

CLAIMS

1. A method of assembling a target polynucleotide, comprising:
partitioning a plurality of polynucleotides into a contained reaction volume, wherein:
the plurality of polynucleotides comprise a first polynucleotide and a second polynucleotide, wherein the second polynucleotide is attached to a support,
the first polynucleotide comprises a first subsequence of a target polynucleotide,
wherein the first polynucleotide comprises a single-stranded 3' end sequence,
the second polynucleotide comprises, in the 3' to 5' direction:

- (i) a single-stranded 3' end sequence,
- (ii) a second subsequence of the target polynucleotide,
- (iii) a Type IIS restriction enzyme recognition sequence, and
- (iv) a complementary sequence capable of hybridizing to all or a portion of

the second subsequence, and

the second polynucleotide is capable of forming a hairpin molecule comprising a 3' overhang, a stem formed by intramolecular nucleotide base pairing between all or a portion of the second subsequence and the complementary sequence, and a loop, wherein the hairpin molecule is in a configuration that is not cleaved by a Type IIS restriction enzyme;

~~wherein the first polynucleotide and/or the second polynucleotide optionally further comprise a tag, a barcode, an amplification site, a unique molecular identifier (UMI), or any combination thereof; and~~

wherein the first and second polynucleotides are connected within the contained reaction volume, thereby assembling the first and second subsequences.

2. The method of claim 1, wherein the first polynucleotide comprises two nucleic acid strands forming a duplex.

3. The method of claim 1 or 2, wherein the first polynucleotide is capable of forming one or more hairpins.

4. The method of any of claims 1-3, wherein the first and/or second polynucleotide comprises a tag, a barcode, an amplification site, a unique molecular identifier (UMI), or any combination thereof. ~~one or more barcodes and/or one or more tags, e.g., a capture tag sequence.~~

~~5. The method of any of claims 1-4, wherein prior to connecting the first and second polynucleotides, the first polynucleotide is not attached to the support.~~

~~6. The method of any of claims 1-4, wherein prior to connecting the first and second polynucleotides, the first polynucleotide is attached to the support.~~

~~7. The method of claim 6, wherein the first polynucleotide is directly or indirectly attached to the support.~~

~~8. The method of claim 6 or 7, wherein the first polynucleotide is covalently or noncovalently attached to the support or a linker, e.g., a cleavable linker.~~

~~9. The method of any of claims 6-8, wherein the first polynucleotide is attached to the support via hybridization (e.g., between a capture probe sequence directly or indirectly on the support and a capture tag sequence of the first polynucleotide), the interaction between a binding pair (e.g., biotin/streptavidin binding), a covalent bond, or any combination thereof.~~

~~10. The method of any of claims 6-9, wherein the first polynucleotide remains attached to the support during and/or after connecting the first and second polynucleotides.~~

~~11. The method of any of claims 6-10, wherein the first polynucleotide is released from the support after the first and second polynucleotides are connected.~~

~~12. The method of any of claims 6-9, wherein the first polynucleotide is released from the support before the first and second polynucleotides are connected.~~

~~13. The method of any of claims 10-12, wherein the releasing comprises heating the contained reaction volume and/or enzymatic cleavage of the first polynucleotide or a linker, e.g., a cleavable linker.~~

~~14. The method of any of claims 1-13, wherein the second polynucleotide comprises one or more barcodes and/or one or more tags, e.g., a capture tag sequence.~~

~~15. The method of any of claims 1-14, wherein the second polynucleotide is directly or indirectly attached to the support.~~

~~16. The method of any of claims 1-15, wherein the second polynucleotide is covalently or noncovalently attached to the support or a linker, e.g., a cleavable linker.~~

~~17. The method of any of claims 1-16, wherein the second polynucleotide is attached to the support via hybridization (e.g., between a capture probe sequence directly or indirectly on the support and a capture tag sequence of the second polynucleotide), the interaction between a binding pair (e.g., biotin/streptavidin binding), a covalent bond, or any combination thereof.~~

~~18. The method of any of claims 1-17, wherein prior to connecting the first and second polynucleotides, the second polynucleotide is not released from the support.~~

~~19. The method of claim 18, wherein the second polynucleotide remains attached to the support during and/or after connecting the first and second polynucleotides.~~

~~20. The method of claim 18 or 19, wherein the second polynucleotide is released from the support after the first and second polynucleotides are connected.~~

~~21. The method of any of claims 1-17, wherein prior to connecting the first and second polynucleotides, the second polynucleotide is released from the support.~~

~~22. The method of claim 20 or 21, wherein the releasing comprises heating the contained reaction volume and/or enzymatic cleavage of the second polynucleotide or a linker, e.g., a cleavable linker.~~

~~23. The method of any of claims 1-22, wherein the first and second polynucleotides are connected in the contained reaction volume when both are not attached to the support.~~

~~24. The method of any of claims 1-23, wherein the second polynucleotide forms the hairpin molecule before and/or during connecting the first and second polynucleotides.~~

~~25. The method of any of claims 1-24, wherein the 5' end of the second polynucleotide is blocked from ligation, extension, and/or hybridization.~~

~~26. The method of any of claims 1-25, wherein the second polynucleotide further comprises, between the second subsequence and the complementary sequence, a sequence comprising one or more barcodes and/or one or more tags, e.g., a capture tag sequence.~~

~~27. The method of claim 26, wherein the sequence comprising one or more barcodes and/or one or more tags is between the Type IIS restriction enzyme recognition sequence and the complementary sequence.~~

~~28. The method of any of claims 1-27, wherein the second polynucleotide further comprises a 5' end sequence that does not hybridize to the single stranded 3' end sequence or the second subsequence.~~

~~29. The method of claim 28, wherein the 5' end sequence comprises one or more barcodes and/or one or more tags, e.g., a capture tag sequence.~~

~~30. The method of claim 28 or 29, wherein the 5' end sequence is blocked from ligation, extension, and/or hybridization.~~

~~31. The method of any of claims 1-30, wherein the stem comprises one or more bulged bases in either one or both strands of the stem.~~

~~32. The method of claim 31, wherein the stem comprises a bulge sequence in the strand comprising the complementary sequence.~~

~~33. The method of claim 31 or 32, wherein the bulge sequence is capable of forming one or more internal hairpins.~~

~~34. The method of any of claims 31-33, wherein the bulge sequence comprises one or more barcodes and/or one or more tags, e.g., a capture tag sequence.~~

~~35. The method of any of claims 31-34, wherein the stem comprises a bulge sequence in the strand comprising the second subsequence.~~

~~36. The method of any of claims 1-35, wherein the second subsequence is capable of forming one or more hairpins internal to the hairpin molecule formed by the second polynucleotide.~~

~~37. The method of any of claims 1-36, wherein the second polynucleotide further comprises an intervening sequence between the second subsequence and the Type IIS restriction enzyme recognition sequence.~~

~~38. The method of claim 37, wherein the intervening sequence is capable of being cleaved from the second subsequence by the Type IIS restriction enzyme when the second polynucleotide forms a duplex with a complementary strand.~~

~~39. The method of any of claims 1-36, wherein there is no intervening sequence between the second subsequence and the Type IIS restriction enzyme recognition sequence.~~

~~40. The method of any of claims 1-39, wherein the 3' end of the 3' overhang is not blocked from ligation, extension, and/or hybridization.~~

~~416. The method of any of claims 1-405, wherein the 3' overhang is between about 1 and about 100 nucleotides in length.~~

~~427. The method of any of claims 1-416, wherein the 3' overhang is between about 2 and about 20 nucleotides in length.~~

~~43. The method of any of claims 1-42, wherein the 3' overhang is between about 2 and about 15 nucleotides in length, e.g., 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides in length.~~

~~448. The method of any of claims 1-437, wherein the contained reaction volume is an emulsion droplet.~~

~~459. The method of any of claims 1-448, wherein the contained reaction volume comprises one or more Type IIS restriction enzymes.~~

~~46. The method of any of claims 1-45, wherein the contained reaction volume comprises one or more polymerases.~~

~~47. The method of any of claims 1-46, wherein the contained reaction volume comprises one or more ligases, and/or~~

~~48. The method of any of claims 1-47, wherein the contained reaction volume comprises one or more nucleases other than a Type IIS restriction enzyme, e.g., one or more exonucleases and/or one or more endonucleases.~~

~~4910. The method of any of claims 1-489, wherein the second polynucleotide forms the hairpin molecule, and all or a portion of the 3' overhang hybridizes to all or a portion of the single-stranded 3' end sequence of the first subsequence to form a hybridization complex.~~

~~50. The method of claim 49, wherein the hybridization complex comprises (i) a nick or gap between the 3' end of the first polynucleotide and the 5' end of the second polynucleotide, and (ii) a nick or gap between the 5' end of the first polynucleotide and the 3' end of the second polynucleotide.~~

~~51. The method of claim 49 or 50, wherein a polymerase is capable of extending the 3' end sequence of the first subsequence in the hybridization complex using the second polynucleotide as template.~~

~~52. The method of claim 49 or 50, wherein a polymerase is incapable of extending the 3' end sequence of the first subsequence in the hybridization complex using the second polynucleotide as template, e.g., when the hybridization complex comprises two nicks, one on each strand, that are between about 1 and about 10 nucleotides apart, e.g., between about 1 and about 6 nucleotides apart.~~

~~53. The method of claim 52, wherein the nick or gap between the 5' end of the first polynucleotide and the 3' end of the second polynucleotide is filled, e.g., by ligation of the nick, or by hybridization of a filler sequence to fill in the gap followed by ligation of the filler sequence.~~

~~54. The method of claim 52 or 53, wherein the nick between the 5' end of the first polynucleotide and the 3' end of the second polynucleotide is ligated by a ligase, whereas the nick between the 3' end of the first polynucleotide and the 5' end of the second polynucleotide is not ligated by the ligase, e.g., wherein the 5' end of the second polynucleotide is blocked from ligation, e.g., wherein the 5' end nucleotide of the second polynucleotide is dephosphorylated.~~

~~55~~11. The method of any of claims 1-10, ~~claims 51-54~~, wherein a double-stranded polynucleotide comprising the first subsequence, the second subsequence, and the Type IIS restriction enzyme recognition sequence, ~~and optionally the complementary sequence~~, is generated by a polymerase that extends the 3' end sequence of the first subsequence using the second polynucleotide as template.

~~56~~12. The method of claim ~~55~~11, wherein a Type IIS restriction enzyme recognizes the Type IIS restriction enzyme recognition sequence and cleaves the double-stranded polynucleotide, thereby generating a cleaved double-stranded polynucleotide comprising the first subsequence connected to the second subsequence.

~~57~~13. The method of claim ~~56~~12, wherein the cleaved double-stranded polynucleotide comprises a single-stranded 3' end sequence.

~~58~~14. The method of claim ~~57~~13, wherein the single-stranded 3' end sequence of the cleaved double-stranded polynucleotide is between about 2 and about 10 nucleotides in length.

~~59~~. ~~The method of any of claims 1-58, wherein the plurality of polynucleotides further comprise a third polynucleotide.~~

~~60~~15. The method of any of claims 1-14, ~~claim 59~~, wherein the plurality of polynucleotides further comprise a ~~the~~ third polynucleotides that is attached to the support and comprises, in the 3' to 5' direction:

- (i) a single-stranded 3' end sequence,
- (ii) a third subsequence of the target polynucleotide,
- (iii) a Type IIS restriction enzyme recognition sequence, and
- (iv) a complementary sequence capable of hybridizing to all or a portion of the third subsequence,

wherein the third polynucleotide is capable of forming a hairpin molecule comprising a 3' overhang, a stem formed by intramolecular nucleotide base pairing between all or a portion of the third subsequence and the complementary sequence, and a loop, wherein the hairpin molecule is in a configuration that is not cleaved by a Type IIS restriction enzyme, and

wherein the first, second, and third polynucleotides are connected sequentially within the contained reaction volume, thereby assembling the first, second, and third subsequences.

~~61~~16. The method of any of claims 1-~~60~~15, wherein the support comprises a particle, a bead, a solid substrate, a plate, a well, an array, a membrane, or a combination thereof.

~~62~~17. The method of any of claims 1-~~61~~16, wherein the target polynucleotide is at least about 100, about 250, about 500, about 1,000, about 2,500, about 5,000, about 10,000, about 25,000, or about 50,000 nucleotides in length.

~~63.~~—The method of any of claims 1-~~62~~, wherein the plurality of polynucleotides comprise 3, 4, 5, 6, 7, 8, 9, 10 or more polynucleotides each comprising a subsequence of the target polynucleotide.

~~64~~18. The method of any of claims 1-~~63~~17, wherein the target polynucleotide is a DNA molecule, ~~and the target polynucleotide optionally comprises a gene or fragment thereof, a gene cluster, a mitochondrial DNA or fragment thereof, a chromosome or fragment thereof, or a genome.~~

~~65.~~—The method of any of claims 1-~~64~~, wherein the first polynucleotide and/or the second polynucleotide further comprise a capture tag sequence, an amplification site, and a UMI, wherein the UMI sequence is complementary to the capture tag sequence and/or the amplification site.

~~66~~19. A method of assembling a plurality of target polynucleotides, comprising:
(a) for each target polynucleotide, partitioning a plurality of polynucleotides into a contained reaction volume, wherein:

the plurality of polynucleotides comprise a first polynucleotide and a second polynucleotide, wherein the second polynucleotide is attached to a support,

the first polynucleotide comprises a first subsequence of the target polynucleotide, wherein the first polynucleotide comprises a single-stranded 3' end sequence,

the second polynucleotide comprises, in the 3' to 5' direction:

(i) a single-stranded 3' end sequence,

- (ii) a second subsequence of the target polynucleotide,
- (iii) a Type IIS restriction enzyme recognition sequence, and
- (iv) a complementary sequence capable of hybridizing to all or a portion of the second subsequence, and

the second polynucleotide is capable of forming a hairpin molecule comprising a 3' overhang, a stem formed by intramolecular nucleotide base pairing between all or a portion of the second subsequence and the complementary sequence, and a loop, wherein the hairpin molecule is in a configuration that is not cleaved by a Type IIS restriction enzyme; and

- (b) within each contained reaction volume, connecting the first and second polynucleotides, thereby assembling the first and second subsequences, wherein the assembly of subsequences of each target polynucleotide is carried out in parallel.

~~67. The method of claim 66, further comprising designing and/or obtaining the plurality of polynucleotides for each target polynucleotide.~~

~~68~~20. The method of claim 19, ~~claim 66 or 67~~, wherein the subsequences in the plurality of polynucleotides for each target polynucleotide are between about 20 and about 200 nucleotides in length.

~~69~~21. The method of claim 19 or 20, ~~any of claims 66-68~~, wherein the plurality of polynucleotides for each target polynucleotide are synthesized, and the synthesis comprises base-by-base synthesis.

~~70. The method of any of claims 66-69, wherein the partitioning comprises enriching polynucleotides comprising subsequences of a given target polynucleotide, but not polynucleotides comprising subsequences of other target polynucleotides, in the contained reaction volume.~~

~~71~~22. The method of any of claims 19-21, ~~claims 66-70~~, wherein the partitioning comprises capturing all or a subset of the plurality of polynucleotides for each target polynucleotide on a bead that is specific for the target polynucleotide.

~~72~~23. The method of claim ~~71~~22, wherein the bead comprises a capture probe that specifically binds to a capture tag that is unique for the target polynucleotide, wherein the capture tag is common in all or a subset of the plurality of polynucleotides comprising subsequences of the target polynucleotide.

~~73~~24. The method of claim ~~71~~22 or ~~72~~23, wherein the partitioning comprises encapsulating the bead in an emulsion droplet, thereby generating a plurality of emulsion droplets for parallel assembly of the plurality of target polynucleotides.

~~74. The method of claim 73, further comprising releasing all or a subset of the polynucleotides captured on the beads into the emulsion droplets.~~

~~75~~25. The method of ~~claim 24, claim 73 or 74,~~ wherein the parallel assembly of the plurality of target polynucleotides is carried out in each emulsion droplet by one or more concerted reaction cycles.

~~76~~26. The method of claim ~~75~~25, wherein the one or more concerted reaction cycles comprise an isothermal reaction.

~~77~~27. The method of claim ~~75~~25 or ~~76~~26, wherein the one or more concerted reaction cycles comprise sequential reactions of hybridization, ligation by a ligase, primer extension by a polymerase, and cleavage by a Type IIS restriction enzyme.

~~78. The method of any of claims 66-77, wherein the assembly of all or a subset of the plurality of target polynucleotides is unidirectional.~~

~~79. The method of any of claims 66-78, wherein the assembly of all or a subset of the plurality of target polynucleotides is bidirectional.~~

~~80~~28. A method of assembling a target polynucleotide, comprising:
(a) partitioning a plurality of polynucleotides into an emulsion droplet, wherein:
the plurality of polynucleotides comprise: (i) a first polynucleotide ~~optionally attached to a bead,~~ and (ii) a second polynucleotide attached to a ~~the~~ bead,
the first polynucleotide comprises a first subsequence of a target polynucleotide, wherein the first polynucleotide comprises a single-stranded 3' end sequence,

the second polynucleotide comprises, in the 3' to 5' direction:

- (i) a single-stranded 3' end sequence capable of hybridizing to the single-stranded 3' end sequence of the first polynucleotide,
- (ii) a second subsequence of the target polynucleotide,
- (iii) a Type IIS restriction enzyme recognition sequence, and
- (iv) a complementary sequence capable of hybridizing to all or a portion of the second subsequence, and

the second polynucleotide further comprises a tag sequence and/or a barcode sequence 5' to the Type IIS restriction enzyme recognition sequence;

(b) in the emulsion droplet, releasing the second polynucleotide from the bead, wherein the second polynucleotide forms a hairpin molecule comprising a 3' overhang, a stem formed by intramolecular nucleotide base pairing between all or a portion of the second subsequence and the complementary sequence, and a loop, wherein the hairpin molecule is in a configuration that is not cleaved by a Type IIS restriction enzyme;

(c) allowing the 3' overhang of the hairpin molecule to hybridize to the single-stranded 3' end sequence of the first polynucleotide, wherein the 5' end of the hairpin molecule is ~~optionally~~ blocked from ligation to the 3' end of the first polynucleotide after hybridization;

(d) ~~optionally~~ ligating the 3' end of the hairpin molecule to the 5' end of the first polynucleotide;

(e) extending the 3' end sequence of the first polynucleotide using the second polynucleotide as template, thereby generating a double-stranded polynucleotide comprising the first subsequence, the second subsequence, and the Type IIS restriction enzyme recognition sequence; ~~and, and optionally the complementary sequence, the tag sequence, and/or the barcode sequence; and~~

(f) cleaving the double-stranded polynucleotide using a Type IIS restriction enzyme, thereby generating a cleaved double-stranded polynucleotide comprising the first subsequence and the second subsequence, wherein the cleaved double-stranded polynucleotide comprises a single-stranded 3' end sequence, ~~and optionally wherein the single-stranded 3' end sequence is between about 2 and about 10 nucleotides in length,~~ thereby assembling the first and second subsequences.

~~81.—The method of claim 80, wherein the first polynucleotide is attached to the bead prior to the partitioning step.~~

~~82.—The method of claim 80, wherein the partitioning step comprises attaching the first polynucleotide and the second polynucleotide to the bead, and the releasing step optionally comprises releasing the first polynucleotide from the bead.~~

~~83.—The method of any of claims 80–82, wherein the first polynucleotide and/or the second polynucleotide are directly or indirectly attached to the bead.~~

~~84.—The method of any of claims 80–83, wherein the first polynucleotide and/or the second polynucleotide are covalently or noncovalently attached to the bead or a linker, e.g., a cleavable linker.~~

~~85.—The method of any of claims 80–84, wherein the first polynucleotide and/or the second polynucleotide are attached to the bead via hybridization (e.g., between a capture probe sequence directly or indirectly on the bead and a capture tag sequence of the first polynucleotide and/or the second polynucleotide), the interaction between a binding pair (e.g., biotin/streptavidin binding), a covalent bond, or any combination thereof.~~

~~86.—The method of claim 80, wherein the first polynucleotide is not attached to the bead prior to, during, or after the partitioning step.~~

~~87.—The method of claim 86, wherein the first polynucleotide is provided in a reaction volume that is partitioned to form the emulsion droplet.~~

~~88.—The method of claim 87, wherein the reaction volume further comprises a ligase, a polymerase, a Type IIS restriction enzyme, and/or a nuclease other than a Type IIS restriction enzyme.~~

~~89.—The method of any of claims 80–88, wherein the first polynucleotide comprises a hairpin.~~

~~90.—The method of claim 89, wherein the first polynucleotide comprises a stem comprising all or a portion of the first subsequence and a loop comprising a tag sequence and/or a barcode sequence.~~

~~91. The method of any of claims 80-90, wherein:
in the partitioning step, the plurality of polynucleotides further comprise (iii) a third polynucleotide attached to the bead,
the third polynucleotide comprises, in the 3' to 5' direction:
(i) a single stranded 3' end sequence capable of hybridizing to the single stranded 3' end sequence of the cleaved double stranded polynucleotide,
(ii) a third subsequence of the target polynucleotide,
(iii) a Type IIS restriction enzyme recognition sequence, and
(iv) a complementary sequence capable of hybridizing to all or a portion of the third subsequence, and
the third polynucleotide further comprises a tag sequence and/or a barcode sequence 5' to the Type IIS restriction enzyme recognition sequence.~~

~~92. The method of claim 91, wherein:
the releasing step further comprises releasing the third polynucleotide from the bead, wherein the third polynucleotide forms a hairpin molecule comprising a 3' overhang, a stem formed by intramolecular nucleotide base pairing between all or a portion of the third subsequence and the complementary sequence, and a loop, wherein the hairpin molecule is in a configuration that is not cleaved by a Type IIS restriction enzyme.~~

~~93. The method of claim 92, further comprising:
(g) hybridizing the 3' overhang of the hairpin molecule formed by the third polynucleotide to the single stranded 3' end sequence of the cleaved double stranded polynucleotide, wherein the 5' end of the hairpin molecule formed by the third polynucleotide is blocked from ligation to the 3' end of the first polynucleotide after hybridization.~~

~~94. The method of claim 93, further comprising:
(h) ligating the 3' end of the hairpin molecule formed by the third polynucleotide to the 5' end of the cleaved double stranded polynucleotide.~~

~~95. The method of claim 94, further comprising:
(i) extending the 3' end sequence of the cleaved double stranded polynucleotide using the third polynucleotide as template, thereby generating a double stranded~~

polynucleotide comprising the first subsequence, the second subsequence, the third subsequence, the Type IIS restriction enzyme recognition sequence of the third polynucleotide, and optionally the complementary sequence, the tag sequence, and/or the barcode sequence of the third polynucleotide.

96. — The method of claim 95, further comprising:

(j) cleaving the double-stranded polynucleotide using a Type IIS restriction enzyme, thereby generating a cleaved double-stranded polynucleotide comprising the first subsequence, the second subsequence, and the third subsequence, wherein the cleaved double-stranded polynucleotide comprises a single-stranded 3' end sequence, and optionally wherein the single-stranded 3' end sequence is between about 2 and about 10 nucleotides in length,

thereby assembling the first, second, and third subsequences.

97. — The method of any of claims 80-96, wherein:

in the partitioning step, the plurality of polynucleotides further comprise an n^{th} polynucleotide attached to the bead, wherein n is an integer of 4 or greater,

the n^{th} polynucleotide comprises, in the 3' to 5' direction:

(i) a single-stranded 3' end sequence capable of hybridizing to the single-stranded 3' end sequence of a cleaved double-stranded polynucleotide comprising the first, second, ..., and the $(n-1)^{\text{th}}$ subsequences of the target polynucleotide,

(ii) an n^{th} subsequence of the target polynucleotide,

(iii) a Type IIS restriction enzyme recognition sequence, and

(iv) a complementary sequence capable of hybridizing to all or a portion of the n^{th} subsequence, and

the n^{th} polynucleotide further comprises a tag sequence and/or a barcode sequence 5' to the Type IIS restriction enzyme recognition sequence.

98. — The method of claim 97, wherein:

the releasing step further comprises releasing the n^{th} polynucleotide from the bead, wherein the n^{th} polynucleotide forms a hairpin molecule comprising a 3' overhang, a stem formed by intramolecular nucleotide base pairing between all or a portion of the n^{th}

~~subsequence and the complementary sequence, and a loop, wherein the hairpin molecule is in a configuration that is not cleaved by a Type IIS restriction enzyme.~~

~~99. The method of claim 98, further comprising repeating a concerted reaction cycle comprising sequential reactions of hybridization, ligation by a ligase, primer extension by a polymerase, and cleavage by a Type IIS restriction enzyme, thereby assembling the first, second, ..., and the $(n-1)^{\text{th}}$ subsequences.~~

10029. A method of assembling a target polynucleotide, comprising:

(a) partitioning a plurality of polynucleotides into an emulsion droplet, wherein:

the plurality of polynucleotides comprise: (i) a first polynucleotide, ~~optionally attached to a bead~~, (ii) a second polynucleotide attached to a ~~the~~ bead, and (iii) a third polynucleotide attached to the bead,

the first polynucleotide comprises a first subsequence of a target polynucleotide and is double-stranded, comprising a single-stranded 3' end sequence in the top strand and a single-stranded 3' end sequence in the bottom strand,

the second polynucleotide comprises, in the 3' to 5' direction:

(i) a single-stranded 3' end sequence capable of hybridizing to the top strand single-stranded 3' end sequence of the first polynucleotide,

(ii) a second subsequence of the target polynucleotide,

(iii) a Type IIS restriction enzyme recognition sequence, and

(iv) a complementary sequence capable of hybridizing to all or a portion of the second subsequence,

~~the second polynucleotide optionally further comprises a tag sequence and/or a barcode sequence 5' to the Type IIS restriction enzyme recognition sequence,~~

the third polynucleotide comprises, in the 3' to 5' direction:

(i) a single-stranded 3' end sequence capable of hybridizing to the bottom strand single-stranded 3' end sequence of the first polynucleotide,

(ii) a third subsequence of the target polynucleotide,

(iii) a Type IIS restriction enzyme recognition sequence, and

(iv) a complementary sequence capable of hybridizing to all or a portion of the third subsequence,

~~the third polynucleotide optionally further comprises a tag sequence and/or a barcode sequence 5' to the Type IIS restriction enzyme recognition sequence;~~

(b) in the emulsion droplet, releasing the second and third polynucleotides, ~~and optionally the first polynucleotide,~~ from the bead, wherein:

the second polynucleotide forms a hairpin molecule comprising a 3' overhang, a stem formed by intramolecular nucleotide base pairing between all or a portion of the second subsequence and the complementary sequence, and a loop, wherein the hairpin molecule is in a configuration that is not cleaved by a Type IIS restriction enzyme, and

the third polynucleotide forms a hairpin molecule comprising a 3' overhang, a stem formed by intramolecular nucleotide base pairing between all or a portion of the third subsequence and the complementary sequence, and a loop, wherein the hairpin molecule is in a configuration that is not cleaved by a Type IIS restriction enzyme;

(c) allowing the 3' overhangs of the hairpin molecules formed by the second and third polynucleotides to hybridize to the top strand single-stranded 3' end sequence and the bottom strand single-stranded 3' end sequence, respectively, of the first polynucleotide, wherein the 5' ends of the hairpin molecules are blocked from ligation to the 3' ends of the first polynucleotide after hybridization;

(d) ligating the 3' ends of the hairpin molecules to the 5' ends of the first polynucleotide;

(e) extending the 3' end sequences of the first polynucleotide using the second and third polynucleotides as template, thereby generating a double-stranded polynucleotide comprising the first subsequence flanked by the second subsequence on one side and the third subsequence on the other side, and the Type IIS restriction enzyme recognition sequences; and, ~~and optionally the complementary sequences, the tag sequence(s), and/or the barcode sequence(s); and~~

(f) cleaving the double-stranded polynucleotide using a Type IIS restriction enzyme, thereby generating a cleaved double-stranded polynucleotide comprising the first subsequence flanked by the second subsequence on one side and the third subsequence on the other side, wherein the cleaved double-stranded polynucleotide comprises a single-stranded 3' end sequence in the top strand and a single-stranded 3' end sequence in the bottom strand, ~~and optionally wherein the single-stranded 3' end sequences are between about 2 and about 10 nucleotides in length,~~

thereby assembling the first, second, and third subsequences.

~~101.—The method of claim 100, wherein:~~

~~in the partitioning step, the plurality of polynucleotides further comprise a fourth polynucleotide attached to the bead and optionally a fifth polynucleotide attached to the bead,~~

~~the fourth polynucleotide comprises, in the 3' to 5' direction:~~

- ~~(i) a single-stranded 3' end sequence capable of hybridizing to the top strand single-stranded 3' end sequence of the cleaved double-stranded polynucleotide,~~
- ~~(ii) a fourth subsequence of the target polynucleotide,~~
- ~~(iii) a Type IIS restriction enzyme recognition sequence, and~~
- ~~(iv) a complementary sequence capable of hybridizing to all or a portion of the fourth subsequence, and~~

~~the fourth polynucleotide optionally further comprises a tag sequence and/or a barcode sequence 5' to the Type IIS restriction enzyme recognition sequence,~~

~~the optional fifth polynucleotide comprises, in the 3' to 5' direction:~~

- ~~(i) a single-stranded 3' end sequence capable of hybridizing to the bottom strand single-stranded 3' end sequence of the cleaved double-stranded polynucleotide,~~

- ~~(ii) a fifth subsequence of the target polynucleotide,~~
- ~~(iii) a Type IIS restriction enzyme recognition sequence, and~~
- ~~(iv) a complementary sequence capable of hybridizing to all or a portion of the fifth subsequence, and~~

~~the fifth polynucleotide optionally further comprises a tag sequence and/or a barcode sequence 5' to the Type IIS restriction enzyme recognition sequence.~~

~~102.—The method of claim 101, wherein:~~

~~the releasing step further comprises releasing the fourth and fifth polynucleotides from the bead, wherein the fourth polynucleotide forms a hairpin molecule comprising a 3' overhang, a stem formed by intramolecular nucleotide base pairing between all or a portion of the fourth subsequence and the complementary sequence, and a loop, wherein the hairpin molecule is in a configuration that is not cleaved by a Type IIS restriction enzyme, and~~

the fifth polynucleotide forms a hairpin molecule comprising a 3' overhang, a stem formed by intramolecular nucleotide base pairing between all or a portion of the fifth subsequence and the complementary sequence, and a loop comprising the Type IIS restriction enzyme recognition sequence in a configuration that is not cleaved by a Type IIS restriction enzyme.

103.—The method of claim 102, further comprising:

(g) hybridizing the 3' overhangs of the hairpin molecules formed by the fourth and fifth polynucleotides to the top strand single stranded 3' end sequence and the bottom strand single stranded 3' end sequence, respectively, of the cleaved double stranded polynucleotide, wherein the 5' ends of the hairpin molecules are blocked from ligation to the 3' ends of the cleaved double stranded polynucleotide after hybridization.

104.—The method of claim 103, further comprising:

(h) ligating the 3' ends of the hairpin molecules formed by the fourth and fifth polynucleotides to the 5' ends of the cleaved double stranded polynucleotide.

105.—The method of claim 104, further comprising:

(i) extending the 3' end sequences of the cleaved double stranded polynucleotide using the fourth and fifth polynucleotides as template, thereby generating a double stranded polynucleotide comprising: the first subsequence flanked by the second subsequence on one side and the third subsequence on the other side, which are in turn flanked by the fourth subsequence and the fifth subsequence, respectively; the Type IIS restriction enzyme recognition sequences of the fourth and fifth polynucleotides; and optionally the complementary sequences, the tag sequence(s), and/or the barcode sequence(s) of the fourth and fifth polynucleotides.

106.—The method of claim 105, further comprising:

(j) cleaving the double stranded polynucleotide using a Type IIS restriction enzyme, thereby generating a cleaved double stranded polynucleotide comprising the first subsequence flanked by the second subsequence on one side and the third subsequence on the other side, which are in turn flanked by the fourth subsequence and the fifth subsequence, respectively, wherein the cleaved double stranded polynucleotide comprises a single stranded 3' end sequence in the top strand and a single stranded 3' end sequence in the

~~bottom strand, and optionally wherein the single-stranded 3' end sequences are between about 2 and about 10 nucleotides in length,~~

~~thereby assembling the first, second, third, fourth, and fifth subsequences.~~

10730. A method of assembling a target polynucleotide, comprising:

(a) partitioning a plurality of polynucleotides into an emulsion droplet, wherein:

the plurality of polynucleotides comprise: (i) a first polynucleotide ~~optionally attached to a bead,~~ and (ii) a second polynucleotide attached to a ~~the~~ bead,

the first polynucleotide comprises a first subsequence of a target polynucleotide, wherein the first polynucleotide comprises a single-stranded 3' end sequence,

the second polynucleotide comprises, in the 3' to 5' direction:

(i) a single-stranded 3' end sequence capable of hybridizing to the single-stranded 3' end sequence of the first polynucleotide,

(ii) a second subsequence of the target polynucleotide,

(iii) a Type IIS restriction enzyme recognition sequence, and

(iv) a complementary sequence capable of hybridizing to all or a portion of the second subsequence, and

the second polynucleotide further comprises a tag sequence and/or a barcode sequence 5' to the Type IIS restriction enzyme recognition sequence;

(b) in the emulsion droplet, releasing the second polynucleotide from the bead, wherein the second polynucleotide forms a hairpin molecule comprising a 3' overhang, a stem formed by intramolecular nucleotide base pairing between all or a portion of the second subsequence and the complementary sequence, and a loop, wherein the hairpin molecule is in a configuration that is not cleaved by a Type IIS restriction enzyme;

(c) allowing the 3' overhang of the hairpin molecule to hybridize to the single-stranded 3' end sequence of the first polynucleotide to form a hybridization complex, wherein:

the 5' end of the hairpin molecule is blocked from ligation to the 3' end of the first polynucleotide after hybridization, and

the hybridization complex comprises (i) a nick or gap between the 3' end of the first polynucleotide and the 5' end of the second polynucleotide, and (ii) a nick or gap between the 5' end of the first polynucleotide and the 3' end of the second polynucleotide,

~~optionally wherein the nicks and gaps are more than about 6-10 nucleotides apart;~~

(d) extending the 3' end sequence of the first polynucleotide using the second polynucleotide as template, thereby generating a double-stranded polynucleotide comprising the first subsequence, the second subsequence, and the Type IIS restriction enzyme recognition sequence; ~~and, and optionally the complementary sequence, the tag sequence, and/or the barcode sequence; and~~

(e) cleaving the double-stranded polynucleotide using a Type IIS restriction enzyme, thereby generating a cleaved double-stranded polynucleotide comprising the first subsequence and the second subsequence, wherein the cleaved double-stranded polynucleotide comprises a single-stranded 3' end sequence, ~~and optionally wherein the single-stranded 3' end sequence is between about 2 and about 10 nucleotides in length,~~ thereby assembling the first and second subsequences.

~~108. The method of claim 107, wherein the emulsion droplet comprises a ligase, a polymerase, and a Type IIS restriction enzyme, and optionally a nuclease other than a Type IIS restriction enzyme.~~

~~109~~1. A method, comprising contacting a pool of polynucleotides with a library of beads, wherein:

the pool of polynucleotides comprises polynucleotide sets P11, ..., and P1j₁; ...; Pk1, ..., and Pkj_k; ...; and Pi1, ..., and Pij_i, wherein *i*, *j*₁, ..., *j*_k, ..., *j*_i, and *k* are integers, *i*, *j*₁, ..., *j*_k, ..., and *j*_i are independently 2 or greater, and $1 \leq k \leq i$,

Pk1, ..., and Pkj_k comprise subsequences Sk1, ..., and Skj_k, respectively, which form target sequence S'_k,

at least one of Pk1, ..., and Pkj_k comprises, in the 3' to 5' direction:

(i) a single-stranded 3' end sequence,

(ii) the subsequence of target sequence S'_k,

(iii) a Type IIS restriction enzyme recognition sequence, and

(iv) a complementary sequence capable of hybridizing to all or a portion of the subsequence of target sequence S'_k,

the at least one of Pk1, ..., and Pkj_k further comprises a tag Tk in all or a subset of Pk1, ..., and Pkj_k, and

the at least one of P_{k1} , ..., and P_{kj_k} is capable of forming a hairpin molecule comprising a 3' overhang, a stem formed by intramolecular nucleotide base pairing between all or a portion of the subsequence of target sequence S'_k and the complementary sequence, and a loop, wherein the hairpin molecule is in a configuration that is not cleaved by a Type IIS restriction enzyme;

beads B_1 , ..., B_k , ..., and B_i in the library comprise capture moieties C_1 , ..., C_k , ..., and C_i , respectively, that specifically binds to tags T_1 , ..., T_k , ..., and T_i , respectively, thereby specifically capturing the at least one of P_{k1} , ..., and P_{kj_k} on one of the beads in the library.

~~110.—The method of claim 109, further comprising placing all or a subset of the beads in emulsion droplets, one bead per emulsion droplet.~~

~~111.—The method of claim 110, further comprising releasing all or a subset of the polynucleotides captured on each of all or a subset of the beads in the emulsion droplets.~~

~~112.—The method of claim 111, further comprising within each emulsion droplet, connecting two or more of P_{k1} , ..., and P_{kj_k} , thereby assembling two or more of subsequences S_{k1} , ..., and S_{kj_k} , in the emulsion droplet.~~

~~113.—The method of claim 112, wherein P_{k1} , ..., and P_{kj_k} are assembled in the emulsion droplet by one or more concerted reaction cycles.~~

~~114.—The method of claim 113, wherein the one or more concerted reaction cycles comprise an isothermal reaction.~~

~~115.—The method of claim 113 or 114, wherein the one or more concerted reaction cycles comprise sequential reactions of hybridization, ligation by a ligase, primer extension by a polymerase, and cleavage by a Type IIS restriction enzyme.~~

~~116.—The method of any of claims 113–115, wherein the one or more concerted reaction cycles comprise sequential assembly of all or a subset of P_{k1} , ..., and P_{kj_k} in a predetermined order.~~

~~117. The method of any of claims 112-116, wherein subsequence sets $S_{11}, \dots$, and $S_{1j_1}, \dots$; $S_{k1}, \dots$, and $S_{kj_k}, \dots$; and $S_{i1}, \dots$, and S_{ij_i} comprise one or more common subsequences among two or more of the subsequence sets.~~

~~118. The method of any of claims 112-117, wherein polynucleotide sets $P_{11}, \dots$, and $P_{1j_1}, \dots$; $P_{k1}, \dots$, and $P_{kj_k}, \dots$; and $P_{i1}, \dots$, and P_{ij_i} comprise one or more common polynucleotides among two or more of the polynucleotide sets.~~

~~119. The method of any of claims 112-116, wherein subsequence sets $S_{11}, \dots$, and $S_{1j_1}, \dots$; $S_{k1}, \dots$, and $S_{kj_k}, \dots$; and $S_{i1}, \dots$, and S_{ij_i} do not contain a common subsequence.~~

~~120. The method of any of claims 112-119, wherein $P_{k1}, \dots$, and P_{kj_k} are assembled to form target sequence S'_k or a portion thereof.~~

~~121. The method of any of claims 112-120, wherein polynucleotide sets $P_{11}, \dots$, and $P_{1j_1}, \dots$; $P_{k1}, \dots$, and $P_{kj_k}, \dots$; and $P_{i1}, \dots$, and P_{ij_i} are assembled to form target sequences $S'_1, \dots$, $S'_k, \dots$, and S'_i or a portion thereof, respectively, in parallel.~~

~~122. The method of any of claims 112-121, further comprising breaking the emulsion droplets and pooling all or a subset of the assembled target sequences or portions thereof.~~

~~123. The method of any of claims 112-122, wherein all or a subset of the assembled target sequences or portions thereof are subjected to further assembly.~~

~~124. The method of claim 123, wherein the further assembly comprises higher order assembly of all or a subset of the assembled target sequences or portions thereof.~~

~~125. The method of claim 123 or 124, wherein the further assembly comprises polymerase cycling assembly (PCA), sequence and ligation independent cloning (SLIC), Golden Gate assembly, Gibson assembly, *in vivo* assembly, or any combination thereof.~~

~~126. The method of any of claims 1-125, wherein the target sequence comprises a sequence difficult to synthesize, difficult to amplify, and/or difficult to sequence verify.~~

~~127. The method of any of claims 1-126, wherein the target sequence comprises a sequence difficult to synthesize base-by-base.~~

~~128. The method of any of claims 1-127, wherein the target sequence comprises a homopolymer sequence, *e.g.*, A_n; a homocopolymer sequence, *e.g.*, [AT]_n; a sequence comprising direct repeats; an AT-rich sequence; a GC-rich sequence, or any combination thereof.~~