity on ^{SCS}DNA yield.

[0016] FIGURE 4B. ^{SCS}DNA products generated using a 6.5 kb plasmid as an RCA DNA template and digesting the resulting double-stranded DNA multimer with either 2, 4, 8, or 16 single-cutter restriction enzymes to produce precursor DNA fragments for CHTLA. CHTLA products were digested with T5 exonuclease and analyzed on an agarose gel. Two technical replicates are shown for 2 and 4 REs, three replicates are shown for 8 REs, and 5 replicates are shown for 16 REs. As the number of RE-digested precursors increases, the yield of ^{SCS}DNA increases.

[0017] FIGURE 4C. ^{SCS}DNA products were generated using pMaxGFP (3.4 kb) as the RCA DNA template and the resulting double-stranded DNA multimers were digested with either two or eight single-cutter restriction enzymes. CHTLA reactions at various final DNA concentrations were set up. **2E**, precursor DNA fragments as a result of digestion with two restriction enzymes. **8E**, with eight restriction enzymes. **0.25, 05, 1.0, and 2.0** designates the final precursor DNA fragment concentration in mg/ml in the CHTLA reaction. **Pre**, precursor DNA fragments.

[0018] FIGURE 5A. A schematic of the introduction of paired Type IIS BsaI restriction sites into an RCA DNA template and the digestion of the resulting RCA products with BsaI.

[0019] FIGURE 5B. Introduction of a pair of BsaI sites into pCMV-red. The double-stranded DNA multimerwas digested with BsaI or BamHI (which cuts once in pCMV-red) and agarose gel electrophoresis was performed. **M 1+2,** Mixture of fragment 1 and fragment 2; **F 1+2**, PCR products created using assembled fragments 1 and 2 as a template; **V B+E**, pCMV-red DNA digested with BamHI and EcoRI; **RCA**, RCA products (i.e., the double-stranded DNA multimer); **Bam**, the double-stranded DNA multimer digested with single-cutter BamHI; **Bsa,** double-stranded DNA multimer digested with BsaI; **Lad**, one-kb DNA ladder.

[0020] FIGURE 5C. CHTLA products after T5 digestion from 3 precursor DNA fragments generated by BsaI digestion of double-stranded DNA multimer. BsaI sites were added at three different positions within a GFP expression cassette using PCR. The first PCR fragment started at position one and ended at position 1633. The second PCR started at position 21 and ended at position 20. The third PCR started at position 201 and ended at position 200. A BsaI site was

introduced at each end of each PCR product by including the BsaI site in the PCR primers. The same 20-bp random sequence region was added at each end of the PCR product. Each PCR product was individually circularized by Gibson Assembly and used as a template for RCA. The three double-stranded DNA multimers were digested with BsaI, purified, mixed at 1:1:1 ratio, and ligated in a CHTLA reaction to produce ^{SCS}DNA. **GFP**, GFP ^{SCS}DNA; **Lad**, 1-kb DNA ladder.

[0021] FIGURE 6A. Approximately 3 kb pBluescript (pBs) was amplified by RCA with phi29 polymerase and random hexamers in a reaction. The RCA products were EtOH precipitated, and the concatemeric double-stranded DNA multimer wasdigested with restriction enzymes (RE) KpnI or XmnI to yield two overlapping unit-length pBS molecules offset by ~1kb to generate heteroduplexes with 1 kb sticky ends, and the RE-digested precursors were column purified. Approximately 1 mg purified precursor DNA fragments was obtained from the 1 mL RCA reaction products. RE-digested precursor DNA fragments were mixed 1:1 and a 10-cycle 50 µL CHTLA reaction was performed. CHTLA products were treated with T5 exonuclease (10 units) to remove the linear DNA, and the circular DNA was column purified. ~200 ug circular DNA (white arrow) was obtained and ~80% was supercoiled.

[0022] FIGURE 6B. 16 kb precursors were prepared by RE digestion of a 16 kb plasmid with single-cutting NheI or XmnI. Precursor DNA fragments were mixed (1:1), a 10-cycle CHTLA reaction was done, products were treated with T5 exonuclease and purified. Linear precursor to circular (yellow arrow) conversion is ~15%.

[0023] FIGURE 7. An ^{scs}DNA carrying the Green Fluorescent Protein cassette (^{scs}DNA-GFP) derived from the plasmid pMax-GFP was generated. The difficult-to-transfect cell line (BLN3) derived from a mouse prostate tumor was chosen to directly compare the transfection efficiency of ^{scs}DNA-GFP versus pMax-GFP. After transfection of equimolar amounts of ^{scs}DNA-GFP and pMax-GFP, fluorescence was measured microscopically. At 48 hours post-transfection, the wells transfected with ^{scs}DNA-GFP showed 4-8 fold more GFP-expressing cells compared to those transfected with pMax-GFP.

[0024] FIGURE 8A. Generation of ^{SCS}DNA using sub-unit length precursor DNA fragments. A 2.4 kb firefly luciferase expression cassette was amplified by RCA and the double-stranded DNA multimer digested with either XhoI alone or XmnI alone. XhoI digestion produced a unit length precursor DNA fragment and XmnI digestion produced two precursor DNA fragments of different length. CHTLA using these precursors yielded the desired ^{SCS}DNA product.

[0025] FIGURE 8B: A 6.5 kb pCMV-RED was RE-digested with EcoRI alone, BamHI alone, or BamHI + SalI together. CHTLA was then performed with 2 precursor DNA fragments (EcoRI cut and BamHI cut, Figure 8B, right panel, first lane) or with EcoRI alone cut pCMV-RED and BamHI + SalI cut pCMV-RED (Figure 8B, right panel, second lane). Combining the unit-length precursor with the 2 sub-unit-length precursors yielded the desired ^{SCS}DNA product.

DETAILED DESCRIPTION, AND PREFERRED EMBODIMENTS THEREOF

[0026] Although the claimed subject matter will be described in terms of certain embodiments, other embodiments, including embodiments that do not provide all of the benefits and features set forth herein, are within the scope of this disclosure as well. Various structural and parameter changes may be made without departing from the scope of this disclosure.

[0027] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art. In case of conflict, the present document, including definitions, will control. Preferred methods and materials are described below, although methods and materials similar or equivalent to those described herein can be used in practice or testing of the present disclosure. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. The materials, methods, and examples disclosed herein are illustrative only and not intended to be limiting.

[0028] "About" and "approximately" are used to provide flexibility to a numerical range endpoint by providing that a given value may be "slightly above" or "slightly below" the endpoint without affecting the desired result, for example, +/- 5%.

[0029] The phrase "in one embodiment" or "in some embodiments" as used herein does not necessarily refer to the same embodiment, though it may. Furthermore, the phrase "in another embodiment" as used herein does not necessarily refer to a different embodiment, although it may. Thus, as described below, various embodiments of the invention may be readily combined, without departing from the scope or spirit of the invention.

[0030] The terms "comprise(s)," "include(s)," "having," "has," "can," "contain(s)," and variants thereof, as used herein, are intended to be open-ended transitional phrases, terms, or words that do not preclude the possibility of additional acts or structures. The singular forms "a," "and" and "the" include plural references unless the context clearly dictates otherwise. The present

disclosure also contemplates other embodiments "comprising," "consisting of" and "consisting essentially of," the embodiments or elements presented herein, whether explicitly set forth or not.

[0031] As used herein, the term "heteroduplex" DNA molecule refers to a double-stranded molecule wherein the a first strand originates from one double-stranded or single stranded precursor DNA molecule, and a second strand originates from a different double-stranded or single-stranded DNA molecule, wherein the first strand and the second strand have been joined by Watson-Crick base pairing in the process known as annealing of, or hybridization of, complementary DNA strands (i.e., the first and second strands).

[0032] As used herein, the term "ligase" and "ligation agent" are used interchangeably and refer to any number of enzymatic or non-enzymatic reagents capable of joining DNA molecules, e.g., between two or more adjacent heteroduplexes with annealed compatible single-stranded ends, or within a single heteroduplex molecule with compatible single-stranded ends such that a circular molecule is formed through the establishment of new bonds. In some embodiments, a ligase is an enzymatic ligation reagent that, under appropriate conditions, forms phosphodiester bonds between the 3′-OH and the 5′-phosphate of adjacent nucleotides in DNA molecules, RNA molecules, oligonucleotides, or hybrids. Temperature sensitive ligases include, but are not limited to, bacteriophage T4 ligase and *E. coli* ligase. "Thermostable ligases" or "thermostable DNA ligase enzymes" include, but are not limited to, Afu ligase, Taq ligase, Tfl ligase, Tth ligase, Tth HB8 ligase, *Thermus* species AK16D ligase and Pfu ligase, HiFi Taq Ligase, or Ampilgase. The skilled artisan will appreciate that any number of thermostable ligases, including DNA ligases and RNA ligases, can be obtained from thermophilic or hyperthermophilic organisms, for example, certain species of eubacteria and archaea; and that such ligases can be employed in the disclosed methods and kits. In some embodiments, the thermostable ligases are fused to a DNA-bending protein.

[0033] As used herein, the term "overlapping sequence" refers to a sequence that is complementary in two polynucleotides, wherein a first polynucleotide comprises an overlapping sequence that is single-stranded (ss) and can be hybridized to a second polynucleotide comprising the complementary ss sequence.

[0034] As used herein, the term "overhang," "offset" or "offset region" refers to the single stranded region of double-stranded (ds) DNA at the end thereof and is either of type 5' or 3' due to the inherent directionality of DNA. The overhangs are generally generated in various lengths by treating double stranded DNA with restriction enzymes or exonucleases and/or by the addition of

appropriate dNTPs (e.g., dATP, dTTP, dCTP, dGTP) through the action of an enzyme, i.e., terminal deoxynucleotidyl transferase. In some embodiments, the overhangs are in a range from 2 to 1000s of base pairs in length.

[0035] As used herein, the term "double stranded DNA" or "dsDNA" refers to oligonucleotides or polynucleotides having 3' overhang(s), 5' overhang(s) and/or blunt end(s) and comprise two single strands, all or part of which are complementary to each other, and thus dsDNA may contain a single stranded region at one or both ends and may be synthetic or natural origin derived from cells or tissues. In one embodiment, dsDNA is a product of PCR (Polymerase Chain Reaction) or fragments generated from genomic DNA or plasmids or vectors by a physical or enzyme treatment thereof.

[0036] As used herein, the term "buffering agent" refers to an agent that allows a solution to resist changes in pH when acid or alkali is added to the solution. Examples of suitable non-naturally occurring buffering agents that may be used in the compositions, kits, and methods described herein include, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), TAPS (tris(hydroxymethyl)methylamino]propanesulfonic acid), tricine (N-(2-Hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine), phosphates, citrates, ammonium, acetates, carbonates, tris(hydroxymethyl)aminomethane (TRIS), TRIS-HCl, 3-(N-morpholino) propanesulfonic acid (MOPS), 3-morpholino-2-hydroxypropanesulfonic acid (MOPSO), 2-(N-morpholino)ethanesulfonic acid (MES), N-(2-Acetamido)-iminodiacetic acid (ADA), piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES), N-(2-Acetamido)-2-aminoethanesulfonic acid (ACES), cholamine chloride, N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), 2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethanesulfonic acid (TES), acetamidoglycine, glycinamide, and bicine (2-(Bis(2-hydroxyethyl)amino)acetic acid) buffers. It should be appreciated that the buffering agents can further at least one additional species such as: electrolytes such as $MgCl_2$, NaCl, and KCl; metal ions; type II topoisomerases (e.g., DNA gyrase); single-stranded DNA binding protein or thermostable single-stranded binding protein; crowding agents (e.g. polyethylene glycol); a redox reagent such as dithiothreitol (DTT); nicotinamide adenine dinucleotide (NAD); detergents; and non-ionic surfactants such as TRITON™ X-100 (Octylphenol Decaethylene Glycol Ether).

[0037] As used herein, the terms "DNA" or "RNA" is defined as a "polynucleotide" and may encompass primers, oligonucleotides, nucleic acid strands, etc. The DNA or RNA may be single

stranded or double stranded or an admixture thereof. Such DNA or RNA polynucleotides may be synthetic, for example, synthesized in a DNA synthesizer, or naturally occurring, for example, extracted from a natural source, or derived from cloned or amplified material. Polynucleotides referred to herein may contain modified bases. Additionally, the DNA or RNA sequences may comprise one or more random or variable nucleotides. The use of randomized nucleotides may also include sequence-restricted regions, wherein sequence restricted means limiting the variation at one position to 2 or 3 nucleotide choices (i.e., A or C; A, G, or C etc.) rather than all 4 (ATGC). Typically, a polynucleotide contains a 5′ phosphate at one terminus ("5′ terminus") and a 3′ hydroxyl group at the other terminus ("3′ terminus") of the chain.

[0038] The nucleic acids utilized herein can be any nucleic acid, for example, human nucleic acids, bacterial nucleic acids, or viral nucleic acids. The nucleic acid sample can be, for example, a nucleic acid sample from one or more cells, tissues, or bodily fluids such as blood, urine, semen, lymphatic fluid, cerebrospinal fluid, or amniotic fluid, or other biological samples, such as tissue culture cells, buccal swabs, mouthwashes, stool, tissues slices, biopsy aspiration, and archeological samples such as bone or mummified tissue. Nucleic acids can be, for example, DNA, RNA, or the DNA product of RNA subjected to reverse transcription. Nucleic acids can be derived from any source including, but not limited to, eukaryotes, plants, animals, vertebrates, fish, mammals, humans, non-humans, bacteria, microbes, viruses, biological sources, serum, plasma, blood, urine, semen, lymphatic fluid, cerebrospinal fluid, amniotic fluid, biopsies, needle aspiration biopsies, cancers, tumors, tissues, cells, cell lysates, crude cell lysates, tissue lysates, tissue culture cells, buccal swabs, mouthwashes, stool, mummified tissue, forensic sources, autopsies, archeological sources, infections, nosocomial infections, production sources, drug preparations, biological molecule productions, protein preparations, lipid preparations, carbohydrate preparations, inanimate objects, air, soil, sap, metal, fossils, excavated materials, and/or other terrestrial or extra-terrestrial materials and sources. In some embodiments, the nucleic acids do not comprise bacterial or viral nucleic acids.

[0039] As used herein, "Taq ligase" and "thermostable ligase" are synonymous.

[0040] As used herein, "synthetic circular supercoiled DNA" or ^{SCS}DNA is intended to capture products consisting of only synthetic circular DNA as well as products comprising some portion of synthetic circular DNA and synthetic supercoiled DNA, wherein the DNA precursors that give

rise to the ^{SCS}DNA may be derived in part or wholly from a natural source (e.g., plasmid). In some embodiments, the synthetic supercoiled DNA is substantially supercoiled.

[0041] As used herein, synthetic circular supercoiled DNA and covalently closed circular DNAs are understood as being substantially exonuclease resistant. As used herein, any exonucleases are understood to lack or have minimal endonuclease activity using double-stranded DNA as a substrate.

[0042] As defined herein, "supercoil" or "supercoiling" is understood to be a global contortion of circular DNA. Supercoiling is understood to be the sum of the "twist" and the "writhe," wherein the twist is the number of helical turns in the DNA and the writhe is the number of times the double helix crosses over itself. Positive and negative supercoiling is understood by the skilled artisan, but briefly positive supercoiling comprises extra, or additional, helical twists (i.e., is over wound) relative to the relaxed state while negative supercoiling comprises less, or subtractive, helical twists (i.e., is under wound) relative to the relaxed state. The DNA of most organisms is negatively supercoiled although it is understood that some amount of positive supercoiling is also present. Negative supercoiling advantageously allows processes such as transcription, DNA replication and recombination. Another term common to supercoiling is the "linking number," or "Lk" which is understood to be the sum of the twist (T) and the writhe (W). The linking number is useful because it is a metric to identify changes of topology of DNA, e.g., as a result of enzymatic breakage and rejoining events. Of interest is the ΔLk, which is determined by the formula $\Delta Lk = Lk\text{-}Lk_m$, wherein Lk_m is the linking number for a relaxed circular DNA and Lk is the linking number for the circular DNA that has undergone supercoiling. Notably, Lk and Lk_m are rounded to the nearest whole integer prior to the calculation of ΔLk. For a circular DNA undergoing negative supercoiling, the ΔLk will be negative.

[0043] As used herein, a "scar" in a supercoiled DNA comprising a sequence of interest comprises a variable length phage integrase attachment site required for recombination of short DNA sequences mediated by a bacteriophage recombinase. In other words, a "scar" is an extraneous prokaryotic sequence. Accordingly, the term "scarless" corresponds to the lack of the variable length phage integrase attachment site in supercoiled DNA comprising the sequence of interest.

[0044] As defined herein, "precursor" DNA fragments include dsDNA molecules (i.e., PCR products, restriction enzyme fragments, chemically or enzymatically produced DNA), single-stranded DNA molecules, DNA oligonucleotides, or mixtures thereof. Nucleotide bases within

said precursor DNA fragments can be native adenine, guanine, cytosine, thymine, or any chemically modified form thereof that can be incorporated into a DNA molecule by chemical synthesis or the action of an enzyme, i.e., a DNA polymerase, or that can be chemically or enzymatically caused to appear in a base or bases after synthesis. The precursor DNAs can range from about 20 nucleotides to thousands to millions of nucleotides in length, and more preferably from about 200 to 10000 nucleotides for double-stranded precursors, and 30 to 200 for single-stranded precursors. In some embodiments, the precursors are generated using methods described herein. In some other embodiments, the precursors are generated using methods known in the art.

[0045] A "restriction enzyme" is a protein isolated from bacteria that cleaves DNA sequences at sequence-specific sites, producing DNA fragments with a known sequence at each end.

[0046] A linear "unit length" precursor DNA refers to a linear DNA molecule that upon circularization gives rise to a defined DNA sequence that is the final product of the CHTLA reaction. Any two or more linear unit length precursors will be completely homologous (e.g., identical in sequence) when circularized. For example, if one linear unit length precursor is represented by the number sequence 12345678, and a second linear unit length precursor is represented by 45678123, it is axiomatic that the circular form of each has the same numeric sequence. Similarly, a "sub-unit" length precursor DNA corresponds to a portion of the unit length precursor DNA. In some embodiments, sub-unit length precursor DNA can combine to become an unit length precursor DNA, e.g., 1234 and 56789 combine to form 123456789 giving rise to the defined DNA sequence that is the final product of the CHTLA reaction. In other embodiments, sub-unit length precursor DNA will not combine or will not combine to form an unit length precursor DNA that can circularize and will instead be digested by the 5' to 3' or 3' to 5' exonucleases.

[0047] CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas (CRISPR-associated) systems, or CRISPR systems, are adaptive defense systems originally discovered in bacteria and archaea. Examples of a Cas endonuclease include but are not limited to Cas9 and Cas12a. Cas9 (formerly referred to as Cas5, Csn1, or Csx12) is a Class 2 Type II Cas endonuclease (Makarova et al., Nature Reviews Microbiology Vol. 13:1-15 (2015)). A Cas9-gRNA complex recognizes a 3' PAM sequence (NGG for the *S. pyogenes* Cas9) at the target site, permitting the spacer of the guide RNA to invade the double-stranded DNA target, and, if sufficient homology between the spacer and protospacer exists, generate a double-strand break cleavage. Cas9

endonucleases comprise RuvC and HNH domains that together produce double strand breaks, and separately can produce single strand breaks. For the *S. pyogenes* Cas9 endonuclease, the double-strand break leaves a blunt end. Cas12a (formerly referred to as Cpf1) is a Class 2 Type V Cas endonuclease, and comprises nuclease RuvC domain but lacks an HNH domain (Yamane et al., Cell 165:949-962 (2016)). Cas12a endonucleases create "sticky" overhang ends. Some uses for CRISPR/Cas systems at a genomic target site include but are not limited to insertions, deletions, substitutions, or modifications of one or more nucleotides at the target site; modifying or replacing nucleotide sequences of interest (such as a regulatory elements); insertion of polynucleotides of interest; gene knock-out; gene-knock in; modification of splicing sites and/or introducing alternate splicing sites; modifications of nucleotide sequences encoding a protein of interest; and gene silencing by expressing an inverted repeat into a gene of interest.

[0048] It is well known to the person skilled in the art that each nucleotide in a dsDNA molecule will pair with its Watson-Crick counterpart, also known as the "complementary" nucleotide. Further, it is understood that dsDNA sequences are represented by an upper or first (sense) strand sequence going in the direction from its 5′- to 3′-end and thus the complementary sequence is the sequence of the lower or second (antisense) strand in the same direction as the upper strand. When DNA sequences are stated as complementary, it is understood that when they anneal or hybridize, a double stranded DNA with antiparallel strands form. It is further understood that annealed complementary DNA sequences may include one or more non-canonical (i.e., Watson-Crick) base pairs or modified nucleotides that pair with multiple other nucleotides (e.g., Deoxyinosine may also pair with the three other DNA bases (deoxythymidine (dT), dA and dG).

[0049] As introduced hereinabove, covalently closed circular DNA that are completely devoid of bacterial DNA (e.g. minicircles) have recently emerged as a powerful gene delivery platform, but GMP production is highly problematic. The present inventors previously developed an improved DNA assembly process termed Heteroduplex Thermostable Ligase Assembly (HTLA™) which is capable of building and commercializing large linear DNA molecules completely *in vitro*, as described in International Patent Application No. PCT/US2023/064977 filed on March 27, 2023 in the name of Charles J. Bieberich and Xiang Li, and entitled "HETERODUPLEX THEROMSTABLE LIGATION ASSEMBLY (HTLA) AND/OR CYCLIC HETERODUPLEX THERMOSTABLE LIGATION ASSEMBLY (CHTLA) FOR GENERATING DOUBLE-STRANDED DNA FRAGMENTS WITH SINGLE-STRANDED STICKY ENDS," which is

hereby incorporated herein in its entirety. HTLA is a straightforward assembly platform that generates ligation-ready single-stranded overhangs of user-defined length to create sticky-end blocks (SEBs) for assembly into higher order linear or circular structures. The starting material for HTLA or CHTLA is dsDNA or oligonucleotide precursors that self-assemble precisely to create much longer DNAs. As recited in International Patent Application No. PCT/US2023/064977, at least three precursors were used as reactants.

[0050] Briefly, the HTLA process is an efficient DNA assembly process that generates ligation-ready, user-defined, heteroduplex DNAs having lengths from one to thousands (or more) of nucleotides that comprise 5' or 3' single-stranded overhangs, or "sticky-ends," that can join to form closed circular DNA molecules from double-stranded or single-stranded DNA precursor molecules (see, e.g., Figures 1 and 2). When such process is performed for one cycle, it is referred to as HTLA while for more than one cycle, the process is referred to as Cyclic Heteroduplex Thermostable Ligase Assembly (CHTLA). As recited in International Patent Application No. PCT/US2023/064977, at least three precursors were used as reactants.

[0051] Surprisingly, in addition to making linear DNA, HLTA and CHTLA can also be used to generate closed circular DNA starting with as few as two precursors, e.g., dsDNA precursors or readily available and inexpensive DNA oligonucleotides (oligos). In International Patent Application No. PCT/US2024/026847 filed on April 29, 2024 in the name of Charles J. Bieberich and Xiang Li, and entitled "A METHOD TO GENERATE SUPERCOILED CIRCULAR DNA IN VITRO," the present inventors also disclosed methods of producing HTLA- and CHTLA-based synthetic circular supercoiled DNA in a test tube, using chemically or enzymatically synthesized oligonucleotides, long single stranded DNA, and/or double stranded DNA. Under specific conditions, single or double-stranded DNA precursors were denatured and annealed in the presence of a thermostable DNA ligase, with or without a thermostable type II topoisomerase, to create circular, predominantly supercoiled DNA molecules or "synthetic circular supercoiled DNA (^{SCS}DNA).

[0052] As described in International Patent Application No. PCT/US2024/026847, the methods comprise providing two or more user-designed DNA precursors to create a DNA sequence wherein a portion of a first strand and a second strand of the DNA sequence overlap with each other, and simultaneously complementary 'sticky' ends are generated that can be complimentary to one another and be ligated to form a covalently closed circle. This procedure is shown in Figure 1.

The dsDNA (or oligo) precursor mixture is heated and cooled in a buffer, heteroduplex molecules I and II form, and a DNA ligase joins the heteroduplex molecules to form covalently closed circular, plasmid vector-less, DNA molecules (synthetic circular supercoiled DNA, ^{SCS}DNA). Performing multiple rounds of heating and cooling (i.e., CHTLA) increases ^{SCS}DNA yield. The methods include the formation of intermediary heteroduplexes having sticky-end blocks (SEB), wherein a portion of the heteroduplexes have 5' overhangs and a portion of the heteroduplexes have 3' overhangs. When the 5' overhang of a heteroduplex on one strand is complimentary to the 5' overhang on the other strand of the same heteroduplex, the heteroduplex circularizes (i.e., closes upon itself) upon ligation of the 5' end of each strand with its own 3' end. When the 3' overhang of a heteroduplex on one strand is complimentary to the 3' overhang on the other strand of the same heteroduplex, the heteroduplex circularizes (i.e., closes upon itself) upon ligation of the 5' end of each strand with its own 3' end. Advantageously, the circular and supercoiled DNA described in International Patent Application No. PCT/US2024/026847 are produced without plasmid vectors, and thus are devoid of bacterial DNA.

[0053] As previously described in International Patent Application No. PCT/US2024/026847, the formation of supercoiled DNA using the HTLA and CHTLA processes was surprising because DNA ligases are not known to have topoisomerase activity. Even so, following denaturing, annealing, and ligation, some of the ^{SCS}DNA produced undergoes negative supercoiling in the presence of a DNA ligase. DNA supercoiling, which reduces the space occupied by the DNA is known to be important for DNA packaging within the nucleoid of bacterial cells. Furthermore, transfection of supercoiled DNA into eukaryotic cells (i.e., to genetically modify the cells for some purpose) is substantially more efficient than transfection of the same DNA in its linear or open (relaxed) circular form.

Rolling Circle Amplification

[0054] The present inventors have identified another method of producing precursors for CHTLA using rolling circle amplification (RCA). RCA is the most robust method to amplify DNA. RCA generates double-stranded DNA, and offers substantial advantages. The first is much higher yield: PCR amplification of a ~3 kb product yields ~5-10 μg/100 μL reaction, while random hexamer-primed RCA of a double-stranded template yields ~50-100 μg/100 μL or more. Another advantage is that RCA is an isothermal reaction that occurs at about 30-45°C which can be achieved in a

water bath. Linear CHTLA precursor DNA fragments can be generated in large amounts using RCA, and the CHTLA precursor DNA fragments are devoid of plasmid sequences.

[0055] RCA is initiated when nucleotide triphosphates and an effective catalytic amount of the desired polymerase enzyme are combined with a primed circular RCA DNA template. Amplified run-on synthesis then occurs: the polymerase starts at the primer, elongates it, and continues around the circle, making the desired oligonucleotide product sequence. It continues past the starting point, displacing the synthesized DNA as it goes, and proceeds many times around the circle. This produces a long single multimer strand which is made up of many end-to-end copies of the desired oligonucleotide product. The size of the multimer product can be about 60 to 5×10^6 nucleotides in length. The length of the multimer can be controlled by time, temperature, relative and absolute concentrations of enzyme, triphosphates, template, and primer. For example, longer periods of time, or lower concentrations of template, will tend to increase the average multimer length. In some embodiments, the RCA method uses catalytic amounts of template, primer, and polymerase enzymes and stoichiometric amounts of the nucleotide triphosphates.

[0056] In some embodiments, RCA uses an enzyme with high processivity and strand-displacement activity, such as Phi29 DNA polymerase, to amplify circular DNA to generate long linear concatemers, or "double-stranded DNA multimers." The reaction occurs isothermally and therefore is readily scalable. RCA can robustly amplify DNA from less than 100 base pairs and up to tens of thousands of base pairs without protocol optimization. Furthermore, DNA with complex structures, for example, the Long Terminal Repeat (LTR) in the lentivirus genome, can also be efficiently and accurately amplified [Karbowniczek, 2017]. Moreover, the yield of DNA using RCA is high. These features make RCA an excellent tool for producing ^{SCS}DNA precursors. Digesting the double-stranded DNA multimers with restriction enzymes that only cut the template DNA once, i.e., single-cutters, generates multiple copies of the linear form of the template DNA. In some embodiments, digestion of the double-stranded DNA multimers with different single-cutter restriction enzymes (e.g., RCA reaction 1 is digested with EcoRI and RCA reaction 2 is digested with BamHI, etc.) will generate a pool of linear unit length DNA precursors that share overlapping and offset homologies, but when circularized, yield the same DNA sequence (see, e.g., Figure 3A). For example, if the desired final circular CHTLA ^{SCS}DNA product is represented as 123456789, a pool of three unit-length DNA precursors could hypothetically be 123456789, 234567891, 345678912. When the pool of DNA precursors is denatured and renatured,

heteroduplex DNA forms with overlapping double-stranded homologous regions and offset single-stranded regions. The offset homologous sequences can either anneal intra-molecularly to circularize or anneal inter-molecularly to form a linear concatemer. In the presence of a DNA ligase, the phosphodiester backbone will be sealed to form either a closed circle or a long concatemer. Treating the reaction mixture with T5 exonuclease removes the linear DNA (long concatemers and a small amount of unreacted precursors) leaving only circular DNA (Figure 3A). Surprisingly, most circular DNAs are supercoiled, and hence ^{SCS}DNA is produced.

[0057] In another embodiment, sub-unit length DNA precursors are generated when double-stranded DNA multimers are digested with a restriction enzyme that cuts more than once in the sequence (i.e., a multiple-cutter) or combining two or more single-cutting restriction enzymes cleave the same double-stranded DNA multimer together. Such sub-unit length DNA precursors can be combined with one or more unit length precursor DNA fragments, or with a different sub-unit length precursor DNA fragments, e.g., generated by cutting the double-stranded DNA multimer with a different single-cutter or multiple-cutter restriction enzyme. In another embodiment, two or more double-stranded DNA multimers generated from circular RCA DNA templates that differ only by the position of one or more paired Type IIS RE sites can be cleaved with one or more Type IIS restriction enzymes to generate unit length or sub-unit length precursor DNA fragments. The Type IIS RE sites are engineered into the circular RCA DNA template at different nucleotide positions within the desired final ^{SCS}DNA product. For example, one pair of Type IIS RE sites can be engineered such that cleavage with the Type IIS restriction enzyme (or enzymes if two different Type IIS sites are inserted) yields a linear unit-length precursor DNA fragment that when circularized has the sequence of the final ^{SCS}DNA product. In this example, the unit length precursor DNA fragment thus generated has the structure of a unit length precursor generated by a single-cutter Type II restriction enzyme. By moving the position of the engineered Type IIS sites to create a second circular RCA DNA template (e.g., if the desired ^{SCS}DNA product is represented as a clock face, moving the Type IIS sites from the 12 o'clock to the 3 o'clock position, etc.) a series of unit length precursor DNA fragments with overlapping and offset homology can be generated. Similarly, if more than one paired Type IIS sites is engineered into the circular RCA DNA template (e.g., at both the 12 o'clock and 3 o'clock positions in the same template), the double-stranded DNA multimers can be digested with one or more Type IIS restriction enzymes to generate sub-unit length DNA precursors.

[0058] In a first aspect, a method of generating precursor DNA fragments using rolling circle amplification (RCA) is described, said method comprising:

priming a circular RCA DNA template using oligonucleotides to form a primed circular RCA DNA template;

amplifying the primed circular RCA DNA template to form a double-stranded DNA multimer by preparing a mixture comprising the primed circular RCA DNA template, an effective amount of deoxynucleotide triphosphates (dNTP), a buffer, and an effective amount of strand-displacing DNA polymerase and incubating same;

isolating the double-stranded DNA multimer from the mixture;

cleaving the double-stranded DNA multimer to produce the precursor DNA fragments; and

separating the precursor DNA fragments from a milieu comprising same.

[0059] In some embodiments, the circular RCA DNA template is complimentary to the nucleotide sequence of the precursor DNA fragment. In some embodiments, the circular RCA DNA template is primed to form a primed circular RCA DNA template by annealing an oligonucleotide primer to the circular RCA DNA template. In some embodiments, the primer contains about 4-50 nucleotides, and more preferably about 6-12 nucleotides. In some embodiments, the primer is an oligonucleotide comprising random hexamers and/or one or more oligonucleotides comprised of a specific DNA sequence(s) present in the circular RCA DNA template. This primer is substantially complementary to part of the circular RCA DNA template, preferably to the beginning of the desired oligomer sequence. In some embodiments, the buffer is a phi29 buffer, as known in the art. In some embodiments, the double-stranded DNA multimer comprises multiple copies of the circular RCA DNA template, i.e., the DNA fragment precursor, arranged in series as a concatemer. In some embodiments, the double-stranded DNA multimer comprises 4-4000 copies of the RCA DNA template, i.e., the precursor DNA fragment, arranged in series. In some embodiments, the double-stranded DNA multimer is formed by incubating the mixture comprising the primed circular RCA DNA template, an effective amount of deoxynucleotide triphosphates (dNTP), a buffer, and an effective amount of strand-displacing DNA polymerase at temperature in a range from about 36°C to about 38°C for time in a range from about 6 hours to about 12 hours, which amplifies the primed circular RCA DNA template. In some embodiments, the double-stranded DNA molecule is isolated from the mixture, prior to cleaving, by precipitating in an alcohol such as ethanol or isopropanol, or column-based techniques

known in the art. In some embodiments, the double-stranded DNA multimer is cleaved using one or more restriction enzymes to produce the precursor DNA fragments. In some embodiments, the double-stranded DNA multimer is cleaved using CRISPR to produce the precursor DNA fragments. In some embodiments, the restriction enzymes cleave the double-stranded DNA multimer at RE sites to produce the precursor DNA fragments having well-defined ends. In some embodiments, the precursor DNA fragments are separated from the milieu comprising same using chromatographic, gel filtration, dialysis, ethanol precipitation, or other extraction techniques known in the art. In some embodiments, the dNTPs are selected from at least one of deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), and deoxythymidine triphosphate (dTTP). In some embodiments, the precursor DNA fragments are identical in sequence to one another but have different starting and ending nucleotide positions and may have 5' or 3' overhangs (generated by restriction enzyme digestion) or may be blunt-ended.

[0060] It should be appreciated by the skilled artisan that the circular RCA DNA template can comprise restriction enzyme recognition sites that naturally occur within the sequence of the DNA template, which are recognized by a specific restriction enzyme. In other words, the person skilled in the art can select the restriction enzyme that recognizes a specific RE recognition site or sites and cleaves the DNA molecule within, or nearby, the RE recognition site. In some embodiments, the circular RCA DNA template comprises RE recognition sites that naturally occur once within the template DNA sequence (i.e. single cutter enzymes). In some embodiments, the circular RCA DNA template comprises RE recognition sites that naturally occur more than once within the template DNA sequence (i.e. multiple cutter enzymes). In other embodiments, the RCA DNA template is engineered to contain RE sites that do not naturally occur in the template DNA sequence (i.e., Type IIS REs). The construction of circular RCA DNA templates is well known in the art.

[0061] As shown in Figure 3A, when the double-stranded DNA multimer is digested with 2 single-cutter REs, two precursor DNA fragments that are identical in sequence are generated but having different starting and ending nucleotide positions and may have 5' or 3' overhangs (generated by restriction enzyme digestion). As will be described hereinafter, the 2 precursor DNA fragments are isolated from the milieu comprising same for subsequent CHTLA processing to produce ^{SCS}DNA. In some other embodiments, as shown in Figure 4A, when the double-stranded DNA

multimer is digested with *n* single-cutter REs, *n* precursor DNA fragments that are identical in sequence are generated but having different starting and ending nucleotide positions and may have 5' or 3' overhangs (generated by restriction enzyme digestion). As will be described hereinafter, the *n* precursor DNA fragments are isolated from the milieu comprising same for subsequent CHTLA processing to produce ^{SCS}DNA. Using RCA, the 2 (or *n*) precursor DNA fragments will be generated in approximately an equal ratio, which after isolation from the milieu, is advantageous for the subsequent CHTLA process described below.

[0062] In some embodiments, the double-stranded DNA multimer can be cleaved using CRISPR or the like to produce the precursor DNA fragments, as readily understood by the person skilled in the art.

[0063] In some embodiments, the strand-displacing DNA polymerases that effectuate the synthesis of a double-stranded DNA multimer in RCA have high fidelity, high processivity, and accept single-stranded or double-stranded templates. For DNA polymerization, i.e., formation of the double-stranded DNA multimers, suitable enzymes include, but are not limited to, Phi29 DNA polymerase, DNA Polymerase I, Klenow fragment of DNA Polymerase I, T7 DNA Polymerase (exonuclease-free), T4 DNA Polymerase, Taq Polymerase, and AMV (or MuLV) Reverse Transcriptase or closely homologous mutants thereof. In some embodiments, the strand-displacing DNA polymerase is Phi29 DNA polymerase or a DNA polymerase having equivalent capabilities.

[0064] Restriction enzymes for digesting the products of the RCA process described herein, i.e., double-stranded DNA multimers, include Type I and Type IIS restriction enzymes. In some embodiments, the RE are single-cutters with one RE recognition site that occur naturally within the circular RCA DNA template, and hence the double-stranded DNA multimer. In some embodiments, the RE are capable of cutting more than once within a given circular RCA DNA template sequence, and hence the double-stranded DNA multimer. Restriction enzymes are well known in the art. In another embodiment, one or more Type IIS RE sites are engineered into the circular RCA DNA template such that they can be used to cleave the double-stranded DNA multimer into unit-length or sub-unit length precursor DNA fragments.

[0065] After the RCA and RE digestion reactions, the milieu comprises a large amount of the precursor DNA fragments and only small amounts of the circular RCA DNA template, primer, strand-displacing DNA polymerase, and restriction enzymes. Thus, in some embodiments, the

product precursor DNA fragments are produced in relatively good purity, and can require only gel filtration or dialysis before use.

[0066] In some embodiments, the CHTLA precursor DNA fragments are not unit length. In this embodiment, upon digestion with the appropriate restriction enzyme(s) or other DNA linearizing technology (i.e., CRISPR), precursor DNA fragments consisting of sub-unit length molecules are generated. For example, in Reaction 1, a circular DNA (i.e,. a plasmid) or double-stranded multimeric product derived from a circular DNA template by RCA is digested with two (or more) REs to generate precursor DNA fragments. In Reaction 2 (or reaction 3, or reaction 4, etc) the circular DNA (i.e., a plasmid) or double-stranded multimeric product derived from a circular DNA template by RCA is digested with one (or more) REs to generate precursor DNA fragments with starting and ending nucleotide positions that differ from those produced by the two or more REs used in Reaction 1, such that when the sub-unit length precursor DNA fragments from the two or more Reactions are acted upon during CHTLA, heteroduplexes with complementary single-stranded overhangs still form and can be ligated to create a closed circular DNA.

Circular RCA DNA templates

[0067] For RCA, an isolated circular DNA template is needed. A circular RCA DNA template which is complementary in sequence to the desired ^{SCS}DNA product can be prepared from a linear precursor, i.e., a linear pre-circle. Methods of obtaining an isolated circular RCA DNA template are known in the art.

[0068] In some embodiments, a method is described for designing and generating circular RCA DNA templates that bear precisely-placed paired Type IIS restriction enzyme sites such that when the double-stranded DNA multimers from two or more such templates are digested with one or more Type IIS enzymes, linear unit-length precursor DNA fragments are produced that are identical in sequence but with different starting and ending nucleotide positions that may include 5' or 3' overhangs or be blunt-ended. In some embodiments, the paired Type IIS restriction sites take the place of naturally occurring restriction sites that appear in the sequence of the desired product ^{SCS}DNA. In some embodiments, the circular DNA templates bearing the paired Type IIS sites are first produced by, for example, conventional DNA ligation of restriction enzyme digested DNA, or by Gibson assembly, or exist as minicircle DNAs that have been engineered to contain the Type IIS sites, or notably, by CHTLA of PCR-generated precursors. In some embodiments,

circular DNA templates are produced that have paired Type IIS restriction enzyme sites every *y* nucleotides within, for example, a *z* base pair-long sequence. For example, a circular DNA template can have paired Type IIS restriction enzyme sites every 200 nucleotides. If the sequence is 2000 base pairs, ten DNA templates are produced and are used to generate ten separate double-stranded DNA multimer that are digested with the Type IIS enzyme(s). The ten DNA templates would then be identical in sequence but have different starting and ending nucleotide positions separated by 200 base pairs. In some embodiments, the paired Type IIS sites could be separated by any nucleotide distance.

[0069] In some embodiments, two precisely-placed paired Type IIS restriction enzyme sites are positioned in the circular RCA DNA template such that when the double-stranded DNA multimers are digested with the Type IIS enzyme, a linear unit-length precursor DNA fragment is produced. In some embodiments, *n*, e.g., 2 or more, circular RCA DNA templates are created bearing one precisely-placed pair of Type IIS restriction enzyme sites such that when their separate double-stranded DNA multimers are digested with Type IIS enzymes, *n* linear unit-length precursor DNA fragments are produced that are identical in sequence but with different starting and ending nucleotide positions that may include 5' or 3' overhangs or be blunt-ended. As will be discussed hereinafter, this may be particularly advantageous for larger circular RCA DNA templates such as those employed in the production of LV.

Cyclic Heteroduplex Thermostable Ligation Assembly (CHTLA)

[0070] Following preparation of the *n* precursor DNA fragments, the CHTLA process can be used to prepare ^{SCS}DNA.

[0071] In a second aspect, a method of forming Sticky-End Blocks (SEB) with 5' or 3' overhangs is described, the method comprising:

generating precursor DNA fragments using rolling circle amplification (RCA);

introducing two or more precursor DNA fragments into a buffer medium comprising a thermostable DNA ligase enzyme, wherein the precursor DNA fragments will correctly assemble to generate a defined DNA sequence;

applying heat to a first temperature to cause the two or more precursor DNA fragments to denature; and

lowering the temperature to a second temperature for annealing in the presence of the thermostable DNA ligase enzyme, thereby generating double-stranded DNA heteroduplexes formed by base pairing of complementary regions, a portion of the heteroduplexes having single-stranded 5' overhangs and a portion of the heteroduplexes having 3' overhangs.

[0072] In some embodiments of the second aspect, a method of forming Sticky-End Blocks (SEB) with 5' or 3' overhangs comprises:

generating precursor DNA fragments using rolling circle amplification (RCA), said method comprising:

priming a circular RCA DNA template using oligonucleotides to form a primed circular RCA DNA template;

amplifying the primed circular RCA DNA template to form a double-stranded DNA multimer by preparing a mixture comprising the primed circular RCA DNA template, an effective amount of deoxynucleotide triphosphates (dNTP), a buffer, and an effective amount of strand-displacing DNA polymerase and incubating same;

isolating the double-stranded DNA multimer from the mixture;

cleaving the double-stranded DNA multimer to produce the precursor DNA fragments; and

separating the precursor DNA fragments from a milieu comprising same;

introducing two or more precursor DNA fragments into a buffer medium comprising a thermostable DNA ligase enzyme, wherein the precursor DNA fragments will correctly assemble to generate a defined DNA sequence;

applying heat to a first temperature to cause the two or more precursor DNA fragments to denature; and

lowering the temperature to a second temperature for annealing in the presence of the thermostable DNA ligase enzyme, thereby generating double-stranded DNA heteroduplexes formed by base pairing of complementary regions, a portion of the heteroduplexes having single-stranded 5' overhangs and a portion of the heteroduplexes having 3' overhangs.

[0073] In some embodiments, the second temperature is lower than the first temperature. In some embodiments, the two or more precursor DNA fragments are selected from dsDNA molecules, single-stranded DNA molecules, DNA oligonucleotides, or mixtures thereof. In some embodiments, the two or more precursor DNA fragments are prepared according to the RCA

method of the first aspect. In some embodiments, there are only two precursor DNA fragments that following denaturing, anncaling, and ligation, form four different heteroduplex species, e.g., as shown in Figure 2. In some embodiments, the only two precursor DNA fragments are mixed in approximately an equal ratio for denaturing, annealing, and ligation. In some embodiments, there are n precursor DNA fragments, e.g., 2 or more (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, or more), that following denaturing, annealing, and ligation, form $2n \times (n\text{-}1)$ different heteroduplex species, e.g., as shown in Figure 4A. In some embodiments, the n precursor DNA fragments are mixed in approximately an equal ratio for denaturing, annealing, and ligation. It should be appreciated by the person skilled in the art that the n precursor DNA fragments are identical in sequence but have different starting and ending nucleotide positions and may have 5' or 3' overhangs (generated by restriction enzyme digestion) or may be blunt-ended. In some embodiments, the denature/anneal cycle occurs only once or is repeated for anywhere from 1 to 100 cycles, as readily determined by the person skilled in the art.

[0074] In some embodiments, the desired SEB products of the second aspect may be purified, for example, by agarose gel purification, or any other means of separation, from other reaction components, for subsequent ^{SCS}DNA and/or supercoil production in a third and/or fourth reaction.

[0075] In some embodiments, the buffer medium for the second aspect comprises a buffer to maintain pH. In some embodiments, the buffer comprises a combination of ATP, Tris-HCl, $MgCl_2$, KCl, NaCl, beta-mercaptoethanol, DTT, NAD, ATP, and Triton™ X-100, to maintain a pH of about 4 to about 12, or about 6 to 10, or about 7.5 to about 9. The denaturing (also referred to as "melting" or the first) temperature can be in a range from about 37°C to 100°C, or about 60°C to 100°C, or about 80°C to 100°C, for time in a range from about 0.1 minutes to about 60 minutes, or about 1 minute to about 5 minutes. The annealing is conducted by lowering the temperature (i.e., the second temperature) about 5°C to about 60°C lower than the denaturing temperature, or lowering about 10°C to 40°C lower than the denaturing temperature, for time in a range from about 0.1 minutes to about 60 minutes, or about 4 minutes to 6 minutes.

[0076] In a third aspect, a method of producing synthetic circular supercoiled DNA (^{SCS}DNA) is described, the method comprising:

generating precursor DNA fragments using rolling circle amplification (RCA);

introducing two or more precursor DNA fragments into a buffer medium comprising a thermostable DNA ligase enzyme, wherein the precursor DNA fragments will correctly assemble to generate a defined DNA sequence;

applying heat to a first temperature to cause the two or more precursor DNA fragments to denature; and

lowering the temperature to a second temperature for annealing in the presence of the thermostable DNA ligase enzyme, thereby generating double-stranded DNA heteroduplexes formed by base pairing of complementary regions, a portion of the heteroduplexes having single-stranded 5' overhangs and a portion of the heteroduplexes having single-stranded 3' overhangs, wherein when the 5' intramolecular overhangs on a heteroduplex molecule are complementary and ligation on both DNA strands occurs, ^{SCS}DNA is produced, and when the 3' intramolecular overhangs on a heteroduplex molecule are complementary and ligation on both DNA strands occurs, ^{SCS}DNA is produced.

[0077] In some embodiments of the third aspect, a method of producing synthetic circular supercoiled DNA (^{SCS}DNA) is described, the method comprising:

generating precursor DNA fragments using rolling circle amplification (RCA), said method comprising:

priming a circular RCA DNA template using oligonucleotides to form a primed circular RCA DNA template;

amplifying the primed circular RCA DNA template to form a double-stranded DNA multimer by preparing a mixture comprising the primed circular RCA DNA template, an effective amount of deoxynucleotide triphosphates (dNTP), a buffer, and an effective amount of strand-displacing DNA polymerase and incubating same;

isolating the double-stranded DNA multimer from the mixture;

cleaving the double-stranded DNA multimer to produce the precursor DNA fragments; and

separating the precursor DNA fragments from a milieu comprising same;

introducing two or more precursor DNA fragments into a buffer medium comprising a thermostable DNA ligase enzyme, wherein the precursor DNA fragments will correctly assemble to generate a defined DNA sequence;

applying heat to a first temperature to cause the two or more precursor DNA fragments to denature; and

lowering the temperature to a second temperature for annealing in the presence of the thermostable DNA ligase enzyme, thereby generating double-stranded DNA heteroduplexes formed by base pairing of complementary regions, a portion of the heteroduplexes having single-stranded 5' overhangs and a portion of the heteroduplexes having single-stranded 3' overhangs, wherein when the 5' intramolecular overhangs on a heteroduplex molecule are complementary and ligation on both DNA strands occurs, ^{SCS}DNA is produced, and when the 3' intramolecular overhangs on a heteroduplex molecule are complementary and ligation on both DNA strands occurs, ^{SCS}DNA is produced.

[0078] In some embodiments, the second temperature is lower than the first temperature. In some embodiments, the application of heat to a first temperature to denature and the lowering to a second temperature to anneal is repeatedly performed in cycles. In some embodiments, the number of cycles is in a range from 1 cycle to 100 cycles. In some embodiments, the two or more precursor DNA fragments are selected from dsDNA molecules, single-stranded DNA molecules, DNA oligonucleotides, or mixtures thereof. In some embodiments, the two or more precursor DNA fragments are prepared according to the RCA method of the first aspect. In some embodiments, there are only two precursor DNA fragments that following denaturing, annealing, and ligation, form four different heteroduplex species, e.g., as shown in Figure 2. In some embodiments, the only two precursor DNA fragments are mixed in approximately an equal ratio for denaturing, annealing, and ligation. In some embodiments, there are n precursor DNA fragments, e.g., 2 or more (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, or more), that following denaturing, annealing, and ligation, form $2n \times (n\text{-}1)$ different heteroduplex species, e.g., as shown in Figure 4A. In some embodiments, the n precursor DNA fragments are mixed in approximately an equal ratio for denaturing, annealing, and ligation. It should be appreciated by the person skilled in the art that the n precursor DNA fragments are identical in sequence but have different starting and ending nucleotide positions and may have 5' or 3' overhangs (generated by restriction enzyme digestion) or may be blunt-ended. In some embodiments, a portion of the ^{SCS}DNA undergoes negative supercoiling in the presence of the thermostable DNA ligase enzyme. In some embodiments, a single stranded DNA binding protein or thermostable single stranded DNA binding protein is present in the

reaction, e.g., added to the buffer medium or added to the milieu at some point prior to annealing, to facilitate annealing of DNA strands.

[0079] In some embodiments, following the production of ^{SCS}DNA and/or supercoiled DNA, concatemers and generally tangled DNA, as well as precursors, can be removed by treatment with a 5' to 3' or 3' to 5' exonuclease, for example, T5, T7, or Exo III exonucleases. The exonucleases can be degraded by proteinase K digestion, removed by organic solvent extraction, and the DNA products (i.e., ^{SCS}DNA and/or supercoiled DNA) are precipitated from solution. Alternatively, column-based methods are known in the art to deproteinate the sample and purify the DNA, as understood by the person skilled in the art.

[0080] In some embodiments, as the DNA fragment precursor diversity increases, i.e., *n* is greater than 2, the higher the yield of ^{SCS}DNA. This is particularly relevant for larger circular RCA DNA templates. For example, for smaller circular RCA DNA templates, e.g., less than about 3.5 kb, 2 precursor DNA fragments, generated using 2 restriction enzymes, can yield greater than 20% ^{SCS}DNA. For larger circular RCA DNA templates, e.g., greater than about 3.5 kb, to achieve a yield of >20%, the number of restriction enzymes *n* is preferably at least 3, 4, 5, 6, or greater.

[0081] In some embodiments of the third aspect, the buffer medium comprising a thermostable DNA ligase enzyme comprises a buffer to maintain pH. In some embodiments, the buffer comprises a combination of ATP, Tris-HCl, $MgCl_2$, KCl, NaCl, beta-mercaptoethanol, DTT, NAD, and Triton™ X-100, to maintain a pH of about 4 to about 12, or about 6 to 10, or about 7.5 to about 9. In some embodiments, a DNA bending protein is also present in the buffer medium with the thermostable DNA ligase enzyme, either fused thereto or added separately. The denaturing (also referred to as "melting" or the first) temperature can be in a range from about 37°C to 100°C, or about 60°C to 100°C, or about 80°C to 100°C, for time in a range from about 0.1 minutes to about 60 minutes, or about 1 minute to about 5 minutes. The annealing is conducted by lowering the temperature (i.e., the second temperature) about 5°C to about 60°C lower than the denaturing temperature, or lowering about 10°C to 40°C lower than the denaturing temperature, for time in a range from about 0.1 minutes to about 60 minutes, or about 4 minutes to 6 minutes. Accordingly, in some embodiments, the second temperature is in a range from about 25°C to about 85°C, or about 25°C to about 70°C, or about 25°C to about 65°C, or about 37°C to about 65°C, or 50°C to about 70°C, for time in a range from about 0.1 minutes to about 60 minutes, or about 4 minutes to 6 minutes. As noted, the denaturing/annealing process can be repeatedly cycled about

2 to about 100 times, wherein each cycle increases the yield of ^{SCS}DNA. Accordingly, in some embodiments, the nucleic acid ligation schemes are temperature cycling from, e.g., about 80°C to 100°C, to a lower temperature of about 40°C to about 70°C, for 2 to 100 cycles.

[0082] In some embodiments of the third aspect, the method of producing synthetic circular supercoiled DNA (^{SCS}DNA) comprises:

generating precursor DNA fragments using rolling circle amplification (RCA);

introducing two or more precursor DNA fragments into a buffer medium at a pH of about 7.5 to about 9 and comprising ATP, Tris-HCl, $MgCl_2$, KCl, NaCl, beta-mercaptoethanol, NAD, DTT, Triton X-100, and at least one thermostable ligase, wherein the precursor DNA fragments will correctly assemble to generate a defined DNA sequence;

applying heat at a first temperature to cause the two or more precursor DNA fragments to denature, wherein the first temperature is determined by the size of the DNA sequence and can range from about 37°C to 100°C for time in a range from about 0.1 minutes to about 60 minutes; and

lowering the temperature to a second temperature for annealing in the presence of the thermostable DNA ligase enzyme, wherein the second temperature is from 10°C to 40°C lower than the first temperature for time in a range from about 4 minutes to about 10 minutes, thereby generating double-stranded DNA heteroduplexes formed by base pairing of complementary regions, a portion of the heteroduplexes having single-stranded 5' overhangs and a portion of the heteroduplexes having single-stranded 3' overhangs, wherein when the 5' intramolecular overhangs on a heteroduplex molecule are complementary and ligation on both DNA strands occurs, ^{SCS}DNA is produced, and when the 3' intramolecular overhangs on a heteroduplex molecule are complementary and ligation on both DNA strands occurs, ^{SCS}DNA is produced.

[0083] In some other embodiments of the third aspect, a method of producing synthetic circular supercoiled DNA (^{SCS}DNA) is described, the method comprising:

generating precursor DNA fragments using rolling circle amplification (RCA), said method comprising:

priming a circular RCA DNA template using oligonucleotides to form a primed circular RCA DNA template;

amplifying the primed circular RCA DNA template to form a double-stranded DNA multimer by preparing a mixture comprising the primed circular RCA DNA template, an

effective amount of deoxynucleotide triphosphates (dNTP), a buffer, and an effective amount of strand-displacing DNA polymerase and incubating same;

isolating the double-stranded DNA multimer from the mixture;

cleaving the double-stranded DNA multimer to produce the precursor DNA fragments; and

separating the precursor DNA fragments from a milieu comprising same;

introducing two or more precursor DNA fragments into a buffer medium at a pH of about 7.5 to about 9 and comprising ATP, Tris-HCl, $MgCl_2$, KCl, NaCl, beta-mercaptoethanol, NAD, DTT, Triton X-100, and at least one thermostable ligase, wherein the precursor DNA fragments will correctly assemble to generate a defined DNA sequence;

applying heat at a first temperature to cause the two or more precursor DNA fragments to denature, wherein the first temperature is determined by the size of the DNA sequence and can range from about 37°C to 100°C for time in a range from about 0.1 minutes to about 60 minutes; and

lowering the temperature to a second temperature for annealing in the presence of the thermostable DNA ligase enzyme, wherein the second temperature is from 10°C to 40°C lower than the first temperature for time in a range from about 4 minutes to about 10 minutes, thereby generating double-stranded DNA heteroduplexes formed by base pairing of complementary regions, a portion of the heteroduplexes having single-stranded 5' overhangs and a portion of the heteroduplexes having single-stranded 3' overhangs, wherein when the 5' intramolecular overhangs on a heteroduplex molecule are complementary and ligation on both DNA strands occurs, ^{SCS}DNA is produced, and when the 3' intramolecular overhangs on a heteroduplex molecule are complementary and ligation on both DNA strands occurs, ^{SCS}DNA is produced.

[0084] In some embodiments of the third aspect, the circular RCA DNA template is greater than about 3.5 kb and the double-stranded DNA multimer is cleaved using three or more restriction enzymes to produce three or more precursor DNA fragments to increase the yield of the ^{SCS}DNA using the CHTLA reaction.

[0085] In a fourth aspect, a method of producing substantially supercoiled DNA is described, the method comprising:

generating precursor DNA fragments using rolling circle amplification (RCA);

introducing two or more precursor DNA fragments into a buffer medium comprising a thermostable DNA ligase enzyme and a thermostable type II topoisomerase (e.g., a thermostable DNA gyrase), wherein the precursor DNA fragments will correctly assemble to generate a defined DNA sequence;

applying heat to a first temperature to cause the two or more precursor DNA fragments to denature; and

lowering the temperature to a second temperature for: (i) annealing in the presence of the thermostable DNA ligase enzyme, thereby generating double-stranded DNA heteroduplexes formed by base pairing of complementary regions, a portion of the heteroduplexes having single-stranded 5' overhangs and a portion of the heteroduplexes having 3' overhangs, wherein when the 5' intramolecular overhangs on a heteroduplex molecule are complementary and ligation on both DNA strands occurs, ^{SCS}DNA is produced, and when the 3' intramolecular overhangs on a heteroduplex molecule are complementary and ligation on both DNA strands occurs, ^{SCS}DNA is produced; and (ii) substantial supercoiling of the ^{SCS}DNA in the presence of the thermostable type II topoisomerase.

[0086] In some embodiments of the fourth aspect, a method of producing substantially supercoiled DNA is described, the method comprising:

generating precursor DNA fragments using rolling circle amplification (RCA), said method comprising:

priming a circular RCA DNA template using oligonucleotides to form a primed circular RCA DNA template;

amplifying the primed circular RCA DNA template to form a double-stranded DNA multimer by preparing a mixture comprising the primed circular RCA DNA template, an effective amount of deoxynucleotide triphosphates (dNTP), a buffer, and an effective amount of strand-displacing DNA polymerase and incubating same;

isolating the double-stranded DNA multimer from the mixture;

cleaving the double-stranded DNA multimer to produce the precursor DNA fragments; and

separating the precursor DNA fragments from a milieu comprising same;

introducing two or more precursor DNA fragments into a buffer medium comprising a thermostable DNA ligase enzyme and a thermostable type II topoisomerase, wherein the precursor DNA fragments will correctly assemble to generate a defined DNA sequence;

applying heat to a first temperature to cause the two or more precursor DNA fragments to denature; and

lowering the temperature to a second temperature for: (i) annealing in the presence of the thermostable DNA ligase enzyme, thereby generating double-stranded DNA heteroduplexes formed by base pairing of complementary regions, a portion of the heteroduplexes having single-stranded 5' overhangs and a portion of the heteroduplexes having 3' overhangs, wherein when the 5' intramolecular overhangs on a heteroduplex molecule are complementary and ligation on both DNA strands occurs, ^{SCS}DNA is produced, and when the 3' intramolecular overhangs on a heteroduplex molecule are complementary and ligation on both DNA strands occurs, ^{SCS}DNA is produced; and (ii) substantial supercoiling of the ^{SCS}DNA in the presence of the thermostable type II topoisomerase.

[0087] In some embodiments, the second temperature is lower than the first temperature. In some embodiments, the application of heat to a first temperature to denature and the lowering to a second temperature to anneal/ligase/supercoil is repeatedly performed in cycles. In some embodiments, the number of cycles is in a range from 1 cycle to 100 cycles. In some embodiments, the two or more precursor DNA fragments are selected from dsDNA molecules, single-stranded DNA molecules, DNA oligonucleotides, or mixtures thereof. In some embodiments, the two or more precursor DNA fragments are prepared according to the RCA method of the first aspect. In some embodiments, there are only two precursor DNA fragments that following denaturing, annealing, and ligation, form four different heteroduplex species, e.g., as shown in Figure 2. In some embodiments, the only two precursor DNA fragments are mixed in approximately an equal ratio for denaturing, annealing, and ligation. In some embodiments, there are n precursor DNA fragments, e.g., 2 or more, (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, or more), that following denaturing, annealing, and ligation, form $2n \times (n\text{-}1)$ different heteroduplex species, e.g., as shown in Figure 4A. In some embodiments, the n precursor DNA fragments are mixed in approximately an equal ratio for denaturing, annealing, and ligation. It should be appreciated by the person skilled in the art that the n precursor DNA fragments are identical in sequence but have different starting and ending nucleotide positions and may have 5' or 3' overhangs (generated by restriction enzyme

digestion) or may be blunt-ended. In some embodiments, the thermostable type II topoisomerase comprises DNA gyrase or Topoisomerase IV. In some embodiments, a single stranded DNA binding protein or thermostable single stranded DNA binding protein is present in the reaction, e.g., added to the buffer medium or added to the milieu at some point prior to annealing, to facilitate annealing of DNA strands.

[0088] In some embodiments, following the production of ^{SCS}DNA and/or supercoiled DNA, concatemers and generally tangled DNA, as well as precursors, can be removed by treatment with a 5' to 3' or 3' to 5' exonuclease, for example, T5, T7, or Exo III exonucleases. The exonucleases can be degraded by proteinase K digestion, removed by organic solvent extraction, and the DNA products (i.e., ^{SCS}DNA and/or supercoiled DNA) are precipitated from solution. Alternatively, column-based methods are known in the art to deproteinate the sample and purify the DNA, as understood by the person skilled in the art.

[0089] In some embodiments, as the DNA fragment precursor diversity increases, i.e., n is greater than 2, the higher the yield of ^{SCS}DNA. This is particularly relevant for larger circular RCA DNA templates. For example, for smaller circular RCA DNA templates, e.g., less than about 3.5 kb, 2 precursor DNA fragments, generated using 2 restriction enzymes, can yield greater than 20% ^{SCS}DNA. For larger circular RCA DNA templates, e.g., greater than about 3.5 kb, to achieve a yield of >20%, the number of restriction enzymes n is preferably at least 3, 4, 5, 6, or greater.

[0090] For the purposes of the present application, any enzyme that behaves like a type II topoisomerase that can induce DNA supercoiling is permissible. In some embodiments, the type II topoisomerase is a DNA gyrase or Topoisomerase IV. It was previously reported that DNA gyrase works via a transient double-strand break in the DNA, rather than nicks, and that DNA gyrase changes the linking number of DNA in steps of two (P.O. Brown and N.R. Cozzarelli, *Scince*, 1979, 206(4422), 1081-1083). As discussed herein, the presence of a type II topoisomerase such as DNA gyrase substantially supercoils the ^{scs}DNA, relative to any supercoiling that may occur to the ^{scs}DNA during a CHTLA reaction in the absence of type II topoisomerase. Accordingly, for the purposes of the present application, "substantially supercoiled" or "substantial supercoiling" corresponds to a degree of supercoiling that is induced by type II topoisomerase that is greater than supercoiling in ^{SCS}DNA produced during a CHTLA reaction in the absence of type II topoisomerase. Relating this to the linking number, for the purposes of the present application, $\Delta Lk_{ss} = Lk_{ss} - Lk_x$ can be calculated, wherein the Lk_{ss} is the linking number of the ^{scs}DNA

substantially supercoiled in the presence of type II topoisomerase and Lk is the linking number of the ^{scs}DNA in the absence of the type II topoisomerase, wherein a calculated ΔLk_{ss} (e.g., -2, -4, -6, -8, -10, etc), corresponds to substantial supercoiling.

[0091] It should be appreciated by the person skilled in the art that some type II topoisomerases are thermostable at temperatures of at least about 95°C, while others are not. In some embodiments, the thermostable type II topoisomerase species is thermostable at temperatures in a range from about 37°C to 100°C, or about 60°C to 100°C, or about 80°C to 100°C, for time in a range from about 30 seconds to about 10 minutes, or about 1 minute to about 5 minutes. Accordingly, when "thermostable type II topoisomerase" is used hereinafter, it is understood that the type II topoisomerase can survive the higher temperature of the denaturing/melting process, while maintaining some or all of its activity. In some embodiments, the thermostable type II topoisomerase is a thermostable DNA gyrase. Thermostable DNA gyrase is known in the art and can be engineered to be thermostable over the preferred temperature range.

[0092] In some embodiments of the fourth aspect, the buffer medium comprising a thermostable DNA ligase enzyme and a thermostable type II topoisomerase comprises a buffer to maintain pH. In some embodiments, the buffer comprises a combination of ATP, Tris-HCl, $MgCl_2$, KCl, NaCl, beta-mercaptoethanol, DTT, NAD, and Triton™ X-100, to maintain a pH of about 4 to about 12, or about 6 to 10, or about 7.5 to about 9. In some embodiments, a DNA bending protein is also present in the buffer medium with the thermostable DNA ligase enzyme, either fused thereto or added separately. The denaturing (also referred to as "melting" or the first) temperature can be in a range from about 37°C to 100°C, or about 60°C to 100°C, or about 80°C to 100°C, for time in a range from about 0.1 minutes to about 60 minutes, or about 1 minute to about 5 minutes. The annealing/ligation/supercoiling is conducted by lowering the temperature (i.e., the second temperature) about 5°C to about 60°C lower than the denaturing temperature, or lowering about 10°C to 40°C lower than the denaturing temperature, for time in a range from about 0.1 minutes to about 60 minutes, or about 4 minutes to 6 minutes. Accordingly, in some embodiments, the second temperature is in a range from about 25°C to about 85°C, or about 25°C to about 70°C, or about 25°C to about 65°C, or about 37°C to about 65°C, or 50°C to about 70°C, for time in a range from about 0.1 minutes to about 60 minutes, or about 4 minutes to 6 minutes. As noted, the process or raising the temperature to the first temperature and lowering to the second temperature can be repeatedly cycled about 2 to about 100 times, wherein each cycle increases the yield of supercoiled

DNA molecules and the extent of supercoiling of individual molecules. Accordingly, in some embodiments of the third aspect, the supercoiling schemes are temperature cycling from, e.g., a first temperature of about 80°C to 100°C to a second temperature of about 40°C to about 70°C, for 2 to 100 cycles.

[0093] In some embodiments of the fourth aspect, the method of producing substantially supercoiled DNA comprises:

generating precursor DNA fragments using rolling circle amplification (RCA);

introducing two or more precursor DNA fragments into a buffer medium at a pH of about 7.5 to about 9 and comprising ATP, Tris-HCl, $MgCl_2$, KCl, NaCl, beta-mercaptoethanol, NAD, DTT, Triton X-100, at least one thermostable ligase and a thermostable type II topoisomerase, wherein the precursor DNA fragments will correctly assemble to generate a defined DNA sequence;

applying heat at a first temperature to cause the two or more precursor DNA fragments to denature, wherein the first temperature for denaturing is determined by the size of the DNA sequence and can range from about 37°C to 100°C for time in a range from about 0.1 minutes to about 60 minutes; and

lowering the temperature to a second temperature for: (i) annealing in the presence of the thermostable DNA ligase enzyme, wherein the second temperature is from 10°C to 40°C lower than the first temperature for time in a range from about 4 minutes to about 10 minutes, thereby generating double-stranded DNA heteroduplexes formed by base pairing of complementary regions, a portion of the heteroduplexes having single-stranded 5' overhangs and a portion of the heteroduplexes having single-stranded 3' overhangs, wherein when the 5' intramolecular overhangs on a heteroduplex molecule are complementary and ligation on both DNA strands occurs, ^{SCS}DNA is produced, and when the 3' intramolecular overhangs on a heteroduplex molecule are complementary and ligation on both DNA strands occurs, ^{SCS}DNA is produced; and (ii) substantial supercoiling of the ^{SCS}DNA in the presence of the thermostable type II topoisomerase.

[0094] In some embodiments of the fourth aspect, the method of producing substantially supercoiled DNA comprises:

generating precursor DNA fragments using rolling circle amplification (RCA), said method comprising:

priming a circular RCA DNA template using oligonucleotides to form a primed circular RCA DNA template;

amplifying the primed circular RCA DNA template to form a double-stranded DNA multimer by preparing a mixture comprising the primed circular RCA DNA template, an effective amount of deoxynucleotide triphosphates (dNTP), a buffer, and an effective amount of strand-displacing DNA polymerase and incubating same;

isolating the double-stranded DNA multimer from the mixture;

cleaving the double-stranded DNA multimer to produce the precursor DNA fragments; and

separating the precursor DNA fragments from a milieu comprising same;

introducing two or more precursor DNA fragments into a buffer medium at a pH of about 7.5 to about 9 and comprising ATP, Tris-HCl, $MgCl_2$, KCl, NaCl, beta-mercaptoethanol, NAD, DTT, Triton X-100, at least one thermostable ligase and a thermostable type II topoisomerase, wherein the precursor DNA fragments will correctly assemble to generate a defined DNA sequence;

applying heat at a first temperature to cause the two or more precursor DNA fragments to denature, wherein the first temperature for denaturing is determined by the size of the DNA sequence and can range from about 37°C to 100°C for time in a range from about 0.1 minutes to about 60 minutes; and

lowering the temperature to a second temperature for: (i) annealing in the presence of the thermostable DNA ligase enzyme, wherein the second temperature is from 10°C to 40°C lower than the first temperature for time in a range from about 4 minutes to about 10 minutes, thereby generating double-stranded DNA heteroduplexes formed by base pairing of complementary regions, a portion of the heteroduplexes having single-stranded 5' overhangs and a portion of the heteroduplexes having single-stranded 3' overhangs, wherein when the 5' intramolecular overhangs on a heteroduplex molecule are complementary and ligation on both DNA strands occurs, ^{SCS}DNA is produced, and when the 3' intramolecular overhangs on a heteroduplex molecule are complementary and ligation on both DNA strands occurs, ^{SCS}DNA is produced; and (ii) substantial supercoiling of the ^{SCS}DNA in the presence of the thermostable type II topoisomerase.

[0095] In some embodiments of the fourth aspect, the circular RCA DNA template is greater than about 3.5 kb and the double-stranded DNA multimer is cleaved using three or more restriction enzymes to produce three or more precursor DNA fragments to increase the yield of the substantially supercoiled DNA using the CHTLA reaction.

[0096] In a fifth aspect, a method of producing substantially supercoiled DNA is described, the method comprising:

generating precursor DNA fragments using rolling circle amplification (RCA);

introducing the two or more precursor DNA fragments into a buffer medium comprising a thermostable DNA ligase enzyme, wherein the precursor DNA fragments will correctly assemble to generate a defined DNA sequence;

applying heat to a first temperature to cause the two or more precursor DNA fragments to denature;

lowering the temperature to a second temperature for annealing in the presence of the thermostable DNA ligase enzyme, thereby generating double-stranded DNA heteroduplexes formed by base pairing of complementary regions, a portion of the heteroduplexes having single-stranded 5' overhangs and a portion of the heteroduplexes having single-stranded 3' overhangs, wherein when the 5' intramolecular overhangs on a heteroduplex molecule are complementary and ligation on both DNA strands occurs, ^{SCS}DNA is produced, and when the 3' intramolecular overhangs on a heteroduplex molecule are complementary and ligation on both DNA strands occurs, ^{SCS}DNA is produced; and

lowering the temperature to a third temperature and adding a bolus of type II topoisomerase to initiate substantial supercoiling of the ^{SCS}DNA in the presence of the type II topoisomerase.

[0097] In some embodiments of the fifth aspect, a method of producing substantially supercoiled DNA is described, the method comprising:

generating precursor DNA fragments using rolling circle amplification (RCA), said method comprising:

priming a circular RCA DNA template using oligonucleotides to form a primed circular RCA DNA template;

amplifying the primed circular RCA DNA template to form a double-stranded DNA multimer by preparing a mixture comprising the primed circular RCA DNA template, an

effective amount of deoxynucleotide triphosphates (dNTP), a buffer, and an effective amount of strand-displacing DNA polymerase and incubating same;

isolating the double-stranded DNA multimer from the mixture;

cleaving the double-stranded DNA multimer to produce the precursor DNA fragments; and

separating the precursor DNA fragments from a milieu comprising same;

introducing the two or more precursor DNA fragments into a buffer medium comprising a thermostable DNA ligase enzyme, wherein the precursor DNA fragments will correctly assemble to generate a defined DNA sequence;

applying heat to a first temperature to cause the two or more precursor DNA fragments to denature;

lowering the temperature to a second temperature for annealing in the presence of the thermostable DNA ligase enzyme, thereby generating double-stranded DNA heteroduplexes formed by base pairing of complementary regions, a portion of the heteroduplexes having single-stranded 5' overhangs and a portion of the heteroduplexes having single-stranded 3' overhangs, wherein when the 5' intramolecular overhangs on a heteroduplex molecule are complementary and ligation on both DNA strands occurs, ^{SCS}DNA is produced, and when the 3' intramolecular overhangs on a heteroduplex molecule are complementary and ligation on both DNA strands occurs, ^{SCS}DNA is produced; and

lowering the temperature to a third temperature and adding a bolus of type II topoisomerase to initiate substantial supercoiling of the ^{SCS}DNA in the presence of the type II topoisomerase.

[0098] In some embodiments, the second temperature is lower than the first temperature. In some embodiments, the third temperature is lower than the second temperature. In some embodiments, the application of heat to a first temperature to denature, the lowering to a second temperature to anneal, and the further lowering to a third temperature to supercoil is repeatedly performed in cycles. In some embodiments, the number of cycles is in a range from 1 cycle to 100 cycles. In some embodiments, the two or more precursor DNA fragments are selected from dsDNA molecules, single-stranded DNA molecules, DNA oligonucleotides, or mixtures thereof. In some embodiments, the two or more precursor DNA fragments are prepared according to the RCA method of the first aspect. In some embodiments, there are only two precursor DNA fragments that following denaturing, annealing, and ligation, form four different heteroduplex species, e.g.,

as shown in Figure 2. In some embodiments, the only two precursor DNA fragments are mixed in approximately an equal ratio for denaturing, annealing, and ligation. In some embodiments, there are n precursor DNA fragments, e.g., 2 or more, (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, or more), that following denaturing, annealing, and ligation, form $2n \times (n\text{-}1)$ different heteroduplex species, e.g., as shown in Figure 4A. In some embodiments, the n precursor DNA fragments are mixed in approximately an equal ratio for denaturing, annealing, and ligation. It should be appreciated by the person skilled in the art that the n precursor DNA fragments are identical in sequence but have different starting and ending nucleotide positions and may have 5' or 3' overhangs (generated by restriction enzyme digestion) or may be blunt-ended. In some embodiments, the type II topoisomerase comprises DNA gyrase or Topoisomerase IV. In some embodiments, there are 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more precursor DNA fragments, as understood by the person skilled in the art. In some embodiments, a single stranded DNA binding protein or thermostable single stranded DNA binding protein is present in the reaction, e.g., added to the buffer medium or added to the milieu at some point prior to annealing, to facilitate annealing of DNA strands.

[0099] In some embodiments, following the production of ^{SCS}DNA and/or supercoiled DNA, concatemers and generally tangled DNA, as well as precursors, can be removed by treatment with a 5' to 3' or 3' to 5' exonuclease, for example, T5, T7, or Exo III exonucleases. The exonucleases can be degraded by proteinase K digestion, removed by organic solvent extraction, and the DNA products (i.e., ^{SCS}DNA and/or supercoiled DNA) are precipitated from solution. Alternatively, column-based methods are known in the art to deproteinate the sample and purify the DNA, as understood by the person skilled in the art.

[00100] In some embodiments, as the DNA fragment precursor diversity increases, i.e., n is greater than 2, the higher the yield of ^{SCS}DNA. This is particularly relevant for larger circular RCA DNA templates. For example, for smaller circular RCA DNA templates, e.g., less than about 3.5 kb, 2 precursor DNA fragments, generated using 2 restriction enzymes, can yield greater than 20% ^{SCS}DNA. For larger circular RCA DNA templates, e.g., greater than about 3.5 kb, to achieve a yield of >20%, the number of restriction enzymes n is preferably at least 3, 4, 5, 6, or greater.

[00101] In some embodiments of the fifth aspect, the buffer medium comprising a thermostable DNA ligase enzyme comprises a buffer to maintain pH. In some embodiments, the buffer comprises a combination of ATP, Tris-HCl, $MgCl_2$, KCl, NaCl, beta-mercaptoethanol, DTT, NAD, and Triton™ X-100, to maintain a pH of about 4 to about 12, or about 6 to 10, or

about 7.5 to about 9. In some embodiments, a DNA bending protein is also present in the buffer medium with the thermostable DNA ligase enzyme, either fused thereto or added separately. The denaturing (also referred to as "melting" or the first) temperature can be in a range from about 37°C to 100°C, or about 60°C to 100°C, or about 80°C to 100°C, for time in a range from about 0.1 minutes to about 60 minutes, or about 1 minute to about 5 minutes. The annealing is conducted by lowering the temperature (i.e., the second temperature) about 5°C to about 60°C lower than the denaturing temperature, or lowering about 10°C to 40°C lower than the denaturing temperature, for time in a range from about 0.1 minutes to about 60 minutes, or about 4 minutes to 6 minutes. Accordingly, in some embodiments, the second temperature is in a range from about 25°C to about 85°C, or about 25°C to about 70°C, or about 25°C to about 65°C, or about 37°C to about 65°C, or 50°C to about 70°C, for time in a range from about 0.1 minutes to about 60 minutes, or about 4 minutes to 6 minutes. It should be appreciated by the person skilled in the art that the second temperature is greater than the third temperature. The third temperature is dependent on the nature of the type II topoisomerase and is selected to ensure that the type II topoisomerase does not undergo heat degradation and is available to supercoil the ^{SCS}DNA. As noted, the denaturing/annealing/supercoiling process can be repeatedly cycles about 2 to about 100 times, wherein each cycle increases the yield of supercoiled DNA molecules and the extent of supercoiling of individual molecules. Accordingly, in some embodiments, the nucleic acid supercoiling schemes are temperature cycling from, e.g., a first temperature of about 80°C to 100°C, to a second temperature of about 40°C to about 70°C, to a third temperature that is lower than the second temperature, for 2 to 100 cycles.

[00102] In some embodiments of the fifth aspect, the circular RCA DNA template is greater than about 3.5 kb and the double-stranded DNA multimer is cleaved using three or more restriction enzymes to produce three or more precursor DNA fragments to increase the yield of the substantially supercoiled DNA using the CHTLA reaction.

[00103] Referring to Figure 2, it is noted that the four possible heteroduplexes that can form upon denaturation and reannealing of two overlapping and offset precursor DNA fragments, two bearing 5' overhangs, and two bearing 3' overhangs, can and do ligate to one another to form concatemers. However, without being bound by theory, under certain reaction conditions, intramolecular (or self) ligation to circularize is substantially favored over intermolecular ligation to form concatemers.

[00104] In some embodiments, the circular and supercoiled DNA described herein are produced without plasmid vectors, and thus are devoid of bacterial DNA. For example, in some embodiments, the precursor DNA fragments can be prepared using RCA, and then a complete closed circular vector can be obtained using the methods described herein, wherein the circular and supercoiled DNA product is devoid of bacterial DNA.

[00105] In some other embodiments, the circular and supercoiled DNA described herein are produced with plasmid (or cosmid, BAC, YAC, etc.) vectors and a DNA sequence of interest, and thus have the structure of a conventional cloning vector plus insert but are generated completely synthetically through the process of HTLA or CHTLA.

[00106] Accordingly, in a sixth aspect, a synthetic circular supercoiled DNA (^{SCS}DNA) is described, wherein the ^{SCS}DNA is devoid of any DNA of bacterial or viral origin. In some embodiments, the ^{SCS}DNA is plasmid vector-less. In some embodiments, the ^{SCS}DNA of the sixth aspect is substantially supercoiled. The ^{SCS}DNA sequence can be completely user-defined (i.e. scarless) and produced in quantities useful for gene therapy, cell engineering (i.e. CAR-T therapy), vaccines, as well as genome engineering in bacteria, yeast or other organisms. For example, in some embodiments, the ^{SCS}DNA is a vaccine, and the precursor DNA fragments are user-designed to direct expression of a vaccine antigen, the precursor DNA fragments are produced in large quantities, e.g., using RCA, and then the precursor DNA fragments undergo CHTLA, as described herein, to produce ^{SCS}DNA that is plasmid vector-less and directs vaccine antigen expression. In another example, ^{SCS}DNA is used to carry a payload gene in a gene therapy setting, as naked DNA or in a formulation designed to enhance cellular uptake generally or in a cell or tissue-directed manner. In another example, the ^{SCS}DNA encodes one or more components required to produce lentiviruses, adeno-associated viruses, or other viruses containing specific payloads for gene therapy in living animals including humans or for modification of cells in culture. In another example, ^{SCS}DNA that has a structure that is identical to a conventional plasmid (or cosmid, BAC, YAC, etc.) but is produced completely synthetically, e.g. to produce a large quantity of low copy conventional vector with insert.

[00107] Advantageously, a 1 ml overnight RCA reaction yields ~1 mg of precursors, and subsequent CHTLA reactions yield 0.25-0.30 mg of ^{scs}DNA (25-30% yield). Using a standard 96-well thermocycler, 15 mgs of ^{scs}DNA can be produced in a 1.5-day workflow. Importantly, by eliminating bacterial fermentation, the ^{scs}DNA produced by CHTLA is essentially free of

endotoxins and further, is devoid of an antibiotic resistance gene or genes. Additionally, ^{scs}DNA is free of bacterial chromosomal DNA contamination, which obviates the need for onerous purification protocols and further improves its safety profile.

[00108] The production of high-quality DNA vectors required to commercially generate viral gene therapies is an acknowledged industry bottleneck. Although conventional, full-length plasmid backbone-based vectors are the current industry standard, minimized DNAs offer substantial safety and efficiency advantages. Disadvantageously, currently no minimized DNA technology capable of scalable production of supercoiled and completely scarless DNAs exists. The current state of the art for minimized DNA production includes: minicircles or minivectors (e.g., Nanoplasmid, Minivec^{TM}), which are generated in bacteria and require extensive and expensive purification to obtain a "scarred" supercoiled final product; or dbDNAs, which are free of bacterial contaminants but are not supercoiled [Karbowniczek, 2017]. The methods described herein represents a substantial departure from the prior art by developing a robust new technology to synthetically produce DNAs with a supercoiled topology that are comprised exclusively of the sequence of interest and are scarless. ^{SCS}DNAs are commercially scalable and cost-effective and are a more efficient agent for transient transfection of LV and AAV packaging cells that yield higher viral titers. Moreover, ^{SCS}DNAs can also be used as non-viral gene delivery agents.

[00109] In some embodiments, ^{scs}DNAs are used instead of bacterially-sourced plasmids in virtually every application including, but not limited to, viral-based gene therapy, DNA vaccines, non-viral gene/cell therapy, and pre-clinical research use. From the vaccine performance perspective, there is an abundant literature demonstrating that the bacterial vector DNA sequences (i.e., the plasmid vector backbone) which are essential for propagation in *E. coli*, are highly undesirable in patient cells. Bacterial DNA can activate intracellular signals that lead to plasmid degradation as well as transcriptional silencing of the antigen expression cassette. These two factors can conspire to result in low antigen production, limiting vaccine potency and lowering the antibody response [Chen, 2008]. Because ^{scs}DNA is completely synthetic and has no plasmid backbone, these limitations are completely obviated. Moreover, the lack of a plasmid backbone means that ^{scs}DNAs are always smaller, and a plethora of studies have demonstrated that smaller DNAs enter cells more readily than larger ones. The fact that the ^{scs}DNA manufacturing workflow is completely synthetic and does not require bacterial fermentation also dramatically reduces the likelihood of endotoxin contamination, obviates safety concerns surrounding accidental delivery

of antibiotic resistance genes into patient cells, and simplifies the purification process. This translates into lower production costs. Further, ^{scs}DNA manufacturing is scalable, technically simple and does not involve bacterial fermentation and its incumbent purification steps.

EXAMPLES

Example 1 – Workflow for ^{SCS}DNA production

[00110] To generate CHTLA precursor DNA fragments, a RCA reaction was set up using pMaxGFP (3.4 kb) as a template (~100 ng plasmid DNA + 50 uM random hexamer oligo in 1X annealing buffer, volume = 100 µL). The reaction was heated to 95°C for 3 min and cooled to 25°C (ramp = 0.1°C/sec) on a PCR block. dNTP (1 mM) and phi29 polymerase were added, the volume was increased to 250 µL and the reaction was incubated in a 37°C water bath overnight. double-stranded DNA multimer were purified by isopropanol precipitation and DNA pellets were washed (70% ethanol) and dissolved in 500 µL of H_2O. This protocol is sufficient to amplify all circular DNA templates proposed herein. The double-stranded DNA multimers were then digested with single-cutter restriction enzymes followed by phenol/chloroform extraction. A substantially equal amount of precursor DNA fragments from two or more restriction enzyme digestions was mixed as precursors for CHTLA reactions. CHTLA was done on a PCR block with a cycling program: 95°C for 3 min, then 20 cycles of 95°C for 1 min, followed by 60°C for 5 min. Although the recommended reaction volume is 100 µL/tube for the PCR block, no yield loss was observed when the volume was increased to 265 µL/tube. Therefore, the maximum reaction volume is approximately 25 mL for a 96-tube PCR block. After the ligation reaction was complete, 100 units of T5 exonuclease were added to each tube and incubated at 37°C overnight to digest linear concatemers and unreacted precursors. The process is illustrated in Figure 3A. Circular DNA was purified by phenol/chloroform extraction and isopropanol precipitation. Reaction products were then treated with T5 exonuclease at 37°C for one hour and analyzed on a gel containing ethidium bromide and compared to untreated reaction products. Figure 3B illustrates the formation of the ^{SCS}DNA, wherein the white arrow designates the concatemers and products from non-specific ligation and the green arrowhead designates the supercoiled form of ^{SCS}DNA.

Example 2 - The effect of precursor diversity and concentration on ^{scs}DNA yield

[00111] During CHTLA, both circular and concatemeric DNAs are generated. Identifying parameters that tilt the balance toward circular DNA could lead to higher ^{SCS}DNA yields, particularly for long products. When a circular DNA (or a double-stranded DNA multimer) is digested (separately) by single-cut restriction enzymes to generate CHTLA precursor DNA fragments, a series of linear molecules with identical overall sequences, but different starting and ending nucleotide positions, are generated. When these linear precursor DNA fragments are pooled and denatured, upon annealing, any first (or "top") strand can anneal with any second (or "bottom") strand. In some cases, homoduplexes form, where, for example, a first strand "A" anneals to a second strand "a," regenerating a completely double-stranded "Aa" precursor. In contrast, whenever a heteroduplex (i.e., Ab or Ba) forms, that molecule is only partially double-stranded, and has single-stranded overhangs that are perfectly complementary. If those overhangs anneal and are intra-molecularly ligated, a circular product results. However, since their overhangs are complementary, it is also possible for Ab heteroduplexes to anneal to each other and be intermolecularly ligated to form an AbAb dimer or higher order concatemer. This competition between intra- and intermolecular ligation is a key determinant of CHTLA reaction efficiency. In the simplest case, with two linear precursors, four heteroduplexes are possible (2 with 5' overhangs, and 2 with 3' overhangs). In an attempt to artificially weight the reaction conditions in favor of intra-molecular ligation, the diversity of possible heteroduplexes was increased (i.e., Ab, Ac, Ad, Ae) which decreases concatemerization by reducing the probability of perfectly complementary overhangs locating each other during annealing and intermolecularly ligating. It should be understood by the skilled artisan that although heteroduplexes with imperfectly complementary overhangs can also anneal, they cannot be ligated and are simply melted in the next cycle. For example, if the single-cutter enzyme number is n, the number of heteroduplexes = $2n \times (n\text{-}1)$ so when n equals 16, the number of possible heteroduplexes = 480. An illustration of this is shown in Figure 4A.

[00112] To determine the effect of increasing the diversity of the CHTLA precursor DNA fragment pool on circular DNA yield, the double-stranded DNA multimer from an approximately 6.5 kb template was digested with anywhere from 2-16 single-cut restriction enzymes to produce an equivalent number of CHTLA precursor DNA fragments. CHTLA products were digested with T5 exonuclease and analyzed on an agarose gel. The ^{SCS}DNA concentration was measured by UV

absorbance. As shown in Figure 3B, when the number of single-cut restriction enzymes increased from 2 to 16, increasing the diversity of precursor DNAs for 2-16, the CHTLA yield for the ~6.5 kb ^{SCS}DNA more than doubled.

[00113] In another experiment, double-stranded DNA multimers were generated using pMaxGFP (3.4 kb) as the template and digestion with either two or eight single-cutter restriction enzymes was effectuated. CHTLA reactions at various final DNA concentrations were set up. It was observed that when the double-stranded DNA multimers were digested with two single-cutters, increasing the DNA concentration from 0.5 to 1.0 mg/ml did not increase the final ^{SCS}DNA yield. In contrast, when the double-stranded DNA multimers were digested with eight single-cutters, the ^{SCS}DNA yield nearly doubled when the DNA concentration increased from 1 to 2 mg/ml (Figure 4C). Advantageously, this demonstrates that increasing the diversity of precursor DNA fragments permits increased performance of the CHTLA reaction at a higher precursor DNA concentration.

[00114] Transfer DNA constructs for lentivirus vector preparation are often 8-10 kb. For ^{SCS}DNA to be commercially viable in this market, it is imperative to improve the conversion rate for constructs in this range. While the strategy described herein for increasing conversion by diversification of the heteroduplex pool is promising, it can be challenging to identify more than sixteen commonly used single-cutters for any given DNA. Moreover, the positions of such naturally occurring restriction sites may not be optimal. Therefore, in some embodiments heteroduplex diversity in CHTLA is increased by inserting paired Type IIS restriction sites [Szybalski, 1991] at precise locations to generate a panel of RCA products that can each be cut by the Type IIS restriction enzyme to generate linear precursor DNA fragments. In addition, it is believed that DNA flexibility is potentially a major factor controlling intra-vs. intermolecular ligation balance, and that higher flexibility would favor intramolecular ligation [Ngo, 2016; Peters, 2011]. DNA flexibility is a function of both intrinsic and extrinsic factors: double-stranded DNA is less flexible than single-stranded DNA [Bao, 2017], and DNA bending proteins increase DNA flexibility by inducing partial melting [Vliet, 1993; Vámosi, 2018]. Accordingly, in some embodiments, heteroduplex flexibility is increased, and hence intramolecular ligation is favored, by using a thermostable ligase fused with a DNA-bending protein or by the addition of a separate DNA bending protein to the CHTLA reaction mixture.

Example 3 - ^{SCS}DNA production from Type IIS restriction enzyme-digested precursors

[00115] In CHTLA, the desired ^{SCS}DNA product forms when heteroduplex DNAs with compatible ends are circularized by intramolecular ligation. However, linear concatemers also form by intermolecular ligation of those same heteroduplexes. At high precursor concentrations, intermolecular concatemer formation is favored, while low concentrations favor intramolecular circularization [Guo, 2015; Thibault, 2017]. However, lower DNA concentration limits the final yield of circular DNA, increasing production costs and complicating the scale-up required for commercialization and profitability. Our preliminary data demonstrates that increasing precursor diversity by cutting the same bulk double-stranded DNA multimer into distinct forms with single-cut restriction enzymes increases intramolecular ligation doubling ^{SCS}DNA yield. Without being bound by theory, it is believed that this occurs because increasing heteroduplex diversity reduces the likelihood heteroduplexes with compatible ends will encounter each other in the reaction milieu and ligate. Unfortunately, it is difficult to test the limits of this effect due to the limited number of available single-cutting enzymes and their naturally occurring locations.

[00116] In the present example, two circular RCA DNA templates were prepared with precisely placed paired Type IIS restriction sites. Type IIS restriction enzymes are ideal tools to generate the precursor species [Oliynyk, 2022; Cheng, 2019]. These enzymes recognize asymmetric sequences and cut the DNA outside the recognition sequences [Szybalski, 1991; Lippow, 2009], providing the ability to create a linear precursor pool with identical sequences with distinct starting and ending nucleotide positions.

[00117] CHTLA precursor DNA fragments can be systematically generated that are identical in sequence but linearized at precisely spaced nucleotide positions. To achieve this, a combination of restriction enzyme digestion and Gibson assembly can be used to insert two Type IIS restriction enzyme sites at regular intervals. For proof-of-principle, paired BsaI sites were placed within the sequence of 6.5 kb pCMV-RED, according to the workflow schematic shown in Figure 5A. A deletion was first created by restriction enzyme digestion. Separately, the deleted sequence was regenerated by Gibson assembly of two PCR products, each containing a newly introduced (and precisely placed) BsaI site and homology to the digested parental plasmid. A second Gibson reaction joined the modified (i.e., BsaI site-containing) insert to the digested parental plasmid. Upon BsaI digestion, a completely scarless linear form of pCMV-RED was generated. In Figure 5B, the double-stranded DNA multimer was digested with BsaI or BamHI

(which cuts once within pCMV-RED) and electrophoresis was run, where **M 1+2,** Mixture of fragment 1 and fragment 2; **F 1+2**, PCR products created using assembled fragments 1 and 2 as a template; **V B+E**, vector DNA digested with BamHI and EcoRI; **RCA**, RCA products; **Bam**, the double-stranded DNA multimerdigested with BamHI; **Bsa,** the double-stranded DNA multimer digested with BsaI; **Lad**, one-kb DNA ladder.

[00118] In Figure 5C, it was demonstrated that BsaI-digested DNA can be used as precursors in CHTLA to produce ^{SCS}DNA. A 1633-bp GFP expression cassette was amplified using a circular RCA DNA template. The first PCR fragment started at position one and ended at position 1633. The second PCR started at position 21 and ended at position 20. The third PCR started at position 201 and ended at position 200. One BsaI site was introduced at each end of the PCR product. The same 20-bp random region was added at each end of the PCR product. Each PCR product was circularized by Gibson Assembly and used as a template for RCA. The three RCA DNAs were digested with BsaI, purified, mixed at 1:1:1 ratio, and ligated in CHTLA reactions to produce ^{SCS}DNA. **GFP**, GFP ^{SCS}DNA; **Lad**, 1-kb DNA ladder**.**

[00119] Accordingly, in some embodiments, paired BsaI sites will be introduced at ~40 positions, and CHTLA reactions will be performed with 20, 25, 30, 35, and 40 precursors and ^{SCS}DNA conversion efficiency will be quantified spectroscopically. Using an identical strategy, fifty paired PaqCI sites at ~220 bp intervals throughout an ~11kb lentivirus transfer plasmid are proposed to be introduced. This plasmid carries a GFP expression cassette, which can be used to quantify expression. No natural PaqCI sites exist on this plasmid. These fifty templates will allow for the generation of 2×50×49 = 4900 heteroduplexes. It is believed that the high number of heteroduplex precursors will allow for an increase in the DNA concentration in the CHTLA reaction to up to 5 mg/ml without a conversion rate drop.

Example 4

[00120] Approximately 3 kb pBluescript (pBs) was chosen as a paradigm and amplified by RCA with phi29 polymerase and random hexamers in a reaction. Specifically, random hexamers (100 μM) were mixed with ~0.1 μg plasmid in 50 μL, heated to 95°C for 3 min, and cooled to 25°C (rate = 0.1°C/sec). Ten 50 μL annealing reactions were pooled and phi29 buffer, dNTPs, and phi29 polymerase were added (final vol =1 mL) and incubated at 37°C for RCA. The double-stranded DNA multimers were EtOH precipitated, and the concatemeric double-stranded DNA

multimers were digested with restriction enzymes (RE) KpnI (i.e., RE1) or XmnI (i.e., RE2) to yield two overlapping unit-length pBS molecules offset by ~1kb to generate heteroduplexes with 1 kb sticky ends, and the RE-digested precursors were column purified. Approximately 1 mg purified precursor DNA fragments was obtained from the 1 mL RCA reaction products. RE-digested precursor DNA fragments were mixed 1:1 and a 10-cycle 50 µL CHTLA reaction was performed. CHTLA products were treated with T5 exonuclease (10 units) to remove the linear DNA, and the circular DNA was column purified. ~200 ug circular DNA (white arrow) was obtained and ~80% was supercoiled (Figure 6A). Linear precursor to circular conversion is ~20%.

[00121] To demonstrate CHTLA can generate larger vectorless supercoiled DNAs, 16 kb precursors were prepared by RE digestion of a 16 kb plasmid with single-cutting NheI or XmnI. Precursors were mixed (1:1), a 10-cycle CHTLA reaction was done, products were treated with T5 exonuclease and purified. Linear precursor to circular (yellow arrow) conversion is ~15%, as illustrated in Figure 6B. Although the yield is lower (~15% conversion of linear precursor to circular product), these data demonstrate that relatively large circular DNAs can be generated by CHTLA. Similarly, circular CHTLA products of 7 and 14 kb were generated (data not shown).

Example 5

[00122] To test the performance of ^{scs}DNAs vs. plasmids in mammalian cells, an ^{scs}DNA carrying the Green Fluorescent Protein cassette (^{scs}DNA-GFP) derived from the plasmid pMax-GFP was generated. The difficult-to-transfect cell line (BLN3) derived from a mouse prostate tumor was chosen to directly compare the transfection efficiency of ^{scs}DNA-GFP versus pMax-GFP. After transfection of equimolar amounts of ^{scs}DNA-GFP and pMax-GFP, fluorescence was measured microscopically. At 48 hours post-transfection, the wells transfected with ^{scs}DNA-GFP showed 4-8 fold more GFP-expressing cells compared to those transfected with pMax-GFP (Figure 7). These data strongly support the hypothesis that ^{scs}DNAs will outperform conventional plasmids in animal tissues.

Example 6

[00123] This example demonstrates the performance of CHTLA in which one precursor DNA fragment was comprised of a unit-length DNA molecule with the DNA sequence of the desired ^{SCS}DNA product, and two other precursor DNA fragments were comprised of sub-unit-

length DNA molecules that together contained the sequence of the desired ^{SCS}DNA product. In one case, a 2.4 kb firefly luciferase expression cassette was amplified by RCA and the double-stranded DNA multimer digested with either XhoI alone or XmnI alone. XhoI digestion produced a unit length precursor DNA fragment and XmnI digestion produced two precursor DNA fragments of different length (Figure 8A). CHTLA using these precursors yielded the desired ^{SCS}DNA product. In the second case (Figure 8B) 6.5 kb pCMV-RED was RE-digested with EcoRI alone, BamHI alone, or BamHI + SalI together. CHTLA was then performed with 2 precursor DNA fragments (EcoRI cut and BamHI cut, Figure 8B, right panel, first lane) or with EcoRI alone cut pCMV-RED and BamHI + SalI cut pCMV-RED (Figure 8B, right panel, second lane). Combining the unit-length precursor with the 2 sub-unit-length precursors yielded the desired ^{SCS}DNA product.

Example 7

[00124] Although fusing T4 DNA ligase with multiple DNA bending proteins is known to increase circularization of both sticky- and blunt-ended DNA, this strategy has not been tried with thermostable ligases. In some embodiments, highly thermostable DNA-bending protein SSO7D will be fused to taq ligase, bacterially expressed, and the recombinant protein purified. The taq ligase-SSO7D fusion protein will be used in CHTLA reactions, for example for an ~3.4 kb and an ~11 kb DNA precursor, and the yield determined. In some embodiments, the pH will be varied, as well as the ion species and concentration to develop an optimal buffer system to efficiently perform CHTLA using the taq ligase-SSO7D fusion protein.

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What is claimed is:

1. A method of producing synthetic circular supercoiled DNA (^{SCS}DNA), the method comprising:

(a) generating precursor DNA fragments using rolling circle amplification (RCA);

(b) introducing two or more precursor DNA fragments into a buffer medium comprising a thermostable DNA ligase enzyme, wherein the precursor DNA fragments will correctly assemble to generate a defined DNA sequence;

(c) applying heat to a first temperature to cause the at least two precursor DNA fragments to denature; and

(d) lowering the temperature to a second temperature for: (i) annealing in the presence of the thermostable DNA ligase enzyme, thereby generating double-stranded DNA heteroduplexes formed by base pairing of complementary regions, a portion of the heteroduplexes having single-stranded 5' overhangs and a portion of the heteroduplexes having 3' overhangs, wherein when the 5' intramolecular overhangs on a heteroduplex molecule are complementary and ligation on both DNA strands occurs, ^{SCS}DNA is produced, and when the 3' intramolecular overhangs on a heteroduplex molecule are complementary and ligation on both DNA strands occurs, ^{SCS}DNA is produced; and (ii) substantial supercoiling of the ^{SCS}DNA in the presence of the thermostable type II topoisomerase.

2. The method of claim 1, wherein step (a) comprises:

priming a circular RCA DNA template using oligonucleotides to form a primed circular RCA DNA template;

amplifying the primed circular RCA DNA template to form a double-stranded DNA multimer by preparing a mixture comprisingthe primed circular RCA DNA template, an effective amount of deoxynucleotide triphosphates (dNTP), a buffer, and an effective amount of strand-displacing DNA polymerase and incubating same;

isolating the double-stranded DNA multimer from the mixture;

cleaving the double-stranded DNA multimer to produce the precursor DNA fragments; and

separating the precursor DNA fragments from a milieu comprising same.

3. The method of claim 2, wherein the circular RCA DNA template is complimentary to the nucleotide sequence of the precursor DNA fragment.

4. The method of claims 2 or 3, wherein the primed circular RCA DNA template is produced by annealing an oligonucleotide to the circular RCA DNA template

5. The method of claim 4, wherein the oligonucleotide comprises random hexamers and/or one or more oligonucleotides of a specific DNA sequence(s) present in the circular RCA DNA template.

6. The method of any of claims 2-5, wherein the double-stranded DNA multimer comprises multiple copies of the circular RCA DNA template arranged in series as a concatemer.

7. The method of any of claims 2-6, wherein the mixture is incubated at temperature in a range from about 36°C to about 38°C for time in a range from about 6 hours to about 12 hours.

8. The method of any of claims 2-7, wherein the double-stranded DNA multimer is cleaved using two or more restriction enzymes.

9. The method of claim 8, wherein *n* precursor DNA fragments are isolated when *n* restriction enzymes are used, wherein *n* = 2, 3, 4, 5, 6, 7, 8, 9, 10, or more.

10. The method of claim 9, wherein the *n* precursor DNA fragments are identical in sequence to one another but have different starting and ending nucleotide positions and (i) have 5' or 3' overhangs or (ii) are blunt-ended.

11. The method of claim 8, wherein the double-stranded DNA multimer is not identical in sequence and the precursor DNA fragments are non-identical.

12. The method of any of claims 2-11, wherein the circular RCA DNA template comprises restriction enzyme (RE) recognition sites that naturally occur within the sequence of the circular RCA DNA template.

13. The method of any of claims 2-11, wherein the RCA DNA template is engineered to contain RE sites that do not naturally occur in the circular RCA DNA template.

14. The method of claim 13, wherein a non-naturally occurring RE site includes Type IIS restriction sites.

15. The method of any of claims 8-14, wherein the restriction enzymes are single-cutters or multiple cutters.

16. The method of any of claims 2-15, wherein the strand-displacing DNA polymerases are selected from Phi29 DNA polymerase, DNA Polymerase I, Klenow fragment of DNA Polymerase I, T7 DNA Polymerase (exonuclease-free), T4 DNA Polymerase, Taq Polymerase, and AMV (or MuLV) Reverse Transcriptase, or closely homologous mutants thereof, preferably Phi29 DNA polymerase.

17. The method of any of the preceding claims, wherein steps (b)-(d) are performed in a single container.

18. The method of any of the preceding claims, wherein two precursor DNA fragments are used and they are introduced in an approximately equal ratio.

19. The method of any of claims 1-17, wherein more than two precursor DNA fragments are used and they are introduced in an approximately equal ratio.

20. The method of any of the preceding claims, wherein the second temperature is lower than the first temperature.

21. The method of any of the preceding claims, wherein the first temperature is in a range from about 37°C to about 100°C, preferably about 80°C to about 100°C.

22. The method of claim 21, wherein the first temperature is applied for time in a range from about 30 seconds to about 10 minutes, preferably about 1 minute to about 5 minutes.

23. The method of any of the preceding claims, wherein the second temperature is about 25°C to about 85°C, preferably 50°C to about 70°C.

24. The method of claim 23, wherein the second temperature is maintained for time in a range from about 0.1 minutes to about 60 minutes.

25. The method of any of the preceding claims, wherein the application of heat to a first temperature to denature and the lowering to a second temperature to anneal is repeatedly performed in cycles, preferably 2 cycles to 100 cycles.

26. The method of any of the preceding claims, wherein during annealing, heteroduplex double-stranded DNA sequences are generated, formed by base pairing of complementary regions, wherein the heteroduplexes comprise 5' or 3' overhangs.

27. The method of claim 26, wherein ^{SCS}DNA is produced when the 5' or 3' overhangs are complementary.

28. The method of any of the preceding claims, wherein the buffer medium comprises a combination of ATP, Tris-HCl, $MgCl_2$, KCl, NaCl, DTT, beta-mercaptoethanol, NAD, and TRITON X-100.

29. The method of claim 28, wherein the pH is maintained at about 4 to about 12, preferably about 7.5 to about 9.

30. The method of claim 27, wherein a portion of the ^{SCS}DNA undergoes negative supercoiling in the presence of the thermostable DNA ligase enzyme.

31. The method of any of the preceding claims, wherein the buffer medium further comprises a type II topoisomerase, preferably a thermostable type II topoisomerase.

32. The method of claim 31, wherein the ^{SCS}DNA are substantially supercoiled.

33. The method of claims 31 or 32, wherein the type II topoisomerase is thermostable and the thermostable type II isomerase can be cycled through the denaturing and annealing processes while maintaining some or all of its activity.

34. The method of claims 31 or 32, wherein the type II topoisomerase is thermostable or not thermostable and the method further comprises lowering the temperature to a third temperature and adding a bolus of type II topoisomerase to initiate substantial supercoiling of the ^{SCS}DNA in the presence of the type II topoisomerase.

35. The method of any of claims 31-34, wherein the type II topoisomerase is a DNA gyrase.

36. The method of any of claims 31-34, wherein the type II topoisomerase is a Topoisomerase IV.

37. The method of any of the preceding claims, wherein the at least two precursor DNA fragments do not comprise intentional nicks.

38. The method of any of the preceding claims, wherein the ^{SCS}DNA produced is devoid of bacterial DNA.

39. A synthetic circular supercoiled DNA (^{SCS}DNA) produced according to the method of any of claims 1-38.