

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, andthe 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 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.
5. The method of any of claims 1-4, wherein the 5' end of the second polynucleotide is blocked from ligation, extension, and/or hybridization.

6. The method of any of claims 1-5, wherein the 3' overhang is between about 1 and about 100 nucleotides in length.
7. The method of any of claims 1-6, wherein the 3' overhang is between about 2 and about 20 nucleotides in length.
8. The method of any of claims 1-7, wherein the contained reaction volume is an emulsion droplet.
9. The method of any of claims 1-8, wherein the contained reaction volume comprises one or more Type IIS restriction enzymes, one or more polymerases, one or more ligases, and/or one or more nucleases other than a Type IIS restriction enzyme.
10. The method of any of claims 1-9, 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.
11. The method of any of claims 1-10, wherein a double-stranded polynucleotide comprising the first subsequence, the second subsequence, and the Type IIS restriction enzyme recognition sequence is generated by a polymerase that extends the 3' end sequence of the first subsequence using the second polynucleotide as template.
12. The method of claim 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.
13. The method of claim 12, wherein the cleaved double-stranded polynucleotide comprises a single-stranded 3' end sequence.
14. The method of claim 13, wherein the single-stranded 3' end sequence of the cleaved double-stranded polynucleotide is between about 2 and about 10 nucleotides in length.

15. The method of any of claims 1-14, wherein the plurality of polynucleotides further comprise a third polynucleotide 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.

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

17. The method of any of claims 1-16, 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.

18. The method of any of claims 1-17, wherein the target polynucleotide is a DNA molecule.

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.

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

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

22. The method of any of claims 19-21, 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.

23. The method of claim 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.

24. The method of claim 22 or 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.

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

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

27. The method of claim 25 or 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.

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 and (ii) a second polynucleotide attached to a 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 blocked from ligation to the 3' end of the first polynucleotide after hybridization;

(d) 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

(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, thereby assembling the first and second subsequences.

29. 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, (ii) a second polynucleotide attached to a 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 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,
- (b) in the emulsion droplet, releasing the second and third polynucleotides 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
- (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,
- thereby assembling the first, second, and third subsequences.

30. 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 and (ii) a second polynucleotide attached to a 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,

(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

(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, thereby assembling the first and second subsequences.

31. A method, comprising contacting a pool of polynucleotides with a library of beads, wherein:

the pool of polynucleotides comprises polynucleotide sets $P1_1, \dots, \text{and } P1_{j_1}; \dots; Pk_1, \dots, \text{and } Pk_{j_k}; \dots; \text{and } Pi_1, \dots, \text{and } Pij_i$, wherein $i, j_1, \dots, j_k, \dots, j_i$, and k are integers, $i, j_1, \dots, j_k, \dots, \text{and } j_i$ are independently 2 or greater, and $1 \leq k \leq i$,

$Pk_1, \dots, \text{and } Pk_{j_k}$ comprise subsequences $Sk_1, \dots, \text{and } Sk_{j_k}$, respectively, which form target sequence $S'k$,

at least one of $Pk_1, \dots, \text{and } Pk_{j_k}$ 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 $Pk_1, \dots, \text{and } Pk_{j_k}$ further comprises a tag Tk in all or a subset of $Pk_1, \dots, \text{and } Pk_{j_k}$, and

the at least one of $Pk_1, \dots, \text{and } Pk_{j_k}$ 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 $B1, \dots, Bk, \dots, \text{and } Bi$ in the library comprise capture moieties $C1, \dots, Ck, \dots, \text{and } Ci$, respectively, that specifically binds to tags $T1, \dots, Tk, \dots, \text{and } Ti$, respectively,

thereby specifically capturing the at least one of $Pk_1, \dots, \text{and } Pk_{j_k}$ on one of the beads in the library.