

CLAIMS

1. A method of assembling a double-stranded polynucleotide having a predefined sequence, the method comprising:

(A) performing an in vitro method to synthesise a first double-stranded polynucleotide having a predefined sequence and performing the in vitro method to synthesise one or more additional double-stranded polynucleotides having a predefined sequence, in which each in vitro method for synthesising the double-stranded polynucleotides comprises performing cycles of synthesis in which in each cycle, a first strand is extended by the incorporation of a nucleotide of the predefined sequence and the second strand which is hybridized to the first strand is extended by the incorporation of a nucleotide thereby forming a nucleotide pair with the incorporated nucleotide of the first strand; in which each cycle comprises extending the first strand by incorporating the nucleotide of the predefined sequence together with an attached reversible terminator group followed by extending the second strand; further in which in each cycle the nucleotides are incorporated into a scaffold polynucleotide comprising a synthesis strand and a support strand hybridized thereto, in which the synthesis strand comprises a primer strand portion and in which each cycle comprises:

- (1) providing the scaffold polynucleotide;
- (2) incorporating into the scaffold polynucleotide by the action of polymerase a nucleotide of the predefined sequence, the nucleotide comprising a reversible terminator group which prevents further extension by polymerase;
- (3) cleaving the scaffold polynucleotide at a cleavage site;
- (4) ligating a ligation polynucleotide to the cleaved scaffold polynucleotide, the ligation polynucleotide comprising a partner nucleotide for the nucleotide of the predefined sequence, in which upon ligation the nucleotide of the predefined sequence pairs with the partner nucleotide; and
- (5) removing the reversible terminator group from the nucleotide of the predefined sequence after step (4) or removing the reversible terminator group from the nucleotide of predefined sequence after step (2) and before step (3), or after step (3) and before step (4); and

(B) joining together the first and the one or more additional polynucleotides.

2. A method according to Claim 1, in which in step (1) the support strand comprises a universal nucleotide; in which step (3) comprises cleaving the scaffold polynucleotide at a cleavage site, the site defined by a sequence comprising the universal nucleotide in the support strand, in which cleavage comprises cleaving the support strand and removing the universal nucleotide; and in which in step (4) the ligation polynucleotide comprises a support strand comprising a universal nucleotide which defines a cleavage site for use in the next cycle, and in which the ligation polynucleotide is ligated to the support strand of the cleaved scaffold polynucleotide.

3. A method according to Claim 1 or 2, the method comprising:

(1) providing the scaffold polynucleotide, in which the synthesis strand comprises a primer strand portion and a helper strand portion separated by a single-strand break, and the support strand comprises a universal nucleotide;

(2) incorporating a first nucleotide of the predefined sequence into the synthesis strand by the action of polymerase, the first nucleotide comprising a reversible terminator group which prevents further extension by polymerase;

(3) cleaving the scaffold polynucleotide at a cleavage site, the site defined by a sequence comprising the universal nucleotide in the support strand, in which cleavage comprises cleaving the support strand and removing the universal nucleotide to provide in the synthesis strand an overhanging end comprising the first nucleotide;

(4) ligating a double-stranded ligation polynucleotide to the cleaved scaffold polynucleotide, the ligation polynucleotide comprising a support strand, a helper strand and a complementary ligation end, the ligation end comprising in the support strand a universal nucleotide and a partner nucleotide for the first nucleotide which overhangs the helper strand, and in the helper strand a terminal nucleotide lacking a phosphate group, in which upon ligation of the support strands the first nucleotide pairs with the partner nucleotide,

(5) removing the reversible terminator group from the first nucleotide after step (4) and before step (6), or after step (2) and before step (3), or after step (3) and before step (4);

- (6) incorporating the next nucleotide of the predefined nucleotide sequence into the synthesis strand of the scaffold polynucleotide by the action of polymerase, the next nucleotide comprising a reversible terminator group which prevents further extension by polymerase;
- 5 (7) cleaving the scaffold polynucleotide at a cleavage site, the site defined by a sequence comprising a universal nucleotide in the support strand, in which cleavage comprises cleaving the support strand and removing the universal nucleotide to provide in the synthesis strand an overhanging end comprising the next nucleotide;
- 10 (8) ligating a double-stranded ligation polynucleotide to the cleaved scaffold polynucleotide, the ligation polynucleotide comprising a support strand, a helper strand and a complementary ligation end, the ligation end comprising in the support strand a universal nucleotide and a partner nucleotide for the next nucleotide which overhangs the helper strand, and in the helper strand a terminal nucleotide lacking a phosphate group, in which upon ligation of the support strands the next nucleotide pairs with the
- 15 partner nucleotide;
- (9) removing the reversible terminator group from the next nucleotide after step (8) and before step (10), or after step (6) and before step (7), or after step (7) and before step (8); and
- (10) repeating steps 6 to 9 multiple times to provide the double-stranded polynucleotide
- 20 having a predefined nucleotide sequence.
4. A method according to Claim 2 or 3, in which in a given synthesis cycle the universal nucleotide occupies position n in: (a) the support strand of the scaffold polynucleotide in steps 1 and 6, in which position n is the nucleotide position in the support strand which is opposite the position in the synthesis strand which will be occupied by the nucleotide of the predefined
- 25 sequence upon its incorporation in that cycle, and (b) the support strand of the ligation polynucleotide in steps 4 and 8, in which position n is the nucleotide position in the support strand which is opposite the position in the synthesis strand which will be occupied by the next nucleotide of the predefined sequence upon its incorporation in the next synthesis cycle; in which position $n-1$ is the next nucleotide position in the support strand relative to the
- 30 position occupied by the universal nucleotide in the direction distal to the helper strand/proximal to the primer strand; and in which the support strand of the scaffold polynucleotide is cleaved between positions n and $n-1$ in steps 3 and 7.

5. A method according to Claim 2 or 3, in which in a given synthesis cycle the universal nucleotide occupies position $n+1$ in: (a) the support strand of the scaffold polynucleotide in steps 1 and 6, in which position n is the nucleotide position in the support strand which is opposite the position in the synthesis strand which will be occupied by the nucleotide of the predefined sequence upon its incorporation in that cycle, and (b) the support strand of the ligation polynucleotide in steps 4 and 8, in which position n is the nucleotide position in the support strand which is opposite the position in the synthesis strand which will be occupied by the next nucleotide of the predefined sequence upon its incorporation in the next synthesis cycle; in which position $n-1$ is the next nucleotide position in the support strand relative to position n in the direction distal to the helper strand/proximal to the primer strand, and in which position $n+1$ is the next nucleotide position in the support strand relative to position n in the direction proximal to the helper strand/distal to the primer strand; and in which the support strand of the scaffold polynucleotide is cleaved between positions n and $n-1$ in steps 3 and 7.
6. A method according to Claim 2 or 3, in which in a given synthesis cycle the universal nucleotide occupies position n in: (a) the support strand of the scaffold polynucleotide in steps 1 and 6, in which position n is the nucleotide position in the support strand which is opposite the position in the synthesis strand which will be occupied by the nucleotide of the predefined sequence upon its incorporation in that cycle, and (b) the support strand of the ligation polynucleotide in steps 4 and 8, in which position n is the nucleotide position in the support strand which is opposite the position in the synthesis strand which will be occupied by the next nucleotide of the predefined sequence upon its incorporation in the next synthesis cycle; in which position $n-1$ is the next nucleotide position in the support strand relative to the position occupied by the universal nucleotide in the direction distal to the helper strand/proximal to the primer strand, and in which position $n-2$ is the next nucleotide position in the support strand relative to position $n-1$ in the direction distal to the helper strand/proximal to the primer strand; and in which the support strand of the scaffold polynucleotide is cleaved between positions $n-1$ and $n-2$ in steps 3 and 7.
7. A method according to Claim 3 or 4, in which:
 - a) in steps (1)/(6) the universal nucleotide in the support strand is positioned opposite the terminal nucleotide of the helper strand adjacent the single-strand break and is paired therewith (position n);

b) in step (2)/(6) the first/next nucleotide is incorporated into the synthesis strand at a position opposite the universal nucleotide in the support strand (position n), whereupon the first/next nucleotide pairs with the universal nucleotide;

5 c) in step (3)/(7) the support strand is cleaved at a position between the universal nucleotide position (position n) and the nucleotide next to the universal nucleotide position in the support strand (position n-1, in the direction distal to the helper strand/proximal to the primer strand), in which cleavage generates a single-nucleotide overhang in the scaffold polynucleotide comprising the first/next nucleotide overhanging the support strand; and

10 d) in step (4)/(8), the ligation end of the ligation polynucleotide comprises a single-nucleotide overhang and in which:

i. the universal nucleotide in the support strand is positioned (position n) opposite the terminal nucleotide of the helper strand and is paired therewith;

15 ii. the universal nucleotide is positioned next to the terminal nucleotide of the support strand;

iii. the terminal nucleotide of the support strand (position n-1) overhangs the terminal nucleotide of the helper strand and is the partner nucleotide for the first/next nucleotide of step (2)/(6).

8. A method according to Claim 3 or 5, in which:

20 a) in step (1) the scaffold polynucleotide is provided in the support strand with a nucleotide (position n) which is the partner nucleotide for the first nucleotide of step (2), and the universal nucleotide in the support strand is positioned next to the partner nucleotide (position n+1, in the direction proximal to the helper strand/distal to the primer strand);

25 b) in step (2)/(6) the first/next nucleotide is incorporated into the synthesis strand at the position opposite the partner nucleotide in the support strand (position n), whereupon the first/next nucleotide pairs with the partner nucleotide;

30 c) in step (3)/(7) the support strand is cleaved at a position between the first nucleotide (position n) and the second nucleotide (position n-1) from the universal nucleotide in the support strand in the direction distal to the helper strand/proximal to

the primer strand, in which cleavage removes the universal nucleotide and creates a single-nucleotide overhang in the scaffold polynucleotide comprising the first/next nucleotide overhanging the support strand;

d) in step (4)/(8), the complementary ligation end of the ligation polynucleotide comprises a single-nucleotide overhang and in which:

- i. the universal nucleotide in the support strand is positioned opposite the penultimate nucleotide of the helper strand (position $n+1$) and is paired therewith;
- ii. the universal nucleotide is positioned next to the penultimate nucleotide of the support strand (position n);

iii. the penultimate nucleotide of the support strand (position n) is paired with the terminal nucleotide of the helper strand and is a partner nucleotide for the next nucleotide in step (6) of the next synthesis cycle; and

iv. the terminal nucleotide of the support strand (position $n-1$) overhangs the terminal nucleotide of the helper strand and is a partner nucleotide for the first nucleotide of step (2), or is a partner nucleotide for the newly-incorporated nucleotide of step (6) of the current synthesis cycle.

9. A method according to Claim 3 or 6, in which:

a) in steps (1)/(6) the universal nucleotide in the support strand of the scaffold polynucleotide is positioned opposite the terminal nucleotide of the helper strand adjacent the single-strand break and is paired therewith (position n);

b) in step (2)/(6), the first/next nucleotide is incorporated into the synthesis strand at a position opposite the universal nucleotide in the support strand, whereupon the first/next nucleotide pairs with the universal nucleotide;

c) in step (3)/(7) the support strand is cleaved at a position between the first nucleotide (position $n-1$) and the second nucleotide (position $n-2$) from the universal nucleotide in the support strand in the direction distal to the helper strand/proximal to the primer strand, in which cleavage removes the universal nucleotide and creates a double-nucleotide overhang in the scaffold polynucleotide comprising the first/next nucleotide overhanging the support strand;

d) in step (4)/(8) the complementary ligation end of the ligation polynucleotide comprises a double-nucleotide overhang and in which:

- i. the universal nucleotide in the support strand is positioned (position n) opposite the terminal nucleotide of the helper strand and is paired therewith;
- 5 ii. the universal nucleotide is positioned next to the penultimate nucleotide of the support strand; and
- iii. the penultimate nucleotide of the support strand (position n-1) overhangs the terminal nucleotide of the helper strand and is the partner nucleotide for the first/next nucleotide in step (2)/(6).

10 10. A method according to any preceding claim, in which a nucleotide which pairs with a first/next nucleotide of the predefined sequence is a nucleotide which is complementary with the first/next nucleotide, preferably naturally complementary.

11. A method according to any of Claims 3 to 10, in which in step (1) and/or (6) the synthesis strand is provided without a helper strand.

15 12. A method according to any of Claims 3 to 11, in which in any one or more cycles of synthesis, or in all cycles of synthesis, after the step of ligating the double-stranded ligation polynucleotide to the cleaved scaffold polynucleotide and before the step of incorporating the next nucleotide of the predefined nucleotide sequence into the synthesis strand of the scaffold polynucleotide, the helper strand portion of the synthesis strand is removed from the scaffold
20 polynucleotide.

13. A method according to any of Claims 1 to 4, 7 and 10 to 12, in which each cleavage step comprises a first step comprising removing the universal nucleotide thus forming an abasic site, and a second step comprising cleaving the support strand at the abasic site; optionally in which the first step is performed with a nucleotide-excising enzyme, and/or in which the
25 second step is performed with an enzyme having abasic site lyase activity, optionally in which the enzyme having abasic site lyase activity is Endonuclease VIII.

14. A method according to any of Claims 1 and 10 to 12, in which the cleavage step comprises cleaving the support strand with an enzyme; optionally in which the enzyme cleaves the support strand after the nucleotide which is next to the universal nucleotide,

thereby creating the overhanging end in the synthesis strand comprising the first/next nucleotide, optionally in which the enzyme is Endonuclease V.

15. A method according to Claim 3, in which in step (1) and/or (6):

a) the helper strand and the portion of the support strand hybridized thereto are connected by a hairpin loop; and

b) the synthesis strand comprising the primer strand portion and the portion of the support strand hybridized thereto are connected by a hairpin loop;

optionally in which at least one of the ligation polynucleotides is provided as a single molecule comprising a hairpin loop connecting the support strand and the helper strand at the end opposite the overhanging end; preferably in which the ligation polynucleotides of each synthesis cycle are provided as single molecules each comprising a hairpin loop connecting the support strand and the helper strand at the end opposite the overhanging end.

16. A method according to any preceding claim, in which the synthesis strand comprising the primer strand portion and the portion of the support strand hybridized thereto are tethered to a common surface, optionally via one or more covalent bonds, preferably in which the one or more covalent bonds is formed between a functional group on the common surface and a functional group on the scaffold molecule, in which the functional group on the scaffold molecule is an amine group, a thiol group, a thiophosphate group or a thioamide group; more preferably in which the functional group on the common surface is a bromoacetyl group, optionally in which the bromoacetyl group is provided on a polyacrylamide surface derived using N- (5- bromoacetamidylpentyl) acrylamide (BRAPA).

17. A method according to any preceding claim, in which the step of removing the reversible terminator group from a nucleotide of the predefined sequence is performed before the cleavage step, or before the ligation step.

18. A method according to any preceding claim, in which synthesis cycles are performed in droplets within a microfluidic system, optionally in which the microfluidic system is an electrowetting system, preferably in which the microfluidic system is an electrowetting-on-dielectric system (EWOD).

19. A method according to any preceding claim, in which following synthesis the strands of the double-stranded polynucleotides are separated to provide a single-stranded polynucleotide having a predefined sequence, or in which following synthesis the double-stranded polynucleotide or a region thereof is amplified, preferably by PCR.
- 5 20. A method according to any preceding claim, in which the first polynucleotide and the one or more additional polynucleotides are double-stranded polynucleotides and are cleaved to create compatible termini and joined together by ligation, optionally in which the first polynucleotide and the one or more additional polynucleotides are cleaved by a restriction enzyme at a cleavage site.