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(54) Title: POLYNUCLEOTIDE SYNTHESIS METHOD, SYSTEM AND KIT

(57) Abstract: The invention relates to new methods for synthesising polynucleotide molecules according to a predefined nucleotide sequence. The invention also relates to methods for the assembly of synthetic polynucleotides following synthesis, as well as systems and kits for performing the synthesis and/or assembly methods.



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POLYNUCLEOTIDE SYNTHESIS METHOD, SYSTEM AND KIT

FIELD OF THE INVENTION

5 The invention relates to new methods for synthesising polynucleotide molecules according to a predefined nucleotide sequence. The invention also relates to methods for the assembly of synthetic polynucleotides following synthesis, as well as systems and kits for performing the synthesis and/or assembly methods.

10 **BACKGROUND TO THE INVENTION**

Two primary methods exist for the synthesis and assembly of polynucleotide molecules, particularly DNA.

15 Phosphoramidite chemistry is a synthetic approach that assembles monomers of chemically activated T, C, A or G into oligonucleotides of approximately 100/150 bases in length via a stepwise process. The chemical reaction steps are highly sensitive and the conditions alternate between fully anhydrous (complete absence of water), aqueous oxidative and acidic (Roy and Caruthers, *Molecules*, 2013, 18, 14268-14284). If the reagents from the previous reaction step have not been completely removed this will be
20 detrimental to future steps of synthesis. Accordingly, this synthesis method is limited to the production of polynucleotides of length of approximately 100 nucleotides.

 The Polymerase Synthetic approach uses a polymerase to synthesise a complementary strand to a DNA template using T, C, A and G triphosphates. The reaction conditions are aqueous and mild and this approach can be used to synthesise DNA
25 polynucleotides which are many thousands of bases in length. The main disadvantage of this method is that single- and double-stranded DNA cannot be synthesised *de novo* by this method, it requires a DNA template from which a copy is made. (Kosuri and Church, *Nature Methods*, 2014, 11, 499-507).

 Thus previous methods cannot be used to synthesise double-stranded DNA *de novo*
30 without the aid of a pre-existing template molecule which is copied.

The inventors have developed new methodologies by which single- and double-stranded polynucleotide molecules can be synthesised *de novo* in a stepwise manner without the need to copy a pre-existing template molecule. Such methods also avoid the extreme conditions associated with phosphoramidite chemistry techniques and in contrast are carried out under mild, aqueous conditions around neutral pH. Such methods also enable *de novo* synthesis of single- or double-stranded polynucleotide molecules with a potential 10^8 improvement on current synthesis methods with nucleotide lengths of >100mers to full genomes, providing a wide range of possibly applications in synthetic biology.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present disclosure as it existed before the priority date of each of the appended claims.

SUMMARY OF THE INVENTION

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

The invention provides an *in vitro* method of synthesising a double-stranded polynucleotide having a predefined sequence, the method comprising performing cycles of synthesis wherein each cycle comprises cleaving a double-stranded polynucleotide and extending the cleaved double-stranded polynucleotide by incorporating a nucleotide pair, wherein a terminal end of a first strand of the cleaved double-stranded polynucleotide is extended by the addition of a nucleotide of the predefined sequence and a terminal end of the second strand of the cleaved double-stranded polynucleotide which is hybridized to the first strand is extended by the addition of a partner nucleotide thereby forming a nucleotide pair with the incorporated nucleotide of the first strand. Preferably, the methods are for synthesising DNA.

In a first aspect, the present disclosure provides an *in vitro* method of synthesising a double-stranded polynucleotide having a predefined sequence, the method comprising performing cycles of synthesis wherein each cycle comprises cleaving a double-stranded scaffold polynucleotide and extending the cleaved double-stranded scaffold polynucleotide by incorporating a nucleotide pair, wherein a terminal end of a first strand of the cleaved

double-stranded scaffold polynucleotide is extended by the addition of a nucleotide of the predefined sequence and a terminal end of the second strand of the cleaved double-stranded scaffold polynucleotide which is hybridized to the first strand is extended by the addition of a partner nucleotide thereby forming a nucleotide pair with the incorporated nucleotide of the first strand, wherein in each cycle the nucleotides are incorporated into the cleaved scaffold polynucleotide, and wherein each cycle comprises ligating a ligation polynucleotide to the cleaved scaffold polynucleotide, the ligation polynucleotide comprising a partner nucleotide for the nucleotide of the predefined sequence, wherein upon ligation the nucleotide of the predefined sequence pairs with the partner nucleotide.

In a second aspect, the present disclosure provides a method of assembling a polynucleotide having a predefined sequence, the method comprising performing the method of the first aspect to synthesise a first polynucleotide having a predefined sequence and one or more additional polynucleotides having a predefined sequence and joining together the first and one or more additional polynucleotides; optionally wherein the first polynucleotide and the one or more additional polynucleotides are cleaved to create compatible termini and joined together, preferably by ligation; further optionally wherein the first polynucleotide and the one or more additional polynucleotides are cleaved by a restriction enzyme at a cleavage site.

In any of the methods of the invention described herein the methods may provide for the synthesis of a single-stranded polynucleotide molecule wherein following synthesis of the double-stranded polynucleotide molecule having a predefined sequence one strand of the double-stranded polynucleotide molecule is removed, or copied and/or amplified, to provide the single-stranded polynucleotide molecule.

In any of the methods of the invention described herein the methods may provide for the synthesis of a double-stranded or single-stranded oligonucleotide. Thus all references herein to the synthesis of a double-stranded or single-stranded polynucleotide

[Text continues on page 3]

using any of the methods of the invention apply *mutatis mutandis* to the synthesis of a double-stranded or single-stranded oligonucleotide.

In methods of the invention described above, each cycle may comprise extending the first strand by adding the nucleotide of the predefined sequence together with an
5 attached reversible blocking group followed by extending the second strand, wherein the reversible blocking group is removed before or after the second strand is extended. In any such method, in each cycle the nucleotides may be incorporated into a cleaved scaffold polynucleotide.

The invention provides a method as described above and herein, wherein each
10 cycle comprises:

- (1) providing a scaffold polynucleotide;
- (2) cleaving the scaffold polynucleotide at a cleavage site;
- 15 (3) adding to the cleaved scaffold polynucleotide by the action of a nucleotide transferase or polymerase enzyme a nucleotide of the predefined sequence, the nucleotide comprising a reversible terminator group which prevents further extension by the enzyme;
- 20 (4) removing the reversible terminator group from the nucleotide of the predefined sequence; and
- 25 (5) ligating a ligation polynucleotide to the cleaved scaffold polynucleotide, the ligation polynucleotide comprising a partner nucleotide for the nucleotide of the predefined sequence, wherein upon ligation the nucleotide of the predefined sequence pairs with the partner nucleotide.

In methods involving a scaffold polynucleotide the scaffold polynucleotide may be provided comprising a synthesis strand and a support strand hybridized thereto, wherein
30 the synthesis strand comprises a primer strand portion and a helper strand portion. In any

such methods the helper strand portion may be removed from the scaffold polynucleotide prior to any one, more or all cleavage steps.

In any such method involving a scaffold polynucleotide the synthesis strand may be the first strand and the support strand may be the second strand.

5 In any such method the support strand may be extended by ligating to the support strand a ligation polynucleotide, wherein in each cycle of synthesis the ligation polynucleotide comprises the nucleotide forming the nucleotide pair with the predefined nucleotide incorporated into the first strand in that cycle.

The ligation polynucleotide may be single-stranded or double-stranded. Preferably,
10 the ligation polynucleotide is double-stranded.

In methods wherein the ligation polynucleotide is double-stranded, the ligation polynucleotide may preferably comprise a support strand and a helper strand. The helper strand may be removed from the scaffold polynucleotide before the step of cleavage in the next synthesis cycle, in such methods the helper strand is removed after the ligation step.

15 The invention provides a method as described above, wherein step (1) comprises providing a scaffold polynucleotide comprising a synthesis strand and a support strand hybridized thereto, wherein the synthesis strand comprises a primer strand portion, and the support strand comprises a universal nucleotide; wherein step (2) comprises cleaving the scaffold polynucleotide at a cleavage site, the site defined by a sequence comprising the
20 universal nucleotide in the support strand, wherein cleavage comprises cleaving the support strand and removing the universal nucleotide from the scaffold polynucleotide; and wherein in step (5) the ligation polynucleotide comprises a support strand comprising the partner nucleotide and a universal nucleotide which defines a cleavage site for use in the next cycle, and wherein the ligation polynucleotide is ligated to the support strand of the
25 cleaved scaffold polynucleotide thereby forming the nucleotide pair.

The invention provides a method as described above, comprising:

(1) providing a scaffold polynucleotide comprising a synthesis strand and a support strand hybridized thereto, wherein 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;

5 (2) cleaving the scaffold polynucleotide at a cleavage site, the site defined by a
sequence comprising the universal nucleotide in the support strand, wherein
cleavage comprises cleaving the support strand and removing the universal
nucleotide from the scaffold polynucleotide to provide a cleaved double-
stranded scaffold polynucleotide comprising a support strand and a synthesis
strand comprising the primer strand portion;

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(3) extending the terminal end of the primer strand portion of the synthesis strand
of the cleaved double-stranded scaffold polynucleotide with a first nucleotide of
the predefined sequence by the action of a nucleotide transferase or polymerase
enzyme, the first nucleotide comprising a reversible terminator group which
15 prevents further extension by the enzyme;

(4) removing the terminator group from the first nucleotide;

20 (5) ligating a double-stranded ligation polynucleotide to the cleaved scaffold
polynucleotide, the ligation polynucleotide comprising a support strand and a
helper strand hybridised thereto and further comprising a complementary
ligation end, the ligation end comprising:

25 (i) in the support strand a universal nucleotide and a partner nucleotide
for the first nucleotide, wherein the partner nucleotide for the first
nucleotide overhangs the helper strand; and

(ii) in the helper strand a terminal nucleotide lacking a phosphate group;
wherein upon ligation of the support strands the first nucleotide pairs with the
partner nucleotide;

30 (6) cleaving the scaffold polynucleotide at a cleavage site, the site defined by a
sequence comprising the universal nucleotide in the support strand, wherein

cleavage comprises cleaving the support strand and removing the universal nucleotide from the scaffold polynucleotide to provide a cleaved double-stranded scaffold polynucleotide comprising a support strand and a synthesis strand comprising a primer strand portion;

5

(7) extending the terminal end of the primer strand portion of the synthesis strand of the cleaved double-stranded scaffold polynucleotide with the next nucleotide of the predefined nucleotide sequence by the action of a nucleotide transferase or polymerase enzyme, the next nucleotide comprising a reversible terminator group which prevents further extension by the enzyme;

10

(8) removing the terminator group from the next nucleotide; and

(9) ligating a double-stranded ligation polynucleotide to the cleaved scaffold polynucleotide, the ligation polynucleotide comprising a support strand and a helper strand hybridised thereto and further comprising a complementary ligation end, the ligation end comprising:

15

(i) in the support strand a universal nucleotide and a partner nucleotide for the next nucleotide, wherein the partner nucleotide for the next nucleotide overhangs the helper strand; and

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(ii) in the helper strand a terminal nucleotide lacking a phosphate group;

wherein upon ligation of the support strands the next nucleotide pairs with the partner nucleotide;

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(10) repeating steps 6 to 9 multiple times to provide the double-stranded polynucleotide having a predefined nucleotide sequence.

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Any such method as described above may be performed according to a method of the invention designated synthesis method version 1 wherein:

- 5 a) prior to and at the cleavage step of the first cycle (step 2) the universal nucleotide occupies position n in the support strand of the scaffold polynucleotide, wherein 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 first nucleotide of the predefined sequence upon its addition to the terminal end of the primer strand portion in that cycle, wherein the nucleotide at position n in the support strand is opposite the terminal nucleotide of the helper strand and is paired therewith;
- 10 b) in the cleavage step of the first cycle (step 2) the support strand of the scaffold polynucleotide is cleaved between positions n and $n-1$, wherein 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 portion;
- 15 c) in the ligation step of the first cycle (step 5) the complementary ligation end of the ligation polynucleotide is structured such that the partner nucleotide for the first nucleotide of the predefined sequence is the terminal nucleotide of the support strand and occupies position n , wherein the universal nucleotide occupies position $n+1$ in the support strand and is paired with the terminal nucleotide of the helper strand, wherein position n is the nucleotide position which will be opposite the first
- 20 nucleotide of the predefined sequence upon ligation of the ligation polynucleotide to the cleaved scaffold polynucleotide in step 5;
- d) in the cleavage step of the second cycle (step 6) and in cleavage steps of all subsequent cycles:
- 25 i. the universal nucleotide occupies position n in the support strand of the scaffold polynucleotide, wherein 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 addition to the terminal end of the primer strand portion in that cycle; and
- 30 ii. the support strand of the scaffold polynucleotide is cleaved between positions n and $n-1$, wherein $n-1$ 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 portion; and

- 5 e) in the ligation step of the second cycle (step 9) and in ligation steps of all subsequent cycles the complementary ligation end of the ligation polynucleotide is structured such that the partner nucleotide for the next nucleotide of the predefined sequence in that cycle is the terminal nucleotide of the support strand and occupies position n , and the universal nucleotide occupies position $n+1$ in the support strand and is paired with the terminal nucleotide of the helper strand; wherein position n is
10 the nucleotide position which upon ligation of the ligation polynucleotide to the cleaved scaffold polynucleotide will be opposite the next nucleotide of the predefined sequence incorporated in that cycle (step 7).

Alternatively, any such method as described above may be performed according to
15 a method of the invention designated synthesis method version 2 wherein:

- a) prior to and at the cleavage step of the first cycle (step 2) the universal nucleotide occupies position $n+1$ in the support strand of the scaffold polynucleotide, wherein position n is the nucleotide position in the support strand which is opposite the
20 position in the synthesis strand which will be occupied by the first nucleotide of the predefined sequence upon its addition to the terminal end of the primer strand portion in that cycle, wherein the nucleotide at position n in the support strand is opposite the terminal nucleotide of the helper strand and is paired therewith, and wherein $n+1$ is the next nucleotide position in the support strand relative to position
25 n in the direction proximal to the helper strand/distal to the primer strand portion;
- b) in the cleavage step of the first cycle (step 2) the support strand of the scaffold polynucleotide is cleaved between positions n and $n-1$, wherein position $n-1$ is the
30 next nucleotide position in the support strand relative to position n in the direction distal to the helper strand/proximal to the primer strand portion;

- 5 c) in the ligation step of the first cycle (step 5) the complementary ligation end of the ligation polynucleotide is structured such that the partner nucleotide for the first nucleotide of the predefined sequence is the terminal nucleotide of the support strand and occupies position n , wherein the universal nucleotide occupies position $n+2$ in the support strand and is paired with the penultimate nucleotide of the helper strand, wherein position n is the nucleotide position which will be opposite the first nucleotide of the predefined sequence upon ligation of the ligation polynucleotide to the cleaved scaffold polynucleotide in step 5 and position $n+2$ is the second position in the support strand relative to position n in the direction proximal to the helper strand/distal to the primer strand portion;
- 10 d) in the cleavage step of the second cycle (step 6) and in cleavage steps of all subsequent cycles:
- 15 i. the universal nucleotide occupies position $n+1$ in the support strand of the scaffold polynucleotide, wherein 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 addition to the terminal end of the primer strand portion in that cycle, and wherein $n+1$ 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 portion; and
- 20 ii. the support strand of the scaffold polynucleotide is cleaved between positions n and $n-1$, wherein $n-1$ 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 portion; and
- 25 e) in the ligation step of the second cycle (step 9) and in ligation steps of all subsequent cycles the complementary ligation end of the ligation polynucleotide is structured such that the partner nucleotide for the next nucleotide of the predefined sequence in that cycle is the terminal nucleotide of the support strand and occupies position n , and the universal nucleotide occupies position $n+2$ in the support strand
- 30

and is paired with the penultimate nucleotide of the helper strand; wherein position n is the nucleotide position which upon ligation of the ligation polynucleotide to the cleaved scaffold polynucleotide will be opposite the next nucleotide of the predefined sequence incorporated in that cycle (step 7) and position $n+2$ is the second position in the support strand relative to position n in the direction proximal to the helper strand/distal to the primer strand portion.

Alternatively still, any such method as described above may be performed according to a method of the invention designated synthesis method version 3 wherein:

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- a) prior to and at the cleavage step of the first cycle (step 2) the universal nucleotide occupies position n in the support strand of the scaffold polynucleotide, wherein 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 first nucleotide of the predefined sequence upon its addition to the terminal end of the primer strand portion in that cycle, wherein the nucleotide at position n in the support strand is opposite the terminal nucleotide of the helper strand and is paired therewith;
- b) in the cleavage step of the first cycle (step 2) the support strand of the scaffold polynucleotide is cleaved between positions $n-1$ and $n-2$, wherein positions $n-1$ and $n-2$ are respectively the next and subsequent nucleotide positions in the support strand relative to position n in the direction distal to the helper strand/proximal to the primer strand portion;
- c) in the ligation step of the first cycle (step 5) the complementary ligation end of the ligation polynucleotide is structured such that the partner nucleotide for the first nucleotide of the predefined sequence is the penultimate nucleotide of the support strand and occupies position n , wherein the universal nucleotide occupies position $n+1$ in the support strand and is paired with the terminal nucleotide of the helper strand, wherein position n is the nucleotide position which will be opposite the first nucleotide of the predefined sequence upon ligation of the ligation polynucleotide

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to the cleaved scaffold polynucleotide in step 5, and wherein 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 portion;

- 5 d) in the cleavage step of the second cycle (step 6) and in cleavage steps of all subsequent cycles:
- 10 i. the universal nucleotide occupies position n in the support strand of the scaffold polynucleotide, wherein 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 addition to the terminal end of the primer strand portion in that cycle; and
 - 15 ii. the support strand of the scaffold polynucleotide is cleaved between positions $n-1$ and $n-2$, wherein positions $n-1$ and $n-2$ are respectively the next and subsequent nucleotide positions in the support strand relative to position n in the direction distal to the helper strand/proximal to the primer strand portion; and
- 20 e) in the ligation step of the second cycle (step 9) and in ligation steps of all subsequent cycles the complementary ligation end of the ligation polynucleotide is structured such that the partner nucleotide for the next nucleotide of the predefined sequence in that cycle is the penultimate nucleotide of the support strand and occupies position n , and the universal nucleotide occupies position $n+1$ in the support strand and is paired with the terminal nucleotide of the helper strand; wherein position n is the nucleotide position which upon ligation of the ligation
- 25 polynucleotide to the cleaved scaffold polynucleotide will be opposite the next nucleotide of the predefined sequence incorporated in that cycle (step 7), and wherein 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 portion.

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Alternatively still, any such method as described above may be performed according to a method of the invention designated synthesis method version 4 wherein:

- 5 a) prior to and at the cleavage step of the first cycle (step 2) the universal nucleotide occupies position $n+2$ in the support strand of the scaffold polynucleotide, wherein 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 first nucleotide of the predefined sequence upon its addition to the terminal end of the primer strand portion in that cycle, wherein the nucleotide at position n in the support strand is
- 10 opposite the terminal nucleotide of the helper strand and is paired therewith, and wherein $n+2$ is the second nucleotide position in the support strand relative to position n in the direction proximal to the helper strand/distal to the primer strand portion;
- 15 b) in the cleavage step of the first cycle (step 2) the support strand of the scaffold polynucleotide is cleaved between positions n and $n-1$, wherein 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 portion;
- 20 c) in the ligation step of the first cycle (step 5) the complementary ligation end of the ligation polynucleotide is structured such that the partner nucleotide for the first nucleotide of the predefined sequence is the terminal nucleotide of the support strand and occupies position n , wherein the universal nucleotide occupies position $n+3$ in the support strand and is paired with the nucleotide which is two positions
- 25 removed from the terminal nucleotide of the helper strand in the direction distal to the primer strand portion, wherein position n is the nucleotide position which will be opposite the first nucleotide of the predefined sequence upon ligation of the ligation polynucleotide to the cleaved scaffold polynucleotide in step 5 and position $n+3$ is the third position in the support strand relative to position n in the direction
- 30 proximal to the helper strand/distal to the primer strand portion;

- d) in the cleavage step of the second cycle (step 6) and in cleavage steps of all subsequent cycles:
- i. the universal nucleotide occupies position $n+2$ in the support strand of the scaffold polynucleotide, wherein 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 addition to the terminal end of the primer strand portion in that cycle, and wherein $n+2$ is the second nucleotide position in the support strand relative to position n in the direction proximal to the helper strand/distal to the primer strand portion; and
 - ii. the support strand of the scaffold polynucleotide is cleaved between positions n and $n-1$, wherein 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 portion; and
- e) in the ligation step of the second cycle (step 9) and in ligation steps of all subsequent cycles the complementary ligation end of the ligation polynucleotide is structured such that the partner nucleotide for the next nucleotide of the predefined sequence in that cycle is the terminal nucleotide of the support strand and occupies position n , and the universal nucleotide occupies position $n+3$ in the support strand and is paired with the nucleotide which is two positions removed from the terminal nucleotide of the helper strand in the direction distal to the primer strand portion; wherein position n is the nucleotide position which upon ligation of the ligation polynucleotide to the cleaved scaffold polynucleotide will be opposite the next nucleotide of the predefined sequence incorporated in that cycle (step 7) and position $n+3$ is the third position in the support strand relative to position n in the direction proximal to the helper strand/distal to the primer strand portion.

Any such method may be performed according to a method of the invention which is a variation of synthesis method version 4 wherein:

- 5 (i) in the cleavage step of the first cycle (step 2) the universal nucleotide instead occupies position $n+3$ in the support strand of the scaffold polynucleotide, wherein $n+3$ is the third nucleotide position in the support strand relative to position n in the direction proximal to the helper strand/distal to the primer strand portion; and the support strand of the scaffold polynucleotide is cleaved between positions n and $n-1$;
- 10 (ii) in the ligation step of the first cycle (step 5) the complementary ligation end of the ligation polynucleotide is structured such that the universal nucleotide instead occupies position $n+4$ in the support strand and is paired with the nucleotide which is 3 positions removed from the terminal nucleotide of the helper strand at the complementary ligation end; wherein position $n+4$ is position 4 in the support strand relative to position n in the direction proximal to the helper strand/distal to the primer strand portion;
- 15 (iii) in the cleavage step of the second cycle (step 6) and in cleavage steps of all subsequent cycles the universal nucleotide occupies position $n+3$ in the support strand of the scaffold polynucleotide, and the support strand of the scaffold polynucleotide is cleaved between positions n and $n-1$; and
- 20 (iv) in the ligation step of the second cycle (step 9) and in ligation steps of all subsequent cycles the complementary ligation end of the ligation polynucleotide is structured such that the universal nucleotide occupies position $n+4$ in the support strand and is paired with the nucleotide which is 2 positions removed from the
- 25 terminal nucleotide of the helper strand at the complementary ligation end.

Any such method may be performed according to a method of the invention which is a further variation of synthesis method version 4 wherein:

- 30 (i) in the cleavage step of the first cycle (step 2) the universal nucleotide instead occupies position $n+3+x$ in the support strand of the scaffold polynucleotide,

wherein $n+3$ is the third nucleotide position in the support strand relative to position n in the direction proximal to the helper strand/distal to the primer strand portion; and the support strand of the scaffold polynucleotide is cleaved between positions n and $n-1$;

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(ii) in the ligation step of the first cycle (step 5) the complementary ligation end of the ligation polynucleotide is structured such that the universal nucleotide instead occupies position $n+4+x$ in the support strand and is paired with the nucleotide which is $3+x$ positions removed from the terminal nucleotide of the helper strand at the complementary ligation end; wherein position $n+4$ is position 4 in the support strand relative to position n in the direction proximal to the helper strand/distal to the primer strand portion;

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(iii) in the cleavage step of the second cycle (step 6) and in cleavage steps of all subsequent cycles the universal nucleotide occupies position $n+3+x$ in the support strand of the scaffold polynucleotide and the support strand of the scaffold polynucleotide is cleaved between positions n and $n-1$;

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(iv) in the ligation step of the second cycle (step 9) and in ligation steps of all subsequent cycles the complementary ligation end of the ligation polynucleotide is structured such that the universal nucleotide occupies position $n+4+x$ in the support strand and is paired with the nucleotide which is $2+x$ positions removed from the terminal nucleotide of the helper strand at the complementary ligation end; and

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(v) wherein x is a whole number between 1 to 10 or more, and wherein x is the same whole number in steps (2), (5), (6) and (9).

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Alternatively still, any such method as described above may be performed according to a method of the invention designated synthesis method version 5 wherein:

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- 5 a) prior to and at the cleavage step of the first cycle (step 2) the universal nucleotide occupies position $n+1$ in the support strand of the scaffold polynucleotide, wherein 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 first nucleotide of the predefined sequence upon its addition to the terminal end of the primer strand portion in that cycle, wherein the nucleotide at position n in the support strand is opposite the terminal nucleotide of the helper strand and is paired therewith, and wherein $n+1$ 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 portion;
- 10 b) in the cleavage step of the first cycle (step 2) the support strand of the scaffold polynucleotide is cleaved between positions $n-1$ and $n-2$, wherein positions $n-1$ and $n-2$ are respectively the next and subsequent nucleotide positions in the support strand relative to position n in the direction distal to the helper strand/proximal to the primer strand portion;
- 15 c) in the ligation step of the first cycle (step 5) the complementary ligation end of the ligation polynucleotide is structured such that the partner nucleotide for the first nucleotide of the predefined sequence is the penultimate nucleotide of the support strand and occupies position n , wherein the universal nucleotide occupies position $n+2$ in the support strand and is paired with the penultimate nucleotide of the helper strand, wherein position n is the nucleotide position which will be opposite the first nucleotide of the predefined sequence upon ligation of the ligation polynucleotide to the cleaved scaffold polynucleotide, and wherein position $n+2$ is the second nucleotide position in the support strand relative to position n in the direction proximal to the helper strand/distal to the primer strand portion;
- 20 d) in the cleavage step of the second cycle (step 6) and in cleavage steps of all subsequent cycles:
- 25 i. the universal nucleotide occupies position $n+1$ in the support strand of the scaffold polynucleotide, wherein position n is the nucleotide position in the
- 30

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 addition to the terminal end of the primer strand portion in that cycle, and wherein 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 portion; and

- ii. the support strand of the scaffold polynucleotide is cleaved between positions n-1 and n-2, wherein positions n-1 and n-2 are respectively the next and subsequent nucleotide positions in the support strand relative to position n in the direction distal to the helper strand/proximal to the primer strand portion;

e) in the ligation step of the second cycle (step 9) and in ligation steps of all subsequent cycles the complementary ligation end of the ligation polynucleotide is structured such that the partner nucleotide for the next nucleotide of the predefined sequence in that cycle is the penultimate nucleotide of the support strand and occupies position n , and the universal nucleotide occupies position $n+2$ in the support strand and is paired with the penultimate nucleotide of the helper strand; wherein position n is the nucleotide position which upon ligation of the ligation polynucleotide to the cleaved scaffold polynucleotide will be opposite the next nucleotide of the predefined sequence incorporated in that cycle (step 7), and wherein position $n+2$ is the second nucleotide position in the support strand relative to position n in the direction proximal to the helper strand/distal to the primer strand portion.

Any such method may be performed according to a method of the invention which is a variation of synthesis method version 5 wherein:

(i) in the cleavage step of the first cycle (step 2) the universal nucleotide instead occupies position $n+2$ in the support strand of the scaffold polynucleotide, wherein $n+2$ is the second nucleotide position in the support strand relative to position n in

the direction proximal to the helper strand/distal to the primer strand portion; and the support strand of the scaffold polynucleotide is cleaved between positions n and $n-1$;

- 5 (ii) in the ligation step of the first cycle (step 5) the complementary ligation end of the ligation polynucleotide is structured such that the universal nucleotide instead occupies position $n+3$ in the support strand and is paired with the nucleotide which is 2 positions removed from the terminal nucleotide of the helper strand at the complementary ligation end; wherein position $n+3$ is position 3 in the support
- 10 strand relative to position n in the direction proximal to the helper strand/distal to the primer strand portion;
- (iii) in the cleavage step of the second cycle (step 6) and in cleavage steps of all subsequent cycles the universal nucleotide occupies position $n+2$ in the support
- 15 strand of the scaffold polynucleotide, and the support strand of the scaffold polynucleotide is cleaved between positions n and $n-1$; and
- (iv) in the ligation step of the second cycle (step 9) and in ligation steps of all subsequent cycles the complementary ligation end of the ligation polynucleotide is
- 20 structured such that the universal nucleotide occupies position $n+3$ in the support strand and is paired with the nucleotide which is 2 positions removed from the terminal nucleotide of the helper strand at the complementary ligation end.

Any such method may be performed according to a method of the invention which is a

25 further variation of synthesis method version 5 wherein:

- (i) in the cleavage step of the first cycle (step 2) the universal nucleotide instead occupies position $n+2+x$ in the support strand of the scaffold polynucleotide, wherein $n+2$ is the second nucleotide position in the support strand relative to
- 30 position n in the direction proximal to the helper strand/distal to the primer strand

portion; and the support strand of the scaffold polynucleotide is cleaved between positions n and $n-1$;

- 5 (ii) in the ligation step of the first cycle (step 5) the complementary ligation end of the ligation polynucleotide is structured such that the universal nucleotide instead occupies position $n+3+x$ in the support strand and is paired with the nucleotide which is $2+x$ positions removed from the terminal nucleotide of the helper strand at the complementary ligation end; wherein position $n+3$ is position 3 in the support strand relative to position n in the direction proximal to the helper strand/distal to the primer strand portion;
- 10 (iii) in the cleavage step of the second cycle (step 6) and in cleavage steps of all subsequent cycles the universal nucleotide occupies position $n+2+x$ in the support strand of the scaffold polynucleotide and the support strand of the scaffold polynucleotide is cleaved between positions n and $n-1$;
- 15 (iv) in the ligation step of the second cycle (step 9) and in ligation steps of all subsequent cycles the complementary ligation end of the ligation polynucleotide is structured such that the universal nucleotide occupies position $n+3+x$ in the support strand and is paired with the nucleotide which is $2+x$ positions removed from the terminal nucleotide of the helper strand at the complementary ligation end; and
- 20 (v) wherein x is a whole number between 1 to 10 or more, and wherein x is the same whole number in steps (2), (5), (6) and (9).

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In any of the methods described above and herein, a partner nucleotide which pairs with a first/next nucleotide of the predefined sequence may be a nucleotide which is complementary with the first/next nucleotide, preferably naturally complementary.

30 In any of the methods described above and herein, in any one or more cycles of synthesis, or in all cycles of synthesis, prior to step (2) and/or (6) the scaffold polynucleotide may be provided comprising a synthesis strand and a support strand

hybridized thereto, and wherein the synthesis strand is provided without a helper strand. In any one or more cycles of synthesis, or in all cycles of synthesis, prior to step (2) and/or (6) the synthesis strand may be removed from the scaffold polynucleotide.

In any of the methods described above and herein, 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 may be removed from the scaffold polynucleotide. In any such method the helper strand portion of the synthesis strand may be removed from the scaffold polynucleotide by: (i) heating the scaffold polynucleotide to a temperature of about 80°C to about 95°C and separating the helper strand portion from the scaffold polynucleotide, (ii) treating the scaffold polynucleotide with urea solution, such as 8M urea and separating the helper strand portion from the scaffold polynucleotide, (iii) treating the scaffold polynucleotide with formamide or formamide solution, such as 100% formamide and separating the helper strand portion from the scaffold polynucleotide, or (iv) contacting the scaffold polynucleotide with a single-stranded polynucleotide molecule which comprises a region of nucleotide sequence which is complementary with the sequence of the helper strand portion, thereby competitively inhibiting the hybridisation of the helper strand portion to the scaffold polynucleotide.

In any such method described above and herein wherein the universal nucleotide occupies position n and wherein the support strand of the scaffold polynucleotide is cleaved between positions n and n-1, each cleavage step may comprise a two-step cleavage process wherein each cleavage step may comprise 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. In any such method the first step may be performed with a nucleotide-excising enzyme. The nucleotide-excising enzyme may be a 3-methyladenine DNA glycosylase enzyme. The nucleotide-excising enzyme may be human alkyladenine DNA glycosylase (hAAG) or uracil DNA glycosylase (UDG). In any such method the second step may be performed with a chemical which is a base. The base may be NaOH.

In any such method the second step may be performed with an organic chemical having abasic site cleavage activity. The organic chemical may be N,N'-dimethylethylenediamine. In any such method the second step may be performed with an enzyme having abasic site lyase activity such as AP Endonuclease, Endonuclease III (Nth) or Endonuclease VIII.

In any such method described above and herein wherein the universal nucleotide occupies position n and wherein the support strand of the scaffold polynucleotide is cleaved between positions n and n-1, each cleavage step may comprise a one step cleavage process comprising removing the universal nucleotide with a cleavage enzyme, wherein the enzyme is Endonuclease III, Endonuclease VIII, formamidopirimidine DNA glycosylase (Fpg) or 8-oxoguanine DNA glycosylase (hOGG1).

In any such method described above and herein wherein the universal nucleotide occupies position n+1 and wherein the support strand of the scaffold polynucleotide is cleaved between positions n and n-1, or in any such method described above and herein wherein the universal nucleotide occupies position n and wherein the support strand of the scaffold polynucleotide is cleaved between positions n-1 and n-2, the cleavage step may comprise cleaving the support strand with an enzyme. The enzyme may cleave the support strand after the nucleotide which is next to the universal nucleotide in the direction proximal to the primer strand portion, thereby creating the overhanging end in the synthesis strand comprising the first/next nucleotide. Such an enzyme may be Endonuclease V.

In any of the methods described above and herein, both strands of the synthesised double-stranded polynucleotide may be DNA strands. The synthesis strand and the support strand may be DNA strands. In such cases incorporated nucleotides are preferably dNTPs, preferably dNTPs comprising a reversible terminator group. In any such method any one or more or all of the incorporated nucleotides comprising a reversible terminator group may comprise 3'-O-allyl-dNTPs or 3'-O-azidomethyl-dNTPs.

In any of the methods described above and herein, a first strand of the synthesised double-stranded polynucleotide may be a DNA strand and the second strand of the synthesised double-stranded polynucleotide may be an RNA strand. The synthesis strand

may be an RNA strand and the support strand may be a DNA strand. In such cases incorporated nucleotides are preferably NTPs, preferably NTPs comprising a reversible terminator group. In any such method any one or more or all of the incorporated nucleotides comprising a reversible terminator group may be 3'-O-allyl-NTPs or 3'-O-azidomethyl-NTPs.

In any of the methods described above and herein involving incorporation of a nucleotide into a synthesis strand comprising DNA *e.g.* incorporation of one or more dNTPs, the enzyme may be a polymerase, preferably a DNA polymerase, more preferably a modified DNA polymerase having an enhanced ability to incorporate a dNTP comprising a reversible terminator group compared to an unmodified polymerase. The polymerase may be a variant of the native DNA polymerase from *Thermococcus* species 9°N, preferably species 9°N-7.

In any of the methods described above and herein involving incorporation of a nucleotide into a synthesis strand comprising RNA *e.g.* incorporation of one or more NTPs, the enzyme may be a polymerase, preferably an RNA polymerase such as T3 or T7 RNA polymerase, more preferably a modified RNA polymerase having an enhanced ability to incorporate an NTP comprising a reversible terminator group compared to an unmodified polymerase.

In any of the methods described above and herein, a first strand of the synthesised double-stranded polynucleotide may be a DNA strand and the second strand of the synthesised double-stranded polynucleotide may be an RNA strand. Alternatively, a first strand of the synthesised double-stranded polynucleotide may be an RNA strand and the second strand of the synthesised double-stranded polynucleotide may be a DNA strand.

In any of the methods described above and herein, the enzyme enzyme has a terminal transferase activity, optionally wherein the enzyme is a terminal nucleotidyl transferase, a terminal deoxynucleotidyl transferase, terminal deoxynucleotidyl transferase (TdT), pol lambda, pol micro or Φ 29 DNA polymerase.

In any of the methods described above and herein, the step of removing the reversible terminator group from the first/next nucleotide may be performed with tris(carboxyethyl)phosphine (TCEP).

In any of the methods described above and herein, the step of ligating a double-stranded ligation polynucleotide to the cleaved scaffold polynucleotide is preferably performed with a ligase enzyme. The ligase enzyme may be a T3 DNA ligase or a T4 DNA ligase.

5 In any of the methods described above and herein, in step (1), (5) and/or (9) the helper strand and the portion of the support strand hybridized thereto may be connected by a hairpin loop.

In any of the methods described above and herein, in step (1) the synthesis strand comprising the primer strand portion and the portion of the support strand hybridized
10 thereto may be connected by a hairpin loop.

In any of the methods described above and herein, in step (1), (5) and/or (9):

- a) the helper strand and the portion of the support strand hybridized thereto may be connected by a hairpin loop; and
- b) the synthesis strand comprising the primer strand portion and the portion of the
15 support strand hybridized thereto may be connected by a hairpin loop.

In any of the methods described above and herein, at least one or more or all of the ligation polynucleotides may be provided as a single molecule comprising a hairpin loop connecting the support strand and the helper strand at the end opposite the complementary ligation end. In any of the methods described above and herein, the ligation
20 polynucleotides of each synthesis cycle may be provided as single molecules each comprising a hairpin loop connecting the support strand and the helper strand at the end opposite the complementary ligation end.

In any of the methods described above and herein, in step (1) the synthesis strand comprising the primer strand portion and the portion of the support strand hybridized
25 thereto may be tethered to a common surface. The primer strand portion and the portion of the support strand hybridized thereto may each comprise a cleavable linker, wherein the linkers may be cleaved to detach the double-stranded polynucleotide from the surface following synthesis.

In any of the methods described above and herein, in step (1) the primer strand portion of the synthesis strand and the portion of the support strand hybridized thereto may be connected by a hairpin loop, and wherein the hairpin loop is tethered to a surface.

5 In any of the methods described above and herein, a hairpin loop may be tethered to a surface via a cleavable linker, wherein the linker may be cleaved to detach the double-stranded polynucleotide from the surface following synthesis. The cleavable linker may be a UV cleavable linker.

In any of the methods described above and herein, the surface to which polynucleotides are attached may be the surface of a microparticle or a planar surface.

10 In any of the methods described above and herein, the surface to which polynucleotides are attached may comprise a gel. The surface comprises a polyacrylamide surface, such as about 2% polyacrylamide, preferably wherein the polyacrylamide surface is coupled to a solid support such as glass.

In any of the methods described above and herein, the synthesis strand comprising the primer strand portion and the portion of the support strand hybridized thereto may be tethered to the common surface via one or more covalent bonds. The one or more covalent bonds may be formed between a functional group on the common surface and a functional group on the scaffold molecule, wherein the functional group on the scaffold molecule may be an amine group, a thiol group, a thiophosphate group or a thioamide group. The functional group on the common surface may be a bromoacetyl group, optionally wherein the bromoacetyl group is provided on a polyacrylamide surface derived using N- (5-bromoacetamidylpentyl) acrylamide (BRAPA).

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In any of the methods described above and herein, the step of removing the reversible terminator group from a nucleotide of the predefined sequence may be performed before the cleavage step, or before the ligation step.

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In any of the methods described above and herein, reactions relating to any of the synthesis cycles described above and herein may be performed in droplets within a microfluidic system. The microfluidic system may be an electrowetting system. The microfluidic system may be an electrowetting-on-dielectric system (EWOD).

In any of the methods described above and herein, following synthesis the strands of the double-stranded polynucleotides may be separated to provide a single-stranded polynucleotide having a predefined sequence.

In any of the methods described above and herein, following synthesis the double-
5 stranded polynucleotide or a region thereof is amplified, preferably by PCR.

The invention also provides a method of assembling a polynucleotide having a predefined sequence, the method comprising performing any of the synthesis methods described above and herein to synthesise a first polynucleotide having a predefined sequence and one or more additional polynucleotides having a predefined sequence and
10 joining together the first and the one or more additional polynucleotides. The first and the one or more additional polynucleotides may preferably comprise different predefined sequences. The first polynucleotide and the one or more additional polynucleotides may be double-stranded or may be single-stranded. The first polynucleotide and the one or more additional polynucleotides may first be cleaved to create compatible termini and then
15 joined together, *e.g.* by ligation. The first polynucleotide and the one or more additional polynucleotides may be cleaved by a restriction enzyme at a cleavage site to create compatible termini.

Any of the *in vitro* methods for synthesising a double-stranded polynucleotide having a predefined sequence as described above and herein, and/or any of the *in vitro*
20 methods of assembling a polynucleotide having a predefined sequence as described above and herein may be performed in droplets within a microfluidic system. In any such methods, the assembly methods may comprise assembly steps which comprise providing a first droplet comprising a first synthesised polynucleotide having a predefined sequence and a second droplet comprising an additional one or more synthesised polynucleotides
25 having a predefined sequence, wherein the droplets are brought in contact with each other and wherein the synthesised polynucleotides are joined together thereby assembling a polynucleotide comprising the first and additional one or more polynucleotides. In any such methods the synthesis steps may be performed by providing a plurality of droplets each droplet comprising reaction reagents corresponding to a step of the synthesis cycle,
30 and sequentially delivering the droplets to the scaffold polynucleotide in accordance with

the steps of the synthesis cycles. In any such methods, following delivery of a droplet and prior to the delivery of a next droplet, a washing step may be carried out to remove excess reaction reagents. In any such methods the microfluidic system may be an electrowetting system. In any such methods the microfluidic system may be an electrowetting-on-
5 dielectric system (EWOD). In any such methods the synthesis and assembly steps may be performed within the same system.

In a related aspect, the invention further provides the use of a universal nucleotide in an *in vitro* method of synthesising a double-stranded polynucleotide having a predefined sequence, wherein the universal nucleotide is used to create a polynucleotide cleavage site
10 during each cycle of synthesis, wherein in each synthesis cycle said use comprises: providing a scaffold polynucleotide comprising a synthesis strand and a support strand hybridized thereto, wherein the synthesis strand comprises a primer strand portion and optionally a helper strand portion separated from the primer strand portion by a single-strand break, and wherein the universal nucleotide is provided in the support strand to
15 provide a cleavage site; cleaving the scaffold polynucleotide at the cleavage site whereupon the universal nucleotide is removed from the scaffold polynucleotide and a cleaved end is created in the scaffold polynucleotide; adding to the terminal end of the synthesis strand at the cleaved end of the scaffold polynucleotide by a polymerase or a transferase enzyme a new nucleotide of the predefined sequence comprising a reversible
20 terminator group; ligating to the cleaved end of the scaffold polynucleotide a ligation polynucleotide having a support strand and a helper strand hybridized thereto, the support strand comprising a nucleotide for pairing with the new nucleotide in the synthesis strand of the scaffold polynucleotide and further comprising a new universal nucleotide for use in creating a new polynucleotide cleavage site for use in the next cycle of synthesis, wherein
25 the reversible terminator group is removed from the new nucleotide after the cleavage or ligation step; and optionally wherein the helper strand is removed after the ligation step and before the cleavage step of the next cycle. Such use of a universal nucleotide in a method of synthesising a double-stranded polynucleotide having a predefined sequence may be implemented using any of the specific methods defined and described above and
30 herein.

In a related aspect, the invention further provides an *in vitro* method of extending a synthesis strand of a polynucleotide molecule with a predefined nucleotide, the method comprising: providing a scaffold polynucleotide comprising the synthesis strand and a support strand hybridized thereto, wherein the synthesis strand comprises a primer strand
5 portion and optionally a helper strand portion separated from the primer strand portion by a single-strand break, and wherein the universal nucleotide is provided in the support strand and defines a polynucleotide cleavage site; cleaving the scaffold polynucleotide at the cleavage site whereupon the universal nucleotide is removed from the scaffold polynucleotide and a cleaved end is created in the scaffold polynucleotide; adding to the
10 terminal end of the synthesis strand at the cleaved end of the scaffold polynucleotide by a polymerase or a transferase enzyme a new nucleotide of the predefined sequence comprising a reversible terminator group; wherein the cleaved end of the scaffold polynucleotide acts as a ligation acceptor site for a ligation polynucleotide having a support strand and a helper strand hybridized thereto, the support strand comprising a
15 nucleotide for pairing with the predefined nucleotide in the synthesis strand of the scaffold polynucleotide and further comprising a new universal nucleotide for use in creating a new polynucleotide cleavage site for use in the next cycle of synthesis, wherein the reversible terminator group is removed from the new nucleotide after the cleavage or ligation step and optionally wherein the helper strand is removed after the ligation step and before the
20 cleavage step of the next cycle. In any such method of extending a synthesis strand of a polynucleotide molecule with a predefined nucleotide, the method may be implemented using any of the specific methods defined and described above and herein.

In a related aspect, the invention further provides an *in vitro* method of synthesising a double-stranded polynucleotide having a predefined sequence, the method comprising
25 cycles of synthesis and wherein each synthesis cycle comprises: providing a scaffold polynucleotide comprising a synthesis strand and a support strand hybridized thereto, wherein the synthesis strand comprises a primer strand portion and optionally a helper strand portion separated from the primer strand portion by a single-strand break, and wherein a universal nucleotide is provided in the support strand and defines a
30 polynucleotide cleavage site; cleaving the scaffold polynucleotide at the cleavage site

whereupon the universal nucleotide is removed from the scaffold polynucleotide and a cleaved end is created in the scaffold polynucleotide; adding to the terminal end of the synthesis strand at the cleaved end of the scaffold polynucleotide by a polymerase or a transferase enzyme a new nucleotide of the predefined sequence comprising a reversible terminator group; ligating to the cleaved end of the scaffold polynucleotide a ligation polynucleotide having a support strand and a helper strand hybridized thereto, the support strand comprising a nucleotide for pairing with the new nucleotide in the synthesis strand of the scaffold polynucleotide and further comprising a new universal nucleotide for use in creating a new polynucleotide cleavage site for use in the next cycle of synthesis; removing the reversible terminator group from the new nucleotide after the cleavage or ligation step; and optionally removing the helper strand after the ligation step and before the cleavage step of the next cycle. In any such method of synthesising a double-stranded polynucleotide having a predefined sequence, the method may be implemented using any of the specific methods defined and described above and herein.

In a related aspect, the invention further provides an *in vitro* method of ligating a ligation polynucleotide comprising a universal nucleotide to a double-stranded polynucleotide during a cycle of synthesising a double-stranded polynucleotide having a predefined sequence, wherein during the synthesis cycle the double-stranded polynucleotide is extended with a predefined nucleotide and a partner therefor; the method comprising providing a scaffold polynucleotide comprising a synthesis strand and a support strand hybridized thereto, wherein the synthesis strand comprises a primer strand portion and optionally a helper strand portion separated from the primer strand portion by a single-strand break, and wherein a universal nucleotide is provided in the support strand and defines a polynucleotide cleavage site; cleaving the scaffold polynucleotide at the cleavage site whereupon the universal nucleotide is removed from the scaffold polynucleotide and a cleaved end is created in the scaffold polynucleotide; adding to the terminal end of the synthesis strand at the cleaved end of the scaffold polynucleotide by a polymerase or a transferase enzyme a new nucleotide of the predefined sequence comprising a reversible terminator group ligating to the cleaved end of the scaffold polynucleotide a ligation polynucleotide having a support strand and a helper strand

hybridized thereto, the support strand comprising a nucleotide for pairing with the new nucleotide in the synthesis strand of the scaffold polynucleotide and further comprising a new universal nucleotide for use in creating a new polynucleotide cleavage site for use in the next cycle of synthesis, thereby creating a new scaffold polynucleotide for use in the next synthesis cycle; removing the reversible terminator group from the new nucleotide after the cleavage or ligation step; and optionally removing the helper strand after the ligation step and before the cleavage step of the next cycle. In any such method of ligating a ligation polynucleotide comprising a universal nucleotide to a double-stranded polynucleotide during a cycle of synthesising a double-stranded polynucleotide having a predefined sequence, the method may be implemented using any of the specific methods defined and described above and herein.

The invention additionally provides a polynucleotide synthesis system for carrying out any of the synthesis and/or assembly methods described above and herein, comprising (a) an array of reaction areas, wherein each reaction area comprises at least one scaffold polynucleotide; and (b) means for the delivery of the reaction reagents to the reaction areas and optionally, (c) means to cleave the synthesised double-stranded polynucleotide from the scaffold polynucleotide. Such a system may further comprise means for providing the reaction reagents in droplets and means for delivering the droplets to the scaffold polynucleotide in accordance with the synthesis cycles.

The invention further provides a kit for use with any of the systems described above and herein, and for carrying out any of the synthesis methods described above and herein, the kit comprising volumes of reaction reagents corresponding to the steps of the synthesis cycles.

The invention also provides a method of making a polynucleotide microarray, wherein the microarray comprises a plurality of reaction areas, each area comprising one or more polynucleotides having a predefined sequence, the method comprising:

- a) providing a surface comprising a plurality of reaction areas, each area comprising one or more double-stranded anchor or scaffold polynucleotides, and

- b) performing cycles of synthesis according to any of the methods described above and herein at each reaction area, thereby synthesising at each area one or more double-stranded polynucleotides having a predefined sequence.

5 In such methods, following synthesis the strands of the double-stranded polynucleotides may be separated to provide a microarray wherein each area comprises one or more single-stranded polynucleotides having a predefined sequence.

DESCRIPTION OF THE FIGURES

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Relevant Figures presented herein and described below show some or all of the steps of a cycle of synthesis using methods, including methods of the invention, as well as means for performing aspects of the methods, such as oligonucleotides, surfaces, surface attachment chemistries, linkers etc. These Figures as well as all descriptions thereof and
15 all associated methods, reagents and protocols are presented for illustration only and are not to be construed as limiting.

Relevant Figures, such as *e.g.* Figures 6, 7, 8, 9, 10, 13a, 14a, 15a etc. show some or all of the steps of a cycle of synthesis including incorporation of a nucleotide (*e.g.*, a nucleotide comprising a reversible terminator group), cleavage (*e.g.*, cleaving the scaffold
20 polynucleotide into a first portion and a second portion, wherein the first portion comprises an universal nucleotide, and the second portion comprises the incorporated nucleotide), ligation (*e.g.*, ligating to the second portion of the cleaved scaffold polynucleotide comprising the incorporated nucleotide, a polynucleotide construct comprising a single-stranded portion, wherein the single-stranded portion comprises a partner nucleotide that is
25 complementary to the incorporated nucleotide) and deprotection (*e.g.*, removing the reversible terminator group from the incorporated nucleotide). These methods are provided for illustrative support only and are not within the scope of the claimed invention. Method schemes shown in Figures 1 to 5 are methods of the invention. The data shown in Figure 51, as described in Example 13, relate to methods of the invention consistent with
30 synthesis method versions 1, 2 and 4 as depicted in Figures 1, 2 and 4 as described herein.

Figure 1. Scheme of Exemplary Method Version 1 of the Invention.

Scheme showing a first synthesis cycle according to exemplary method version 1 of the invention. The method comprises a cycle of provision of a scaffold polynucleotide, cleavage, incorporation, deprotection and ligation. The scheme shows the provision of a scaffold polynucleotide (101) comprising a universal nucleotide in the support strand which defines a polynucleotide cleavage site and a single-stranded break (“nick”) in the synthesis strand. The scheme shows cleavage of the support strand by the creation of a single-stranded break in the support strand (102) followed by addition of a thymine nucleotide to the terminal end of the synthesis strand at the cleaved end of the scaffold polynucleotide (103) and removal of the reversible blocking group during a protection step (104). Following ligation of a ligation polynucleotide to the cleaved scaffold polynucleotide (105) the newly incorporated thymine nucleotide is paired opposite its partner nucleotide adenine (104). Following the ligation step a reconstituted scaffold polynucleotide is provided with the newly incorporated pair of nucleotides (106) for use in the next synthesis cycle. This A-T pair is shown for illustration purposes only and is not limiting, it can be any pair depending on the required predefined sequence. Nucleotide pairs at positions H-I can be any pair. Nucleotide X can be any nucleotide. The Figure also shows reference signs corresponding to a second synthesis cycle.

Figure 2. Scheme of Exemplary Method Version 2 of the Invention.

Scheme showing a first synthesis cycle according to exemplary method version 2 of the invention. The method comprises a cycle of provision of a scaffold polynucleotide, cleavage, incorporation, deprotection and ligation. The scheme shows the provision of a scaffold polynucleotide (101) comprising a universal nucleotide in the support strand which defines a polynucleotide cleavage site and a single-stranded break (“nick”) in the synthesis strand. The scheme shows cleavage of the support strand by the creation of a single-stranded break in the support strand (102) followed by addition of a thymine

nucleotide to the terminal end of the synthesis strand at the cleaved end of the scaffold polynucleotide (103) and removal of the reversible blocking group during a protection step (104). Following ligation of a ligation polynucleotide to the cleaved scaffold polynucleotide (105) the newly incorporated thymine nucleotide is paired opposite its partner nucleotide adenine (104). Following the ligation step a reconstituted scaffold polynucleotide is provided with the newly incorporated pair of nucleotides (106) for use in the next synthesis cycle. This A-T pair is shown for illustration purposes only and is not limiting, it can be any pair depending on the required predefined sequence. Nucleotide pairs at positions H-I and J-K can be any pair. Nucleotide X can be any nucleotide. The Figure also shows reference signs corresponding to a second synthesis cycle.

Figure 3. Scheme of Exemplary Method Version 3 of the Invention.

Scheme showing a first synthesis cycle according to exemplary method version 3 of the invention. The method comprises a cycle of provision of a scaffold polynucleotide, cleavage, incorporation, deprotection and ligation. The scheme shows the provision of a scaffold polynucleotide (101) comprising a universal nucleotide in the support strand which defines a polynucleotide cleavage site and a single-stranded break (“nick”) in the synthesis strand. The scheme shows cleavage of the support strand by the creation of a single-stranded break in the support strand (102) followed by addition of a thymine nucleotide to the terminal end of the synthesis strand at the cleaved end of the scaffold polynucleotide (103) and removal of the reversible blocking group during a protection step (104). Following ligation of a ligation polynucleotide to the cleaved scaffold polynucleotide (105) the newly incorporated thymine nucleotide is paired opposite its partner nucleotide adenine (104). Following the ligation step a reconstituted scaffold polynucleotide is provided with the newly incorporated pair of nucleotides (106) for use in the next synthesis cycle. This A-T pair is shown for illustration purposes only and is not limiting, it can be any pair depending on the required predefined sequence. Nucleotide pairs at positions H-I and J-K can be any pair. Nucleotide X can be any nucleotide. The Figure also shows reference signs corresponding to a second synthesis cycle.

Figure 4. Scheme of Exemplary Method Version 4 of the Invention.

Scheme showing a first synthesis cycle according to exemplary method version 4 of the invention. The method comprises a cycle of provision of a scaffold polynucleotide, cleavage, incorporation, deprotection and ligation. The scheme shows the provision of a scaffold polynucleotide (101) comprising a universal nucleotide in the support strand which defines a polynucleotide cleavage site and a single-stranded break (“nick”) in the synthesis strand. The scheme shows cleavage of the support strand by the creation of a single-stranded break in the support strand (102) followed by addition of a thymine nucleotide to the terminal end of the synthesis strand at the cleaved end of the scaffold polynucleotide (103) and removal of the reversible blocking group during a protection step (104). Following ligation of a ligation polynucleotide to the cleaved scaffold polynucleotide (105) the newly incorporated thymine nucleotide is paired opposite its partner nucleotide adenine (104). Following the ligation step a reconstituted scaffold polynucleotide is provided with the newly incorporated pair of nucleotides (106) for use in the next synthesis cycle. This A-T pair is shown for illustration purposes only and is not limiting, it can be any pair depending on the required predefined sequence. Nucleotide pairs at positions H-I, J-K and L-M can be any pair. Nucleotide X can be any nucleotide. The Figure also shows reference signs corresponding to a second synthesis cycle.

Figure 5. Scheme of Exemplary Method Version 5 of the Invention.

The scheme shows the provision of a scaffold polynucleotide (101) comprising a universal nucleotide in the support strand which defines a polynucleotide cleavage site and a single-stranded break (“nick”) in the synthesis strand. The scheme shows cleavage of the support strand by the creation of a single-stranded break in the support strand (102) followed by addition of a thymine nucleotide to the terminal end of the synthesis strand at the cleaved end of the scaffold polynucleotide (103) and removal of the reversible blocking group during a protection step (104). Following ligation of a ligation polynucleotide to the

cleaved scaffold polynucleotide (105) the newly incorporated thymine nucleotide is paired opposite its partner nucleotide adenine (104). Following the ligation step a reconstituted scaffold polynucleotide is provided with the newly incorporated pair of nucleotides (106) for use in the next synthesis cycle. This A-T pair is shown for illustration purposes only and is not limiting, it can be any pair depending on the required predefined sequence. Nucleotide pairs at positions H-I, J-K and L-M can be any pair. Nucleotide X can be any nucleotide. The Figure also shows reference signs corresponding to a second synthesis cycle.

10 **Figure 6. Scheme of Exemplary Method Version 1.**

Scheme showing a first synthesis cycle according to exemplary method version 1 of the Examples section. This method is provided for illustrative support only and is not within the scope of the claimed invention. The method comprises a cycle of provision of a scaffold polynucleotide, incorporation, cleavage, ligation and deprotection. The scheme shows the incorporation of a thymine nucleotide in the first synthesis cycle (101, 102) and its pairing opposite a partner adenine nucleotide (104), as well as the provision of a scaffold polynucleotide (106) for use in the next synthesis cycle. This pair is shown for illustration purposes only and is not limiting, it can be any pair depending on the required predefined sequence. Nucleotide Z can be any nucleotide. Nucleotide X can be any appropriate nucleotide. The Figure also shows reference signs corresponding to a second synthesis cycle.

25 **Figure 7. Scheme of Exemplary Method Version 2.**

Scheme showing a first synthesis cycle according to exemplary method version 2 of the Examples section. This method is provided for illustrative support only and is not within the scope of the claimed invention. The method comprises a cycle of provision of a scaffold polynucleotide, incorporation, cleavage, ligation and deprotection. The scheme shows the incorporation in the first cycle (201, 202) of a thymine nucleotide and its pairing

opposite a partner adenine nucleotide (204), as well as the provision of a scaffold polynucleotide (206) comprising a guanine for pairing with a cytosine in the next synthesis cycle. These pairs are shown for illustration purposes only and are not limiting, they can be any pairs depending on the required predefined sequence. Nucleotide Z can be any nucleotide. Nucleotide X can be any appropriate nucleotide. The Figure also shows reference signs corresponding to a second synthesis cycle.

Figure 8. Scheme of Exemplary Method Version 3.

Scheme showing a first synthesis cycle according to exemplary method version 3 of the Examples section. This method is provided for illustrative support only and is not within the scope of the claimed invention. The method comprises a cycle of provision of a scaffold polynucleotide, incorporation, cleavage, ligation and deprotection. The scheme shows the incorporation in the first cycle (301, 302) of a thymine nucleotide and its pairing opposite a partner adenine nucleotide (304), as well as the provision of a scaffold polynucleotide (306) for use in the next synthesis cycle. This pair is shown for illustration purposes only and is not limiting, it can be any pair depending on the required predefined sequence. The scheme also shows a cytosine-guanine pair as a component of the scaffold polynucleotide and which is not part of the predefined sequence. This pair is also shown for illustration purposes only and is not limiting, it can be any pair. Nucleotide Z can be any nucleotide. Nucleotide X can be any appropriate nucleotide.

Figure 9. Scheme of Exemplary Method Version 4.

Scheme showing a first synthesis cycle according to exemplary method version 4 of the Examples section. This method is provided for illustrative support only and is not within the scope of the claimed invention. The method comprises a cycle of provision of a scaffold polynucleotide, incorporation, cleavage, ligation and deprotection. The scheme shows the incorporation in the first cycle (401, 402) of a thymine nucleotide and its pairing opposite a partner universal nucleotide (404), as well as the provision of a scaffold

polynucleotide (406) comprising a guanine for pairing with a cytosine in the next synthesis cycle. These pairs are shown for illustration purposes only and are not limiting, they can be any pairs depending on the required predefined sequence. Nucleotides X, Y and Z can be any nucleotide.

5

Figure 10. Scheme of Exemplary Method Version 5.

Scheme showing a first synthesis cycle according to exemplary method version 5 of the Examples section. This method is provided for illustrative support only and is not within the scope of the claimed invention. The method comprises a cycle of provision of a scaffold polynucleotide, incorporation, cleavage, ligation and deprotection. The scheme shows the incorporation in the first cycle (501, 502) of a thymine nucleotide and its pairing opposite a partner adenine nucleotide (504), as well as the provision of a scaffold polynucleotide (506) comprising a guanine for pairing with a cytosine in the next synthesis cycle. The scheme also shows a cytosine-guanine pair (position n-2) as a component of the scaffold polynucleotide and which is not part of the predefined sequence. These pairs are shown for illustration purposes only and are not limiting, they can be any pairs depending on the required predefined sequence. Nucleotides X, Y and Z can be any nucleotide.

20 **Figure 11. Scheme Showing Surface Immobilization of Scaffold Polynucleotides.**

Schemes show (a to h) possible example hairpin loop configurations of scaffold polynucleotides and their immobilisation to surfaces.

Schemes (i and j) show examples of surface chemistries for attaching polynucleotides to surfaces. The examples show double-stranded embodiments wherein both strands are connected via a hairpin, but the same chemistries may be used for attaching one or both strands of an unconnected double-stranded polynucleotide.

Figure 12. Absence of Helper Strand – Incorporation.

a) Scheme showing incorporation step highlighted in dashed box.

b) Evaluation of DNA polymerases for incorporation of 3'-*O*-modified-dTTPs opposite inosine. The Figure depicts a gel showing results of incorporation of 3'-*O*-modified-dTTPs by various DNA polymerases (Bst, Deep Vent (Exo-), Terminator I and Terminator IX) in presence of Mn²⁺ ions at 50°C. Lane 1: Incorporation of 3'-*O*-allyl-dTTPs using Bst DNA polymerase. Lane 2: Incorporation of 3'-*O*-azidomethyl-dTTPs using Bst DNA polymerase. Lane 3: Incorporation of 3'-*O*-allyl-dTTPs using Deep vent (exo-) DNA polymerase. Lane 4: Incorporation of 3'-*O*-azidomethyl-dTTPs using Deep vent (exo-) DNA polymerase. Lane 5: Incorporation of 3'-*O*-allyl-dTTPs using Terminator I DNA polymerase. Lane 6: Incorporation of 3'-*O*-azidomethyl-dTTPs using Terminator I DNA polymerase. Lane 7: Incorporation of 3'-*O*-allyl-dTTPs using Terminator IX DNA polymerase. Lane 8: Incorporation of 3'-*O*-azidomethyl-dTTPs using Terminator IX DNA polymerase.

c) Evaluation of DNA polymerases for incorporation of 3'-*O*-modified-dTTPs opposite inosine. Results of incorporation using various DNA polymerases.

d) Evaluation of the temperature on the incorporation using Terminator IX DNA polymerase. The Figure depicts a gel showing results of incorporation of 3'-modified-dTTP opposite inosine in presence of Mn²⁺ ions using Terminator IX DNA polymerase at various temperatures. Lane 1: Incorporation of 3'-*O*-allyl-dTTPs at 37°C. Lane 2: Incorporation of 3'-*O*-azidomethyl-dTTPs at 37°C. Lane 3: Incorporation of 3'-*O*-allyl-dTTPs at 50°C. Lane 4: Incorporation of 3'-*O*-azidomethyl-dTTPs at 50°C. Lane 5: Incorporation of 3'-*O*-allyl-dTTPs at 65°C. Lane 6: Incorporation of 3'-*O*-azidomethyl-dTTPs at 65°C.

e) Evaluation of the temperature on the incorporation using Terminator IX DNA polymerase. Results of incorporation performed at different temperatures.

f) Evaluation of the presence of Mn²⁺ on the incorporation using Terminator IX DNA polymerase. The Figure depicts a gel showing results of incorporation of 3'-*O*-modified-dTTP opposite inosine at 65°C. Lane S: Standards. Lane 1: Incorporation of 3'-

O-allyl-dTTPs without Mn^{2+} ions. Lane 2: Incorporation of 3'-*O*-azidomethyl-dTTPs without Mn^{2+} ions. Lane 3: Incorporation of 3'-*O*-allyl-dTTPs in presence of Mn^{2+} ions. Lane 4: Incorporation of 3'-*O*-azidomethyl-dTTPs in presence of Mn^{2+} ions.

- g) Evaluation of the presence of Mn^{2+} on the incorporation using Terminator IX DNA polymerase. Results of incorporation in presence and absence of Mn^{2+} ions.
- h) Oligonucleotides used for study of the incorporation step.

Figure 13. Absence of Helper Strand – Cleavage.

- a) Scheme showing cleavage of hybridized polynucleotide strands in the absence of a helper strand. Cleavage step is highlighted in dashed box.
- b) Gel showing cleavage of oligonucleotide with hAAG and 0.2M NaOH (strong base) at 37°C and room temperature 24°C respectively. Lane 1. Starting oligonucleotide. Lane 2 which was a positive control that contained both full length strands showed a higher yield of cleaved to uncleaved DNA ratio of 90% : 10%. Lane 3 which included the cleavage reaction without a helper strand showed a low percentage yield of cleaved to uncleaved DNA ratio of 10 % : 90%.
- c) Gel showing cleavage of oligonucleotide with hAAG and Endo VIII at 37°C. Lane 2 which was a positive control that contained both full length strands showed a higher yield of cleaved to uncleaved DNA ratio of ~ 90% : 10%. Lane 3 which included the cleavage reaction without a helper strand showed a low percentage yield of cleaved to uncleaved DNA ratio of ~7% : 93%.
- d) A summary of cleavage of oligonucleotide with hAAG/Endo VIII and hAAG/Chemical base.
- e) Oligonucleotides used for study of the cleavage step.

Figure 14. Absence of Helper Strand – Ligation.

a) Scheme showing ligation of hybridized polynucleotide strands in the absence of
 5 a helper strand. Ligation step highlighted in dashed box.

b) Gel showing ligation of Oligonucleotides with Quick T4 DNA ligase at room
 temperature (24°C) in the absence of a helper strand. Lane 1 contained a mixture of the
 36mers TAMRA single stranded oligos and 18mers TAMRA single stranded oligos. These
 oligos served reference bands.

10 c) Oligonucleotides used for study of the ligation step.

Figure 15. Version 1 Chemistry with Helper Strand – Incorporation.

a) Scheme showing incorporation step highlighted in dashed box.

15

b) Oligonucleotides applicable for study of the incorporation step.

Figure 16. Version 1 Chemistry with Helper Strand – Cleavage.

20 a) Scheme showing cleavage of hybridized polynucleotide strands in the absence of
 a helper strand. Cleavage step is highlighted in dashed box.

b) Gel showing cleavage of Oligonucleotide with hAAG and 0.2M NaOH (strong
 base) at 37°C and room temperature 24°C respectively. Lane 1. Starting oligonucleotide.
 Lane 2 which was a positive control that contained both full length strands showed a
 25 higher yield of cleaved to uncleaved DNA ratio of 90% : 10%. Lane 3 which included the
 cleavage reaction without a helper strand showed a low percentage yield of cleaved to
 uncleaved DNA ratio of 10 % : 90%. Lane 4 which included the cleavage reaction with a
 helper strand showed an equal percentage yield of cleaved to uncleaved DNA ratio of 50 %
 : 50%.

c) Evaluation of Endonuclease VIII for cleavage of abasic sites. Gel shows cleavage of oligonucleotide with hAAG and Endo VIII at 37°C. Lane 2 which was a positive control that contained both full length strands showed a higher yield of cleaved to uncleaved DNA ratio of ~ 90% : 10%. Lane 3 which included the cleavage reaction
 5 without a helper strand showed a low percentage yield of cleaved to uncleaved DNA ratio of ~7% : 93%. Lane 4 which included the cleavage reaction with a helper strand showed an low percentage yield of cleaved to uncleaved DNA ratio of 10 % : 90%.

d) Evaluation of N,N'-dimethylethylenediamine for cleavage of abasic sites. Gel shows cleavage of oligonucleotide with hAAG and 100mM N,N'-
 10 dimethylethylenediamine at 37°C. Lane 1. Starting oligonucleotide. Lane 2 which was a positive control that contained both full length strands showed a 100% cleaved DNA. Lane 3 which included the cleavage reaction with a helper strand showed a higher percentage yield of cleaved to uncleaved DNA ratio of 90 % : 10%.

e) A summary of cleavage of oligonucleotide with hAAG/Endo VIII,
 15 hAAG/chemical base and hAAG/ alternative chemical base.

f) Oligonucleotides used for study of the cleavage step.

Figure 17. Version 1 Chemistry with Helper Strand – Ligation.

20 a) Scheme showing ligation of hybridized polynucleotide strands in the presence of a helper strand. Ligation step highlighted in dashed box.

b) Gel showing ligation of oligonucleotides with Quick T4 DNA ligase at room temperature (24°C) in the presence of a helper strand. Lane 1 contained a mixture of the 36mers TAMRA single stranded oligos and 18mers TAMRA single stranded oligos. These
 25 oligos served reference bands. In lane 2 there was an observable ligation product of expected band size 36mers after 20 minutes.

c) Gel showing ligation of oligonucleotides with Quick T4 DNA ligase at room temperature (24°C) after overnight incubation in the presence of a helper strand. Lane 1 contained a mixture of the 36mers TAMRA single stranded oligos and 18mers TAMRA

single stranded oligos. These oligos served as reference bands. In lane 2 there was an observable completely ligated product of expected band size of 36mers.

d) Oligonucleotides used for study of the ligation step.

5 **Figure 18. Version 2 Chemistry with Helper Strand – Incorporation.**

a) Scheme showing incorporation step highlighted in orange dashed box

b) Gel showing results of incorporation of 3'-*O*-modified-dTTPs by Terminator IX DNA polymerase at 27°C. Lane 1: Starting material. Lane 2: Incorporation after 1
10 minute, conversion 5%. Lane 3: Incorporation after 2 minutes, conversion 10%. Lane 4: Incorporation after 5 minutes, conversion 20%. Lane 5: Incorporation after 10 minutes, conversion 30%. Lane 6: Incorporation after 20 minutes, conversion 35%.

c) The Figure depicts a gel showing results of incorporation of 3'-*O*-modified-dTTPs by Terminator IX DNA polymerase at 37°C. Lane 1: Starting material. Lane 2:
15 Incorporation after 1 minute, conversion 30%. Lane 3: Incorporation after 2 minutes, conversion 60%. Lane 4: Incorporation after 5 minutes, conversion 90%. Lane 5: Incorporation after 10 minutes, conversion 90%. Lane 6: Incorporation after 20 minutes, conversion 90%.

d) Gel showing results of incorporation of 3'-*O*-modified-dTTPs by Terminator
20 IX DNA polymerase at 47°C. Lane 1: Starting material. Lane 2: Incorporation after 1 minute, conversion 30%. Lane 3: Incorporation after 2 minutes, conversion 65%. Lane 4: Incorporation after 5 minutes, conversion 90%. Lane 5: Incorporation after 10 minutes, conversion 90%. Lane 6: Incorporation after 20 minutes, conversion 90%.

e) Gel showing results of incorporation of 3'-*O*-modified-dTTPs by Terminator
25 IX DNA polymerase at 27°C. Lane 1: Starting material. Lane 2: Incorporation after 1 minute, conversion 70%. Lane 3: Incorporation after 2 minutes, conversion 85%. Lane 4: Incorporation after 5 minutes, conversion 92%. Lane 5: Incorporation after 10 minutes, conversion 96%. Lane 6: Incorporation after 20 minutes, conversion 96%.

f) Gel showing results of incorporation of 3'-*O*-modified-dTTPs by Terminator IX
30 DNA polymerase at 37°C. Lane 1: Starting material. Lane 2: Incorporation after 1 minute,

conversion 85%. Lane 3: Incorporation after 2 minutes, conversion 95%. Lane 4: Incorporation after 5 minutes, conversion 96%. Lane 5: Incorporation after 10 minutes, conversion 96%. Lane 6: Incorporation after 20 minutes, conversion 96%.

g) Gel showing results of incorporation of 3'-*O*-modified-dTTPs by Terminator IX DNA polymerase at 47°C. Lane 1: Starting material. Lane 2: Incorporation after 1 minute, conversion 85%. Lane 3: Incorporation after 2 minutes, conversion 90%. Lane 4: Incorporation after 5 minutes, conversion 96%. Lane 5: Incorporation after 10 minutes, conversion 96%. Lane 6: Incorporation after 20 minutes, conversion 96%.

h) Summary of incorporation of 3'-*O*-azidomethyl-dTTP at various temperatures and presence of Mn²⁺ ions.

i) Gel showing results of incorporation of 3'-*O*-modified-dNTPs opposite complementary base by Terminator IX DNA polymerase in presence of Mn²⁺ at 37°C. Lane 1: Starting material. Lane 2: Incorporation of 3'-*O*-azidomethyl-dTTP for 5 minutes. Lane 3: Incorporation of 3'-*O*-azidomethyl-dATP for 5 minutes. Lane 4: Incorporation of 3'-*O*-azidomethyl-dCTP for 5 minutes. Lane 5: Incorporation of 3'-*O*-azidomethyl-dGTP for 5 minutes.

j) Oligonucleotides used for study of the incorporation step.

Figure 19. Version 2 Chemistry with Helper Strand – Cleavage.

20

a) Scheme showing cleavage of hybridized polynucleotide strand in the presence of a helper strand. Cleavage step is highlighted in orange dashed box.

b) Gel shows cleavage of Oligonucleotide with Endo V at 37°C. Lane 1. Starting oligonucleotide. Lane 2 which was a positive control that contained both full length strands showed a yield of cleaved to uncleaved DNA ratio of 80% : 20%. Lane 3 which included the cleavage reaction without a helper strand showed a much higher yield of cleaved DNA of >99%. Lane 4 which included the cleavage reaction with a helper strand also showed a DNA cleavage yield of >99%.

c) A summary of cleavage study with Endonuclease V.

d) Oligonucleotides used for study of the cleavage step.

Figure 20. Version 2 Chemistry with Helper Strand – Ligation.

a) Scheme showing ligation of hybridized polynucleotide strands in the absence of a helper strand. Ligation step highlighted in orange dashed box.

5 b) Oligonucleotides for study of the ligation step.

Figure 21. Version 2 Chemistry with Helper Strand – Deprotection.

a) Scheme showing deprotection step highlighted in orange dashed box.

10 b) The Figure depicts a gel showing results of 3'-O-azidomethyl group deprotection by 50mM TCEP after incorporation of 3'-O-azidomethyl-dTTP. Lane 1: Starting primer
Lane 2: Incorporation of 3'-O-azidomethyl-dTTPs in presence Mn^{2+} . Lane 3: Extension of the product in lane 2 by addition of all natural dNTPs. Lane 4: Deprotection of the product (0.5 μ M) in lane 2 by 50 mM TCEP. Lane 5: Extension of the product in lane 4 by
15 addition of all natural dNTPs.

c) The Figure depicts a gel showing results of 3'-O-azidomethyl group deprotection by 300mM TCEP after incorporation of 3'-O-azidomethyl-dTTP. Lane 1: Starting primer. Lane 2: Incorporation of 3'-O-azidomethyl-dTTPs in presence Mn^{2+} . Lane 3: Extension of the product in lane 2 by addition of all natural dNTPs. Lane 4: Deprotection of the product
20 (0.5 μ M) in lane 2 by 300mM TCEP. Lane 5: Extension of the product in lane 4 by addition of all natural dNTPs.

d) The Figure depicts a gel showing results of 3'-O-azidomethyl group deprotection by 50mM TCEP after incorporation of 3'-O-azidomethyl-dCTP. Lane 1: Starting primer. Lane 2: Incorporation of 3'-O-azidomethyl-dCTPs in presence Mn^{2+} . Lane 3: Extension of
25 the product in lane 2 by addition of all natural dNTPs. Lane 4: Deprotection of the product (0.5 μ M) in lane 2 by 300mM TCEP. Lane 5: Extension of the product in lane 4 by addition of all natural dNTPs.

e) The Figure depicts a gel showing results of 3'-O-azidomethyl group deprotection by 300mM TCEP after incorporation of 3'-O-azidomethyl-dCTP. Lane 1: Starting primer

Lane 2: Incorporation of 3'-*O*-azidomethyl-dCTPs in presence Mn^{2+} . Lane 3: Extension of the product in lane 1 by addition of all natural dNTPs. Lane 4: Deprotection of the product (0.5 μM) in lane 1 by 300mM TCEP. Lane 5: Extension of the product in lane 3 by addition of all natural dNTPs.

- 5 f) . The Figure depicts a gel showing results of 3'-*O*-azidomethyl group deprotection by 300mM TCEP after incorporation of 3'-*O*-azidomethyl-dATP.

Lane 1: Starting primer

- Lane 2: Incorporation of 3'-*O*-azidomethyl-dATPs in presence Mn^{2+} . Lane 3: Extension of the product in lane 2 by addition of all natural dNTPs. Lane 4: Deprotection of the product (0.5 μM) in lane 2 by 300mM TCEP. Lane 5: Extension of the product in lane 4 by addition of all natural dNTPs.

- g) The Figure depicts a gel showing results of 3'-*O*-azidomethyl group deprotection by 300mM TCEP after incorporation of 3'-*O*-azidomethyl-dGTP. Lane 1: Starting primer. Lane 2: Incorporation of 3'-*O*-azidomethyl-dGTPs in presence Mn^{2+} . Lane 3: Extension of the product in lane 2 by addition of all natural dNTPs. Lane 4: Deprotection of the product (0.5 μM) in lane 2 by 300mM TCEP. Lane 5: Extension of the product in lane 4 by addition of all natural dNTPs.

- h) Efficiency of deprotection by TCEP on 0.2 μM DNA.

- i) Oligonucleotides used for study of the cleavage step.

20

Figure 22. Version 2 Chemistry with Double Hairpin Model – Incorporation.

- a) Scheme showing incorporation step highlighted in dashed box.

- b) Evaluation of DNA polymerases for incorporation of 3'-*O*-modified-dTTPs opposite its natural counterpart. The Figure depicts a gel showing results of incorporation of 3'-*O*-modified-dTTPs by Terminator IX DNA polymerase at 37°C. Lane 1: Starting material. Lane 2: Incorporation of natural dNTP mix. Lane 3: Incorporation of 3'-*O*-azidomethyl-dTTP by Terminator IX DNA polymerase. Lane 4: Extension of the product in lane 3 by addition of all natural dNTPs.

c) Evaluation of DNA polymerases for incorporation of 3'-O-modified-dTTPs opposite its natural counterpart. Oligonucleotides applicable for study of the incorporation step.

5 **Figure 23. Version 2 Chemistry with Double Hairpin Model – Cleavage.**

a) Scheme showing cleavage of a hairpin Oligonucleotide. Cleavage step is highlighted in dashed box.

b) Gel showing cleavage of Hairpin Oligonucleotide with Endo V at 37°C. Lane 1. Starting hairpin oligonucleotide. Lane 2 which was the cleaved hairpin oligonucleotide after 5 minutes showed a high yield of digested DNA with a ratio of ~ 98%. Lane 3 which was the cleaved hairpin oligonucleotide after 10 minutes showed a high yield of digested DNA with a ratio of ~ 99%. Lane 4 which was the cleaved hairpin oligonucleotide after 30 minutes showed a high yield of digested DNA with a ratio of ~ 99% and in lane 5 which was the cleaved hairpin oligonucleotide after 1hr showed a high yield of digested DNA with a ratio of ~ 99%.

c) Oligonucleotides used for study of the cleavage step.

Figure 24. Version 2 Chemistry with Double Hairpin Model – Ligation.

20

a) Scheme showing ligation of hybridized hairpins. Ligation step highlighted in dashed box.

b) The gel shows ligation of Hairpin Oligonucleotides with Blunt/TA DNA ligase at room temperature (24°C) in the presence of a helper strand. Lane 1 contained a starting hairpin Oligonucleotide. Lane 2 which was the ligated hairpin oligonucleotide after 1 minute showed a high yield of ligated DNA product with a ratio of ~ 85%. Lane 3 which was the ligated hairpin oligonucleotide after 2 minutes showed a high yield of digested DNA with a ratio of ~ 85%. Lane 4 which was the ligated hairpin oligonucleotide after 3 minutes showed a high yield of ligated DNA product with a ratio of ~ 85%. Lane 5 which

was the ligated hairpin oligonucleotide after 4 minutes showed a high yield of ligated DNA product with a ratio of $\sim >85\%$.

c) Hairpin Oligonucleotides used for study of the Ligation step.

5 **Figure 25. Version 2 Chemistry - Complete Cycle on Double Hairpin Model.**

a) Scheme showing full cycle involving enzymatic incorporation, cleavage, ligation and deprotection steps.

b) Evaluation of DNA polymerases for incorporation of 3'-O-modified-dTTPs
 10 opposite its natural counterpart. The Figure depicts a gel showing results of incorporation of 3'-O-modified-dTTPs by Terminator IX DNA polymerase at 37°C. Lane 1: Starting material. Lane 2: Incorporation of 3'-O-azidomethyl-dTTP by Terminator IX DNA polymerase. Lane 3: Extension of the product in lane 2 by addition of all natural dNTPs. Lane 4: Cleavage of the product in lane 2 by Endonuclease V. Lane 5: Ligation of the
 15 product in lane 4 by blunt TA ligase kit.

c) Oligonucleotides applicable for study of the incorporation step.

Figure 26. Version 2 Chemistry - Complete Cycle on Single Hairpin Model using Helper Strand.

20

a) Scheme showing full cycle involving enzymatic incorporation, cleavage, ligation and deprotection steps.

b) Oligonucleotides applicable for study of the incorporation step.

25 **Figure 27. Version 3 Chemistry - Complete Cycle on Double-Hairpin Model.**

a) Scheme showing full cycle involving enzymatic incorporation, cleavage, ligation and deprotection steps.

b) Oligonucleotides applicable for study of the incorporation step.

30

Figure 28. Version 2 Chemistry – Complete Two-Cycle on Double-Hairpin Model.

- a) Scheme showing the first full cycle involving enzymatic incorporation, deprotection, cleavage and ligation steps.
- 5 b) Scheme showing the second full cycle, following the first full cycle, involving enzymatic incorporation, deprotection, cleavage and ligation steps.
- c) The Figure depicts a gel showing full two-cycle experiment comprising: incorporation, deprotection, cleavage and ligation steps.
- 10 Lane 1. Starting material.
 Lane 2. Extension of starting material with natural dNTPs.
 Lane 3. Incorporation of 3'-O-azidomethyl-dTTP by Terminator IX DNA polymerase.
 Lane 4. Extension of the product in lane 3 by addition of all natural dNTPs.
 Lane 5. Deprotection of the product in lane 3 by TCEP.
- 15 Lane 6. Extension of the product in lane 5 by addition of all natural dNTPs.
 Lane 7. Cleavage of the product in lane 5 by Endonuclease V.
 Lane 8. Ligation of the product in lane 7 by blunt TA ligase kit.
 Lane 9. Cleavage of the product in lane 8 by Lambda exonuclease.
 Lane 10. Starting material for second cycle – the same material as in lane 9.
- 20 Lane 11. Incorporation of 3'-O-azidomethyl-dTTP by Terminator IX DNA polymerase.
 Lane 12. Extension of the product in lane 11 by addition of all natural dNTPs.
 Lane 13. Deprotection of the product in lane 11 by TCEP.
 Lane 14. Extension of the product in lane 13 by addition of all natural dNTPs.
 Lane 15. Cleavage of the product in lane 13 by Endonuclease V.
- 25 Lane 16. Ligation of the product in lane 15 by blunt TA ligase kit.
- d) Oligonucleotides used for study.

Figure 29.

Example showing a mechanism of release from a scaffold polynucleotide of a polynucleotide of predefined sequence, as synthesised in accordance with the methods
5 described herein.

Figure 30.

Schematic of an exemplary method for the synthesis of RNA according to the
10 invention. The exemplary method shows synthesis in the absence of a helper strand.

Figure 31.

Schematic of an exemplary method for the synthesis of RNA according to the
15 invention. The exemplary method shows synthesis in the presence of a helper strand.

Figure 32.

Schematic of an exemplary method for the synthesis of RNA according to the
20 invention. The exemplary method shows synthesis in the presence of a helper strand.

Figure 33.

Schematic of the 1st full cycle of an exemplary method for the synthesis of DNA
25 according to synthesis method version 2 with single hairpin model, involving a step of denaturing the helper strand prior to the incorporation step.

Figure 34.

Schematic of the 2nd full cycle of an exemplary method for the synthesis of DNA according to synthesis method version 2 with single hairpin model, involving a step of
 5 denaturing the helper strand prior to the incorporation step.

Figure 35.

Schematic of the 3rd full cycle of an exemplary method for the synthesis of DNA
 10 according to synthesis method version 2 with single hairpin model, involving a step of denaturing the helper strand prior to the incorporation step.

Figure 36.

15 Oligonucleotides used in the experiments detailed in Example 9.

Figure 37.

Gel showing reaction products corresponding to a full three-cycle experiment as
 20 detailed in Example 9.

The Figure depicts a gel showing the results of a full three-cycle experiment comprising: incorporation, deblock, cleavage and ligation steps.

25 Lane 1: Starting material.
 Lane 2: Extension of starting material with natural dNTPs
 Lane 3: Incorporation of 3'-O-azidomethyl-dTTP by Terminator X DNA polymerase.
 Lane 4: Extension of the product in lane 3 by addition of all natural dNTPs.↵
 Lane 5: Deblock of the product in lane 3 by TCEP
 30 Lane 6: Extension of the product in lane 5 by addition of all natural dNTPs.↵

- Lane 7: Cleavage of the product in lane 5 by Endonuclease V.
- Lane 8: Ligation of the product in lane 7 by T3 DNA ligase
- Lane 9: Starting material for 2nd cycle – the same material as in lane 9
- Lane 10: Extension of the product in lane 9 by addition of all natural dNTPs.
- 5 Lane 11: Incorporation of 3'-O-azidomethyl-dTTP by Terminator X DNA polymerase.
- Lane 12: Extension of the product in lane 11 by addition of all natural dNTPs.
- Lane 13: Deblock of the product in lane 11 by TCEP
- Lane 14: Extension of the product in lane 13 by addition of all natural dNTPs.
- Lane 15: Cleavage of the product in lane 13 by Endonuclease V
- 10 Lane 16: Ligation of the product in lane 15 by T3 DNA ligase
- Lane 17: Starting material for 3rd cycle – the same material as in lane 16
- Lane 18: Extension of the product in lane 17 by addition of all natural dNTPs.
- Lane 19: Incorporation of 3'-O-azidomethyl-dTTP by Terminator X DNA polymerase.
- Lane 20: Extension of the product in lane 19 by addition of all natural dNTPs.
- 15 Lane 21: Deblock of the product in lane 19 by TCEP
- Lane 22: Extension of the product in lane 21 by addition of all natural dNTPs.
- Lane 23: Cleavage of the product in lane 21 by Endonuclease V
- Lane 24: Ligation of the product in lane 23 by T3 DNA ligase

20 **Figure 38.**

Fluorescence signals from polyacrylamide gel surfaces spiked with different amount of BRAPA exposed to FITC-PEG-SH and FITC-PEG-COOH.

25 **Figure 39.**

Measured fluorescence signals from fluorescein channel on polyacrylamide gel surfaces spiked with different amount of BRAPA that are exposed to FITC-PEG-SH and FITC-PEG-COOH.

30

Figure 40.

(a) Shows sequences of hairpin DNA without linker immobilised on different samples.

5 (b) Shows sequences of hairpin DNA with linker immobilised on different samples.

Figure 41.

Fluorescence signals from hairpin DNA oligomers with and without linker
10 immobilised onto bromoacetyl functionalised polyacrylamide surfaces.

Figure 42.

Measured fluorescence from hairpin DNA oligomers with and without linker
15 immobilised onto bromoacetyl functionalised polyacrylamide surfaces.

Figure 43.

Fluorescence signals from hairpin DNA oligomers with and without linker
20 immobilised onto bromoacetyl functionalised polyacrylamide surfaces following
incorporation of triphosphates.

Figure 44.

25 Measured fluorescence from hairpin DNA oligomers with and without linker
immobilised onto bromoacetyl functionalised polyacrylamide surfaces following
incorporation of triphosphates.

Figure 45.

(a) Experimental overview and outcome for each reaction step as detailed in Example 12.

5 (b) Oligonucleotides used in the experiments detailed in Example 12.

Figure 46.

Shows fluorescence signals from hairpin DNA oligomers before and after cleavage
10 reactions (Example 12).

Figure 47.

Shows measured fluorescence signals from hairpin DNA oligomers before and after
15 cleavage reactions (Example 12).

Figure 48.

Shows the sequences for the inosine-containing strand and the complimentary
20 'helper' strand for ligation reactions (Example 12).

Figure 49.

Results relating to fluorescence signals from hairpin DNA oligomers corresponding
25 to the monitoring of ligation reactions (Example 12).

Figure 50.

Results relating to measured fluorescence from hairpin DNA oligomers
30 corresponding to the monitoring of ligation reactions (Example 12).

Figure 51.

Results relating to incorporation of 3'-*O*-modified-dNTPs by Terminator X DNA
 5 polymerase using an incorporation step according to methods of the invention, *e.g.*
 synthesis method versions of the invention 1, 2 and 4 (Figures 1, 2 and 4 respectively and
 Example 13).

Figure 51a provides the nucleic acid sequences of primer strand (primer strand
 portion of synthesis strand; SEQ ID NO: 68) and template strand (support strand; SEQ ID
 10 NO: 69).

Figure 51b depicts a gel showing the results of incorporation of 3'-*O*-modified-
 dNTPs by Terminator X DNA polymerase in presence of Mn²⁺ ions at 37°C.

Lane 1: Starting oligonucleotide.

Lane 2: Incorporation of 3'-*O*-azidomethyl-dTTP (>99% efficiency)

15 Lane 3: Incorporation of 3'-*O*-azidomethyl-dATP (>99% efficiency).

Lane 4: Incorporation of 3'-*O*-azidomethyl-dCTP (>90% efficiency).

Lane 5: Incorporation of 3'-*O*-azidomethyl-dGTP (>99% efficiency).

Upon addition, the newly added 3'-*O*-modified-dNTP occupies position *n* in the
 primer strand portion. The next nucleotide position in the primer strand portion is
 20 consequently designated *n*-1, *e.g.* in accordance with step 3 of invention method versions
 1, 2 and 4 as depicted in Figures 1, 2 and 4 respectively.

Interpretation of Figures.

25 The structures depicted in Figures 11, 12a, 13a, 14a, 15a, 16a, 17a, 18a, 19a, 20a,
 21a, 22a, 23a, 24a, 25a, 26a, 27a, 28a, 28b, 29, 30, 31, 32, 33, 34, and 35 are to be
 interpreted consistently with those depicted in Figures 6, 7, 8, 9 and 10. Thus in these
 Figures, each left hand strand of a double-stranded scaffold polynucleotide molecule
 relates to the support strand (corresponding to strand "a" in Figures 6 to 10); each right
 30 hand strand of a double-stranded scaffold polynucleotide molecule relates to the synthesis

strand (corresponding to strand “b” in Figures 6 to 10); all scaffold polynucleotide molecules comprise a lower synthesis strand which corresponds to a strand comprising a primer strand portion (corresponding to the solid and dotted line of strand “b” in Figures 6 to 10); certain scaffold polynucleotide molecules (*e.g.* in Figures 15a and 23a) are shown, prior to incorporation of the new nucleotide, with an upper synthesis strand which corresponds to a strand comprising a helper strand portion (corresponding to the dashed line of strand “b” in Figures 6 to 10); certain scaffold polynucleotide molecules (*e.g.* in Figures 12a, 13a and 14a) are shown with no helper strand portion (corresponding to an absence of the dashed line of strand “b” in Figures 6 to 10); and certain scaffold polynucleotide molecules (*e.g.* in Figures 33, 34 and 35) are shown, after the ligation step, with an upper synthesis strand which corresponds to a strand comprising a helper strand portion (corresponding to the dashed line of strand “b” in Figures 6 to 10) and wherein the helper strand portion is removed prior to incorporation of the new nucleotide in the next synthesis cycle.

In addition, in these Figures, where relevant, each new nucleotide is shown to be incorporated together with a reversible terminator group, labelled rtNTP and depicted as a small circular structure (corresponding to the small triangular structure in Figures 6 to 10) and terminal phosphate groups are labelled “p” and depicted as a small elliptical structure.

Figures 11c, 11d, 11g, 11h, 22a, 23a, 24a, 25a, 27a, 28a, 28b, and 29 show scaffold polynucleotide molecules wherein strands comprising a helper strand portion and support strands are connected by a hairpin loop. Figures 11b, 22a, 23a, 24a, 25a, 26a, 27a, 28a, 28b, 29, 33, 34, and 35 show scaffold polynucleotide molecules wherein strands comprising a primer strand portion and support strands are connected by a hairpin loop.

Figures such as Figure 27a and 28a show scaffold polynucleotide molecules wherein the strand comprising a helper strand portion (upper right strand) and the support strand (upper left strand) is connected by a hairpin loop and, in the same molecule, the strand comprising the primer strand portion (lower right strand) and the support strand (lower left strand) are connected by a hairpin loop.

The data shown in Figure 51 are to be interpreted as comprising an incorporation step according to methods of the invention, *e.g.* consistent with synthesis method versions of the invention 1, 2 and 4 (Figures 1, 2 and 4 respectively).

5 **DETAILED DESCRIPTION OF THE INVENTION**

The present invention provides methods for the *de novo* synthesis of polynucleotide molecules according to a predefined nucleotide sequence. Synthesised polynucleotides are preferably DNA and are preferably double-stranded polynucleotide molecules. The invention provides advantages compared with existing synthesis methods. For example, all reaction steps may be performed in aqueous conditions at mild pH, extensive protection and deprotection procedures are not required. Furthermore, synthesis is not dependent upon the copying of a pre-existing template strand comprising the predefined nucleotide sequence.

The present inventors have determined that the use of a universal nucleotide, as defined herein, allows a newly-incorporated nucleotide to be correctly paired with its desired partner nucleotide during each cycle of synthesis. The use of a universal nucleotide allows for the creation of a polynucleotide cleavage site within a synthesised region which facilitates cleavage and repeat cycles of synthesis. The invention provides versatile methods for synthesising polynucleotides, and for assembling large fragments comprising such synthesised polynucleotides.

Certain embodiments of the synthesis methods of the invention will be described in more general detail herein by reference to exemplary methods including five exemplary method versions of the invention (Figures 1 to 5) and certain variants. It is to be understood that all exemplary methods, including the five exemplary method versions of the invention, are not intended to be limiting on the invention. The invention provides an *in vitro* method of synthesising a double-stranded polynucleotide molecule having a predefined sequence, the method comprising performing cycles of synthesis wherein in each cycle a first polynucleotide strand is extended by the incorporation of a nucleotide of the predefined sequence, and then the second polynucleotide 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. Preferably, the methods are for synthesising DNA. Specific methods described herein are provided as embodiments of the invention.

5

Reaction conditions

In one aspect the invention provides a method for synthesising a double-stranded polynucleotide having a predefined sequence.

10 In some embodiments, synthesis is carried out under conditions suitable for hybridization of nucleotides within double-stranded polynucleotides. Polynucleotides are typically contacted with reagents under conditions which permit the hybridization of nucleotides to complementary nucleotides. Conditions that permit hybridization are well-known in the art (for example, Sambrook *et al.*, 2001, Molecular Cloning: a laboratory
15 manual, 3rd edition, Cold Spring Harbour Laboratory Press; and Current Protocols in Molecular Biology, Greene Publishing and Wiley-Interscience, New York (1995)).

Incorporation of nucleotides into polynucleotides can be carried out under suitable conditions, for example using a polymerase (*e.g.*, Terminator IX polymerase) or a terminal deoxynucleotidyl transferase (TdT) enzyme or functional variant thereof to
20 incorporate modified nucleotides (*e.g.*, 3'-*O*-modified-dNTPs) at a suitable temperature (*e.g.*, ~65°C) in the presence of a suitable buffered solution. In one embodiment, the buffered solution can comprise 2 mM Tris-HCl, 1 mM (NH₄)₂SO₄, 1 mM KCl, 0.2 mM MgSO₄ and 0.01% Triton® X-100.

Cleavage of polynucleotides can be carried out under suitable conditions, for
25 example using a polynucleotide cleaving enzyme (*e.g.*, endonuclease) at a temperature that is compatible with the enzyme (*e.g.*, 37°C) in the presence of a suitable buffered solution. In one embodiment, the buffered solution can comprise 5 mM potassium acetate, 2 mM Tris-acetate, 1 mM magnesium acetate and 0.1 mM DTT.

Ligation of polynucleotides can be carried out under suitable conditions, for
30 example using a ligase (*e.g.*, T4 DNA ligase) at a temperature that is compatible with the

enzyme (*e.g.*, room temperature) in the presence of a suitable buffered solution. In one embodiment, the buffered solution can comprise 4.4 mM Tris-HCl, 7mM MgCl₂, 0.7mM dithiothreitol, 0.7mM ATP, 5% polyethylene glycol (PEG6000).

Deprotection can be carried out under suitable conditions, for example using a reducing agent (*e.g.*, TCEP). For example, deprotection can be performed using TCEP in Tris buffer (*e.g.*, at a final concentration of 300mM).

Anchor polynucleotides and scaffold polynucleotides

Double-stranded polynucleotides having a predefined sequence are synthesized by methods of the invention by incorporation of pre-defined nucleotides into a pre-existing polynucleotide, referred to herein as a scaffold polynucleotide, which may be attached to or capable of being attached to a surface as described herein. As described in more detail herein a scaffold polynucleotide forms a support structure to accommodate the newly-synthesised polynucleotide and, as will be apparent from the description herein, does not comprise a pre-existing template strand which is copied as in conventional methods of synthesis. A scaffold polynucleotide may be referred to as an anchor polynucleotide if the scaffold polynucleotide is attached to a surface. Surface attachment chemistries for attaching a scaffold polynucleotide to a surface to form an anchor polynucleotide are described in more detail herein.

In one embodiment a scaffold polynucleotide comprises a synthesis strand hybridized to a complementary support strand. The synthesis strand comprises a primer strand portion and optionally a helper strand portion separated by a single-strand break or “nick” (*e.g.* Figures 1 to 5). Both the primer strand portion and the helper strand portion of the synthesis strand may be provided hybridized to the complementary support strand. Alternatively, the helper strand portion of the synthesis strand may be provided separately. The primer strand portion of the synthesis strand may be provided first, followed by the support strand and helper strand. Alternatively components of the scaffold polynucleotide may be provided separately. For example, the support strand may be provided first, followed by the primer strand portion of the synthesis strand and then the helper strand.

The support strand may be provided first, followed by the helper strand portion of the synthesis strand and then the primer strand. The helper strand portion may be provided before a cleavage step. The helper strand portion may be omitted from a scaffold polynucleotide prior to cleavage and incorporation of a new predefined nucleotide. The helper strand portion may be removed from a scaffold polynucleotide prior to cleavage and incorporation of a new predefined nucleotide, *e.g.* by denaturation, as describe in more detail herein. Upon mixing of the components in suitable conditions the scaffold polynucleotide forms upon hybridization of the separate components.

New synthesis is initiated by polymerase or transferase enzyme at the site of the single-strand break. Thus polymerase or transferase enzyme will act to extend the terminal nucleotide of the primer strand portion at the site of the single-strand break. The single-stranded break or “nick” between the helper strand portion of the synthesis strand and the primer strand portion of the synthesis strand is typically initially achieved by providing both portions of the synthesis strand as separate molecules which will align following hybridization with the support strand. The (5') terminal nucleotide of the helper strand at the single-stranded break site is typically provided lacking a phosphate group. The lack of a terminal phosphate group prevents the terminal nucleotide of the helper strand portion ligating with the terminal nucleotide of the primer strand portion at the single-stranded break site, thus maintaining the single-stranded break. Creation and maintenance of the single-stranded break could be effected by other means. For example, the terminal nucleotide of the helper strand portion may be provided with a suitable blocking group which prevents ligation with the primer strand portion. Preferably the helper strand is provided lacking a terminal phosphate group at the single-stranded break site.

A scaffold polynucleotide may be provided with each of the support and synthesis strands unconnected at adjacent ends. A scaffold polynucleotide may be provided with both support and synthesis strands connected at adjacent ends, such as via a hairpin loop, at both ends of the scaffold polynucleotide. A scaffold polynucleotide may be provided with both support and synthesis strands connected at adjacent ends, such as via a hairpin loop, at one end of the scaffold polynucleotide or any other suitable linker.

Scaffold polynucleotides with or without hairpins may be immobilized to a solid support or surface as described in more detail herein (see Figure 11).

The terms “hairpin” or “hairpin loop” are commonly used in the current technical field. The term “hairpin loop” is also often referred to as a “stem loop”. Such terms refer to a region of secondary structure in a polynucleotide comprising a loop of unpaired nucleobases which form when one strand of a polynucleotide molecule hybridizes with another section of the same strand due to intramolecular base pairing. Thus hairpins can resemble U-shaped structures. Examples of such structures are shown in Figure 11.

10 **Nucleotides and universal nucleotides**

Nucleotides which can be incorporated into synthetic polynucleotides by any of the methods described herein may be nucleotides, nucleotide analogues and modified nucleotides.

15 In any of the synthesis methods of the invention defined and described herein, nucleotides are preferably incorporated as nucleotides comprising a reversible terminator group as described herein.

Nucleotides may comprise natural nucleobases or non-natural nucleobases. Nucleotides may contain a natural nucleobase, a sugar and a phosphate group. Natural nucleobases comprise adenosine (A), thymine (T), uracil (U), guanine (G) and cytosine (C). One of the components of the nucleotide may be further modified.

20 Nucleotide analogues are nucleotides that are modified structurally either in the base, sugar or phosphate or combination therein and that are still acceptable to a polymerase enzyme as a substrate for incorporation into an oligonucleotide strand.

25 A non-natural nucleobase may be one which will bond, *e.g.* hydrogen bond, to some degree to all of the nucleobases in the target polynucleotide. A non-natural nucleobase is preferably one which will bond, *e.g.* hydrogen bond, to some degree to nucleotides comprising the nucleosides adenosine (A), thymine (T), uracil (U), guanine (G) and cytosine (C).

A non-natural nucleotide may be a peptide nucleic acid (PNA), a locked nucleic acid (LNA) and an unlocked nucleic acid (UNA), a bridged nucleic acid (BNA) or a morpholino, a phosphorothioate or a methylphosphonate.

A non-natural nucleotide may comprise a modified sugar and/or a modified
5 nucleobase. Modified sugars include but are not limited to 2'-*O*-methylribose sugar. Modified nucleobases include but are not limited to methylated nucleobases. Methylation of nucleobases is a recognised form of epigenetic modification which has the capability of altering the expression of genes and other elements such as microRNAs. Methylation of nucleobases occurs at discrete loci which are predominately dinucleotide consisting of a
10 CpG motif, but may also occur at CHH motifs (where H is A, C, or T). Typically, during methylation a methyl group is added to the fifth carbon of cytosine bases to create methylcytosine. Thus modified nucleobases include but are not limited to 5-methylcytosine.

Nucleotides of the predefined sequence may be incorporated opposite partner
15 nucleotides to form a nucleotide pair. A partner nucleotide may be a complementary nucleotide. A complementary nucleotide is a nucleotide which is capable of bonding, *e.g.* hydrogen bonding, to some degree to the nucleotides of the predefined sequence.

Typically, a nucleotide of the predefined sequence is incorporated into a polynucleotide opposite a naturally complementary partner nucleobase. Thus adenosine
20 may be incorporated opposite thymine and vice versa. Guanine may be incorporated opposite cytosine and vice versa. Alternatively, a nucleotide of the predefined sequence may be incorporated opposite a partner nucleobase to which it will bond, *e.g.* hydrogen bond, to some degree.

Alternatively a partner nucleotide may be a non-complementary nucleotide. A
25 non-complementary nucleotide is a nucleotide which is not capable of bonding, *e.g.* hydrogen bonding, to the nucleotide of the predefined sequence. Thus a nucleotide of the predefined sequence may be incorporated opposite a partner nucleotide to form a mismatch, provided that the synthesised polynucleotide overall is double-stranded and wherein the first strand is attached to the second strand by hybridization.

The term “opposite” is to be understood as relating to the normal use of the term in the field of nucleic acid biochemistry, and specifically to conventional Watson-Crick base-pairing. Thus a first nucleic acid molecule of sequence 5'-ACGA-3' may form a duplex with a second nucleic acid molecule of sequence 5'-TCGT-3' wherein the G of the first molecule will be positioned opposite the C of the second molecule and will hydrogen bond therewith. A first nucleic acid molecule of sequence 5'-ATGA-3' may form a duplex with a second nucleic acid molecule of sequence 5'-TCGT-3', wherein the T of the first molecule will mismatch with the G of the second molecule but will still be positioned opposite therewith and will act as a partner nucleotide. This principle applies to any nucleotide partner pair relationship disclosed herein, including partner pairs comprising universal nucleotides.

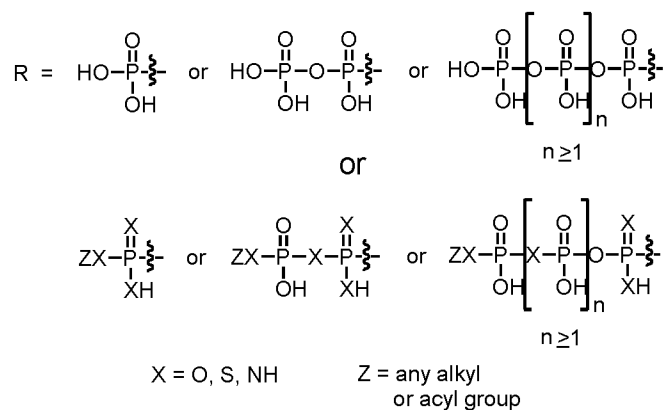
In all of the methods described herein a position in the support strand, and the opposite position in the synthesis strand, is assigned the position number “n”. This position refers to the position of a nucleotide in the support strand of a scaffold polynucleotide which in any given synthesis cycle is opposite the nucleotide position in the synthesis strand which is occupied by or will be occupied by the first/next nucleotide of the predefined sequence upon its addition to the terminal end of the primer strand portion in that cycle at step (3) or in incorporation steps of subsequent cycles. Position “n” also refers to the position in the support strand of a ligation polynucleotide at ligation step (5), or in ligation steps of subsequent cycles, which position is the nucleotide position which will be opposite the first/subsequent nucleotide of the predefined sequence upon ligation of the ligation polynucleotide to the cleaved scaffold polynucleotide in step (5) of the first cycle or in ligation steps of subsequent cycles.

Both the position in the support strand and the opposite position in the synthesis strand may be referred to as position n.

Further details concerning the definition of position “n” are provided with reference to Figures 1 to 5 and the descriptions thereof in relation to the five exemplary synthesis method versions 1 to 5 described in more detail herein.

Nucleotides and nucleotide analogues may preferably be provided as nucleoside triphosphates. Thus in any of the methods of the invention in order to synthesise DNA

polynucleotides, nucleotides may be incorporated from 2'-deoxyribonucleoside-5'-O-triphosphates (dNTPs), *e.g.* via the action of a DNA polymerase enzyme or *e.g.* via the action of an enzyme having deoxynucleotidyl terminal transferase activity. In any of the methods of the invention in order to synthesise RNA polynucleotides, nucleotides may be incorporated ribonucleoside-5'-O-triphosphates (NTPs), *e.g.* via the action of a RNA polymerase enzyme or *e.g.* via the action of an enzyme having nucleotidyl terminal transferase activity. Triphosphates can be substituted by tetraphosphates or pentaphosphates (generally oligophosphate). These oligophosphates can be substituted by other alkyl or acyl groups:



and their salts

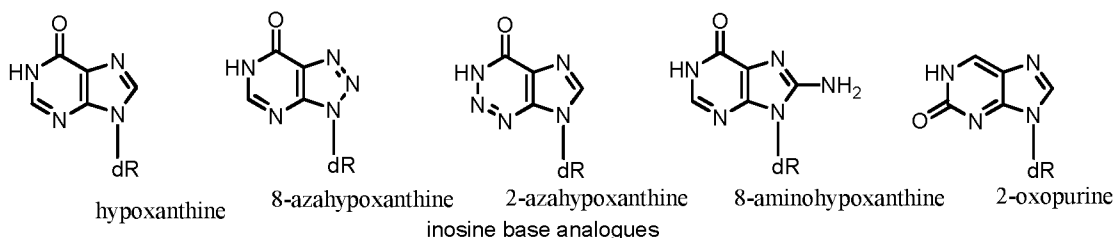
Methods of the invention may use a universal nucleotide. A universal nucleotide may be used as a component of the support strand of a scaffold molecule to facilitate a newly-incorporated nucleotide to be correctly paired with its desired partner nucleotide during each cycle of synthesis. A universal nucleotide may also be incorporated into the synthesis strand as a component of the predefined nucleotide sequence if desired.

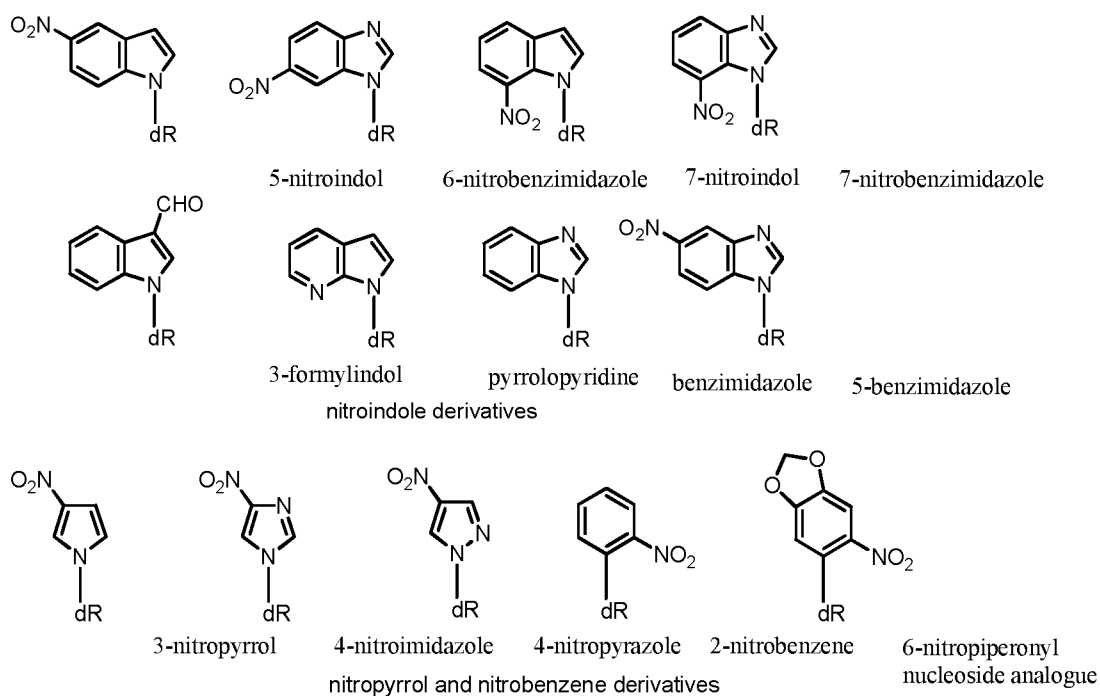
A universal nucleotide is one wherein the nucleobase will bond, *e.g.* hydrogen bond, to some degree to the nucleobase of any nucleotide of the predefined sequence. A universal nucleotide is preferably one which will bond, *e.g.* hydrogen bond, to some degree to nucleotides comprising the nucleosides adenosine (A), thymine (T), uracil (U), guanine (G) and cytosine (C). The universal nucleotide may bond more strongly to some nucleotides than to others. For instance, a universal nucleotide (I) comprising the

nucleoside, 2'-deoxyinosine, will show a preferential order of pairing of I-C > I-A > I-G approximately = I-T.

Examples of possible universal nucleotides are inosines or nitro-indoles. The universal nucleotide preferably comprises one of the following nucleobases: hypoxanthine, 4-nitroindole, 5-nitroindole, 6-nitroindole, 3-nitropyrrole, nitroimidazole, 4-nitropyrazole, 4-nitrobenzimidazole, 5-nitroindazole, 4-aminobenzimidazole or phenyl (C6-aromatic ring). The universal nucleotide more preferably comprises one of the following nucleosides: 2'-deoxyinosine, inosine, 7-deaza-2'-deoxyinosine, 7-deaza-inosine, 2-aza-deoxyinosine, 2-aza-inosine, 4-nitroindole 2'-deoxyribonucleoside, 4-nitroindole ribonucleoside, 5-nitroindole 2' deoxyribonucleoside, 5-nitroindole ribonucleoside, 6-nitroindole 2' deoxyribonucleoside, 6-nitroindole ribonucleoside, 3-nitropyrrole 2' deoxyribonucleoside, 3-nitropyrrole ribonucleoside, an acyclic sugar analogue of hypoxanthine, nitroimidazole 2' deoxyribonucleoside, nitroimidazole ribonucleoside, 4-nitropyrazole 2' deoxyribonucleoside, 4-nitropyrazole ribonucleoside, 4-nitrobenzimidazole 2' deoxyribonucleoside, 4-nitrobenzimidazole ribonucleoside, 5-nitroindazole 2' deoxyribonucleoside, 5-nitroindazole ribonucleoside, 4-aminobenzimidazole 2' deoxyribonucleoside, 4-aminobenzimidazole ribonucleoside, phenyl C-ribonucleoside or phenyl C-2'-deoxyribosyl nucleoside.

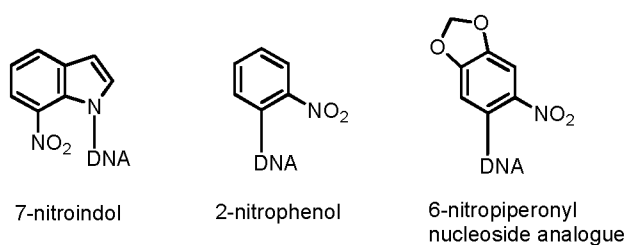
Some examples of universal bases are shown below:





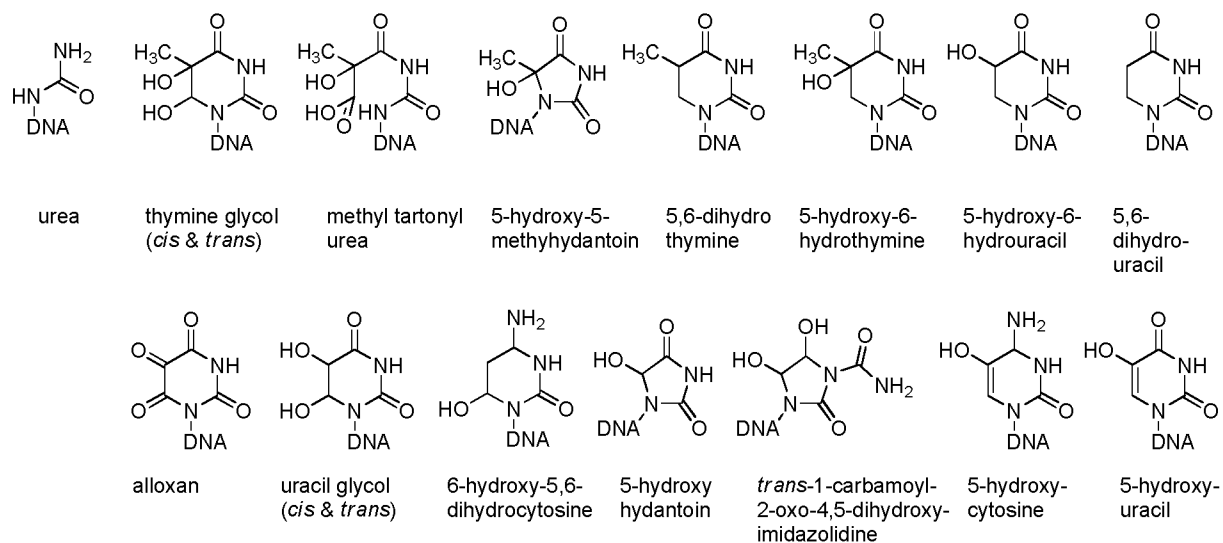
Universal nucleotides incorporating cleavable bases may also be used, including
 5 photo- and enzymatically-cleavable bases, some examples of which are shown below.

Photocleavable bases:

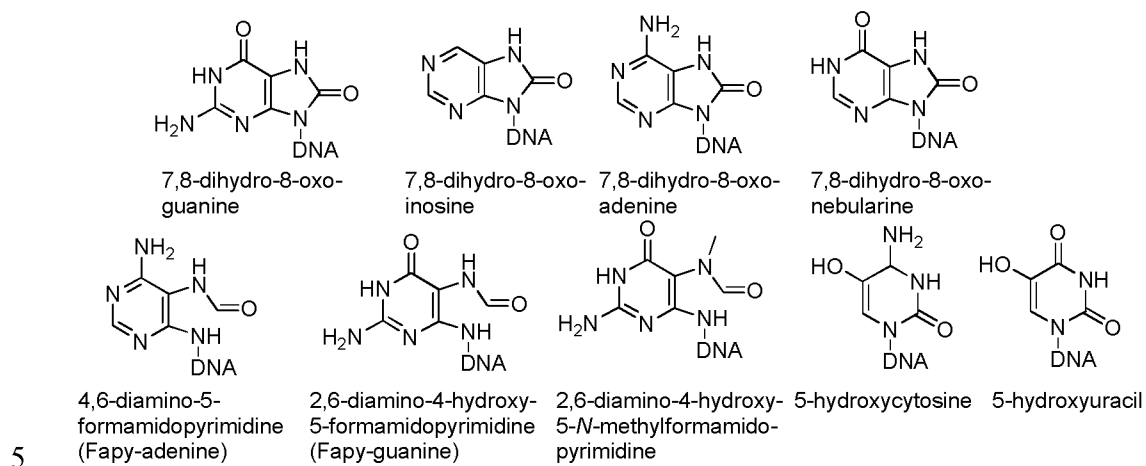


10

Base analogues cleavable by Endonuclease III:

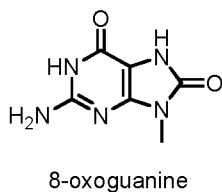


Base analogues cleavable by Formamidopyrimidine DNA glycosylase (Fpg):



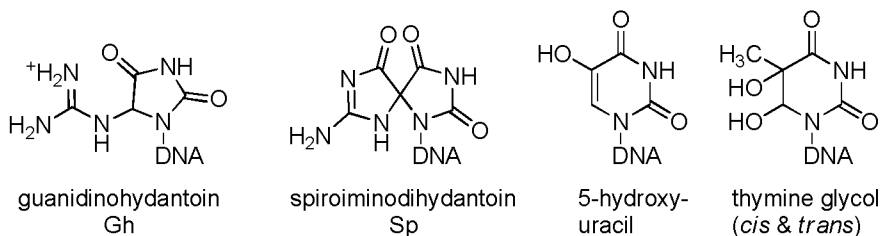
5

Base analogues cleavable by 8-oxoguanine DNA glycosylase (hOGG1):



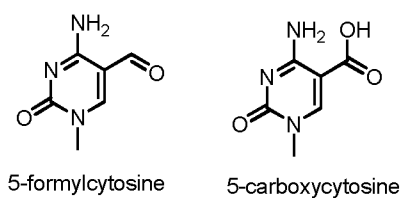
10

Base analogues cleavable by hNeil1:

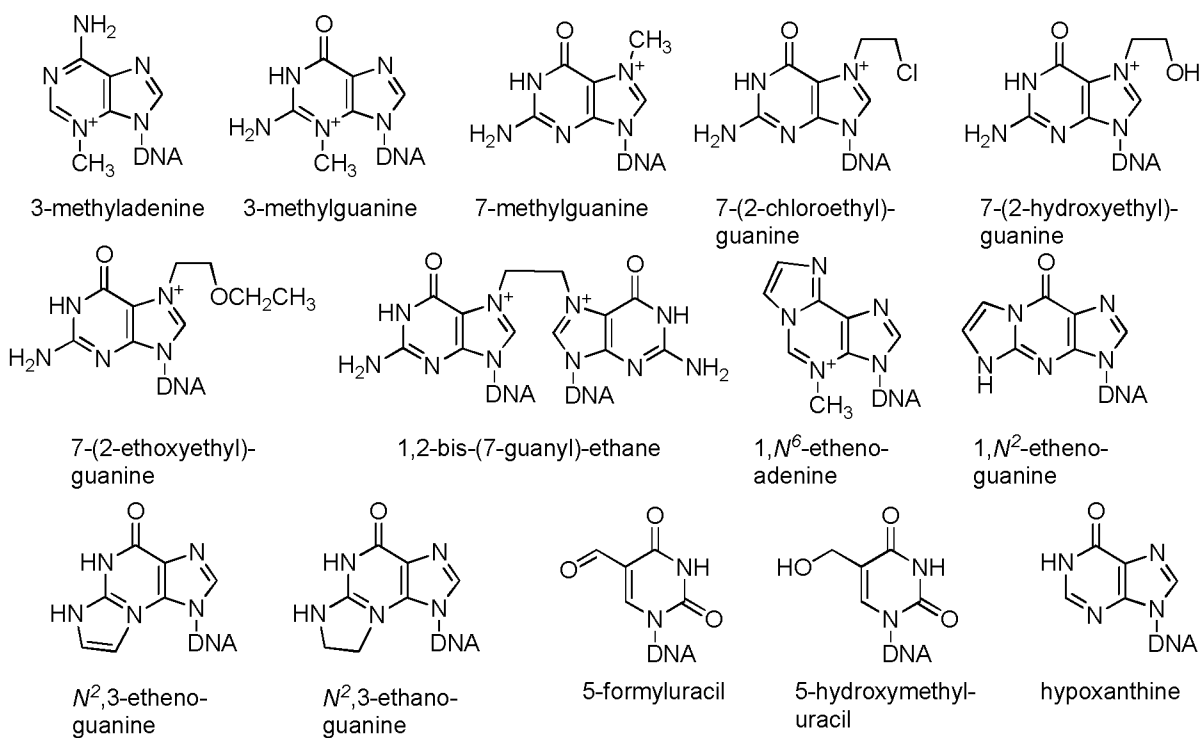


Base analogues cleavable by Thymine DNA glycosylase (TDG):

5

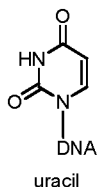


Base analogues cleavable by Human Alkyladenine DNA glycosylase (hAAG):

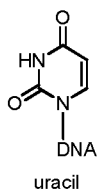


10

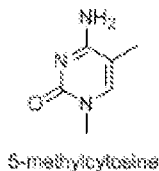
Bases cleavable by uracil DNA glycosylase:



- 5 Bases cleavable by Human single-strand-selective monofunctional uracil-DNA Glycosylase (SMUG1):



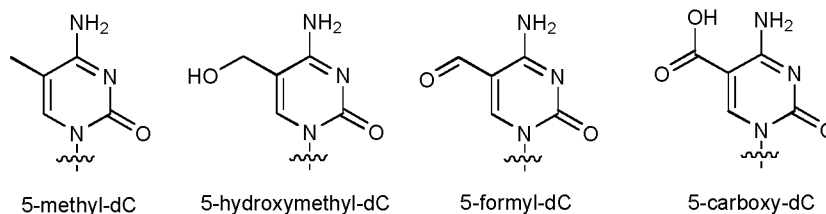
- 10 Bases cleavable by 5-methylcytosine DNA glycosylase (ROS1):



- (see S. S. David, S. D. Williams *Chemical reviews* 1998, 98, 1221-1262 and M. I. Ponferrada-Marín, T. Roldán-Arjona, R. R. Ariza *Nucleic Acids Res* 2009 ,37, 4264–4274).
- 15

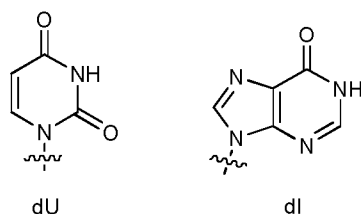
In any of the methods involving scaffold polynucleotides, the universal nucleotide most preferably comprises 2'-deoxyinosine.

- Examples of epigenetic bases which may be incorporated using any of the synthesis
- 20 methods described herein include the following:



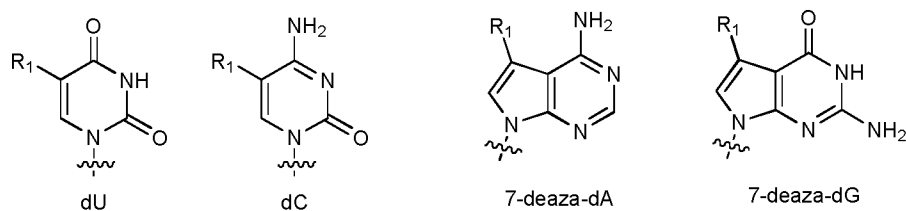
Examples of modified bases which may be incorporated using any of the synthesis methods described herein include the following:

5



Examples of halogenated bases which may be incorporated using any of the synthesis methods described herein include the following:

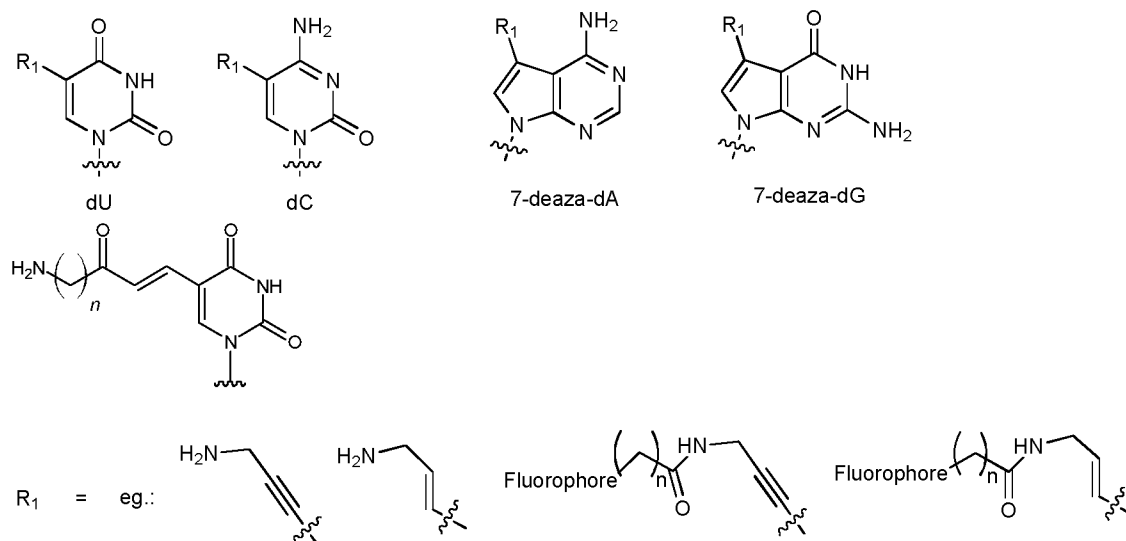
10



where R1 = F, Cl, Br, I, alkyl, aryl, fluorescent label, aminopropargyl, aminoallyl.

15

Examples of amino-modified bases, which may be useful in *e.g.* attachment/linker chemistry, which may be incorporated using any of the synthesis methods described herein include the following:

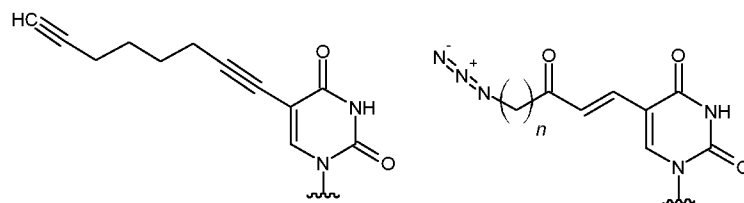


5

where base = A, T, G or C with alkyne or alkene linker.

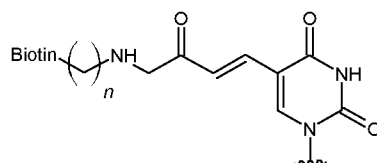
Examples of modified bases, which may be useful in *e.g.* click chemistry, which may be incorporated using any of the synthesis methods described herein include the following:

10



Examples of biotin-modified bases which may be incorporated using any of the synthesis methods described herein include the following:

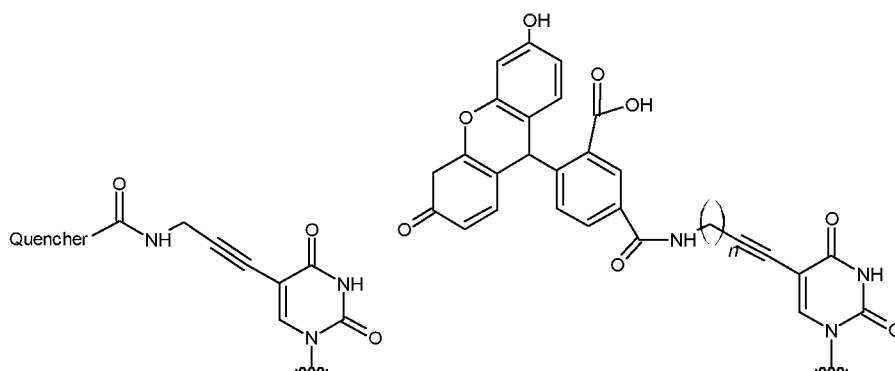
15



where base = A, T, G or C with alkyne or alkene linker.

20

Examples of bases bearing fluorophores and quenchers which may be incorporated using any of the synthesis methods described herein include the following:



5

Nucleotide-incorporating enzymes

Enzymes are available that are capable of extending, by the addition of a nucleotide, a single-stranded polynucleotide portion of a double-stranded polynucleotide molecule and/or that are capable of extending one strand of a blunt-ended double-stranded polynucleotide molecule. This includes enzymes which have template-independent enzyme activity, such as template-independent polymerase or template-independent transferase activity.

Thus in any of the methods described herein the enzyme which is used for the addition of a nucleotide of the predefined sequence, *e.g.* to the terminal end of the synthesis strand of a scaffold polynucleotide, has template-independent enzyme activity, such as template-independent polymerase or template-independent transferase activity.

Any suitable enzyme may be employed to add a predefined nucleotide using the methods described herein. Thus in all methods defined and described herein referring to the use of a polymerase or a transferase enzyme, the polymerase or transferase enzyme may be substituted with another enzyme capable of performing the same function as a polymerase or transferase enzyme in the context of the methods of the invention.

A polymerase enzyme may be employed in the methods described herein. Polymerase enzymes may be chosen based on their ability to incorporate modified nucleotides, in particular nucleotides having attached reversible terminator groups, as

25

described herein. In the exemplary methods described herein all polymerases which act on DNA must not have 3' to 5' exonuclease activity. The polymerase may have strand displacement activity.

Thus preferably the polymerase is a modified polymerase having an enhanced
5 ability to incorporate a nucleotide comprising a reversible terminator group compared to an unmodified polymerase. The polymerase is more preferably a genetically engineered variant of the native DNA polymerase from *Thermococcus* species 9°N, preferably species 9°N-7. Examples of modified polymerases are Terminator IX DNA polymerase and Terminator X DNA polymerase available from New England BioLabs. This enzyme has
10 an enhanced ability to incorporate 3'-O-modified dNTPs.

Examples of other polymerases that can be used for incorporation of reversible terminator dNTPs in any of the methods of the invention are Deep Vent (exo-), Vent (Exo-), 9°N DNA polymerase, Terminator DNA polymerase, Terminator IX DNA polymerase, Terminator X DNA polymerase, Klenow fragment (Exo-), Bst DNA
15 polymerase, Bsu DNA polymerase, Sulfolobus DNA polymerase I, and Taq Polymerase.

Examples of other polymerases that can be used for incorporation of reversible terminator NTPs in any of the methods of the invention are T3 RNA polymerase, T7 RNA polymerase, SP6 RNA polymerase, pol lambda, pol micro or Φ 29 DNA polymerase.

For the extension of such a polynucleotide synthesis molecule comprising DNA, a
20 DNA polymerase may be used. Any suitable DNA polymerase may be used.

The DNA polymerase may be for example Bst DNA polymerase full length, Bst DNA polymerase large fragment, Bsu DNA polymerase large fragment, E. coli DNA polymerase DNA Pol I large (Klenow) fragment, M-MuLV reverse transcriptase, phi29 DNA polymerase, Sulfolobus DNA polymerase IV, Taq DNA polymerase, T4 DNA
25 polymerase, T7 DNA polymerase and enzymes having reverse transcriptase activity, for example M-MuLV reverse transcriptase.

The DNA polymerase may lack 3' to 5' exonuclease activity. Any such suitable polymerase enzyme may be used. Such a DNA polymerase may be, for example, Bst DNA polymerase full length, Bst DNA polymerase large fragment, Bsu DNA polymerase

large fragment, DNA Pol I large (Klenow) fragment (3'→5' exo-), M-MuLV reverse transcriptase, Sulfolobus DNA polymerase IV, Taq DNA polymerase.

The DNA polymerase may possess strand displacement activity. Any such suitable polymerase enzyme may be used. Such a DNA polymerase may be, for example, Bst
5 DNA polymerase large fragment, Bsu DNA polymerase large fragment, DNA Pol I large (Klenow) fragment (3'→5' exo-), M-MuLV reverse transcriptase, phi29 DNA polymerase.

The DNA polymerase may lack 3' to 5' exonuclease activity and may possess strand displacement activity. Any such suitable polymerase enzyme may be used. Such a DNA polymerase may be, for example, Bst DNA polymerase large fragment, Bsu DNA
10 polymerase large fragment, E. coli DNA polymerase DNA Pol I large (Klenow) fragment, M-MuLV reverse transcriptase.

The DNA polymerase may lack 5' to 3' exonuclease activity. Any such suitable polymerase enzyme may be used. Such a DNA polymerase may be, for example, Bst DNA polymerase large fragment, Bsu DNA polymerase large fragment, DNA Pol I
15 large (Klenow) fragment, DNA Pol I large (Klenow) fragment (3'→5' exo-), M-MuLV reverse transcriptase, phi29 DNA polymerase, Sulfolobus DNA polymerase IV, T4 DNA polymerase, T7 DNA polymerase.

The DNA polymerase may lack both 3' to 5' and 5' to 3' exonuclease activities and may possess strand displacement activity. Any such suitable polymerase enzyme may be
20 used. Such a DNA polymerase may be, for example, Bst DNA polymerase large fragment, Bsu DNA polymerase large fragment, DNA Pol I large (Klenow) fragment (3'→5' exo-), M-MuLV reverse transcriptase.

The DNA polymerase may also be a genetically engineered variant. For example, the DNA polymerase may be a genetically engineered variant. of the native DNA
25 polymerase from *Thermococcus* species 9°N, such as species 9°N-7. One such example of a modified polymerase is Terminator IX DNA polymerase or Terminator X DNA polymerase available from New England BioLabs. Other engineered or variant DNA polymerases include Deep Vent (exo-), Vent (Exo-), 9°N DNA polymerase, Terminator DNA polymerase, Klenow fragment (Exo-), Bst DNA polymerase, Bsu DNA polymerase,
30 Sulfolobus DNA polymerase I, and Taq Polymerase.

For the extension of such a polynucleotide synthesis molecule comprising RNA, any suitable enzyme may be used. For example an RNA polymerase may be used. Any suitable RNA polymerase may be used.

The RNA polymerase may be T3 RNA polymerase, T7 RNA polymerase, SP6 RNA polymerase, E. coli RNA polymerase holoenzyme.

The enzyme may have a terminal transferase activity, *e.g.* the enzyme may be a terminal nucleotidyl transferase, or terminal deoxynucleotidyl transferase, and wherein the polynucleotide synthesis molecule is extended to form a polynucleotide molecule comprising DNA or RNA, preferably DNA. Any of these enzymes may be used in the methods of the invention wherein extension of a polynucleotide synthesis molecule is required.

One such enzyme is a terminal nucleotidyl transferase enzyme, such as terminal deoxynucleotidyl transferase (TdT) (see *e.g.* Motea et al, 2010; Minhaz Ud-Dean, Syst. Synth. Biol., 2008, 2(3-4), 67-73). TdT is capable of catalysing the addition to a polynucleotide synthesis molecule of a nucleotide molecule (nucleoside monophosphate) from a nucleoside triphosphate substrate (NTP or dNTP). TdT is capable of catalysing the addition of natural and non-natural nucleotides. It is also capable of catalysing the addition of nucleotide analogues (Motea et al, 2010). Pol lambda and pol micro enzymes may also be used (Ramadan K, et al., J. Mol. Biol., 2004, 339(2), 395-404), as may Φ 29 DNA polymerase.

Techniques for the extension of a single-stranded polynucleotide molecule, both DNA and RNA, in the absence of a template by the action of a terminal transferase enzyme (*e.g.* terminal deoxynucleotidyl transferase; TdT) to create an artificially-synthesised single-stranded polynucleotide molecule has been extensively discussed in the art. Such techniques are disclosed in, for example, Patent application publications WO2016/034807, WO 2016/128731, WO2016/139477 and WO2017/009663, as well as US2014/0363852, US2016/0046973, US2016/0108382, and US2016/0168611. These documents describe the controlled extension of a single-stranded polynucleotide synthesis molecule by the action of TdT to create an artificially-synthesised single-stranded polynucleotide molecule. Extension by natural and non-natural/artificial nucleotides using such enzymes is

described, as is extension by modified nucleotides, for example, nucleotides incorporating blocking groups. Any of the terminal transferase enzymes disclosed in these documents may be applied to methods of the present invention, as well as any enzyme fragment, derivative, analogue or functional equivalent thereof provided that the terminal transferase
5 function is preserved in the enzyme.

Directed evolution techniques, conventional screening, rational or semi-rational engineering/mutagenesis methods or any other suitable methods may be used to alter any such enzyme to provide and/or optimise the required function. Any other enzyme which is capable of extending a single-stranded polynucleotide molecule portion, such as a
10 molecule comprising DNA or RNA, with a nucleotide without the use of a template may be used.

Thus in any of the methods defined herein a single stranded polynucleotide synthesis molecule portion comprising DNA or blunt-ended double-stranded polynucleotide comprising DNA may be extended by an enzyme which has template-
15 independent enzyme activity, such as template-independent polymerase or transferase activity. The enzyme may have nucleotidyl transferase enzyme activity, *e.g.* a deoxynucleotidyl transferase enzyme, such as terminal deoxynucleotidyl transferase (TdT), or an enzyme fragment, derivative, analogue or functional equivalent thereof. A polynucleotide synthesis molecule extended by the action of such an enzyme comprises
20 DNA.

In any of the methods defined herein a single stranded portion of a polynucleotide synthesis molecule comprising RNA, or blunt-ended double-stranded polynucleotide comprising RNA may be extended by an enzyme which has nucleotidyl transferase enzyme (*e.g.* including TdT), or an enzyme fragment, derivative, analogue or functional
25 equivalent thereof. A polynucleotide synthesis molecule extended by the action of such an enzyme may comprise RNA. For the synthesis of a single stranded polynucleotide synthesis molecule comprising RNA, or a single stranded portion of a polynucleotide synthesis molecule comprising RNA, any suitable nucleotidyl transferase enzyme may be used. Nucleotidyl transferase enzymes such as poly (U) polymerase and poly(A)
30 polymerase (*e.g.* from *E. coli*) are capable of template-independent addition of nucleoside

monophosphate units to polynucleotide synthesis molecules. Any of these enzymes may be applied to methods of the present invention, as well as any enzyme fragment, derivative, analogue or functional equivalent thereof provided that the nucleotidyl transferase function is preserved in the enzyme. Directed evolution techniques, conventional screening,
5 rational or semi-rational engineering/mutagenesis methods or any other suitable methods may be used to alter any such enzyme to provide and/or optimise the required function.

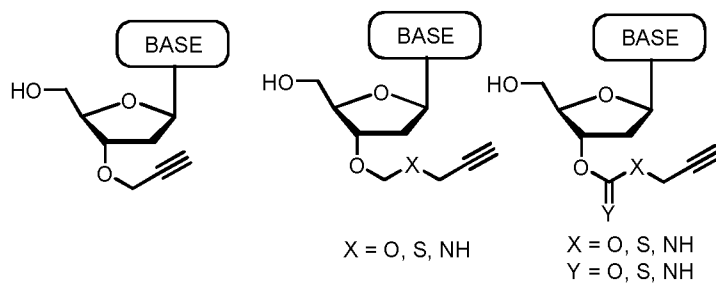
Reversible blocking groups

10 All methods defined and described herein refer to a reversible blocking group or reversible terminator group. Such groups act to prevent further extension by the enzyme in a given synthesis cycle so that only a nucleotide of predefined sequence may controllably be used to extend the synthesis strand, and thus non-specific nucleotide incorporation is prevented. Any functionality which achieves this effect may be used in any of the methods
15 defined and described herein. Reversible blocking groups/reversible terminator groups attached to nucleotides and deblocking steps are preferred means for achieving this effect. However this effect may be achieved by alternative means as appropriate.

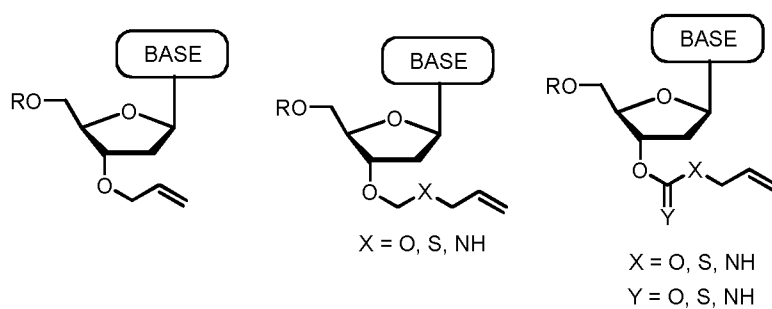
Any suitable reversible blocking group may be attached to a nucleotide to prevent further extension by the enzyme following the incorporation of a nucleotide in a given
20 cycle and to limit incorporation to one nucleotide per cycle. In any the methods of the invention the reversible blocking group is preferably a reversible terminator group which acts to prevent further extension by a polymerase enzyme. Examples of reversible terminators are provided below.

25

Propargyl reversible terminators:

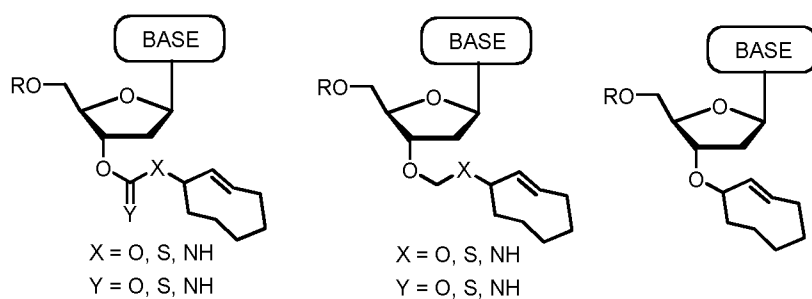


5 Allyl reversible terminators:

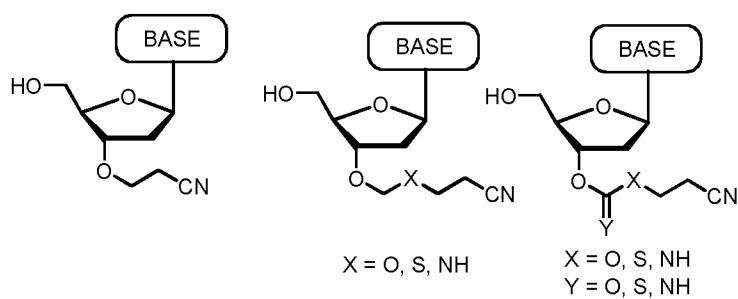


Cyclooctene reversible terminators:

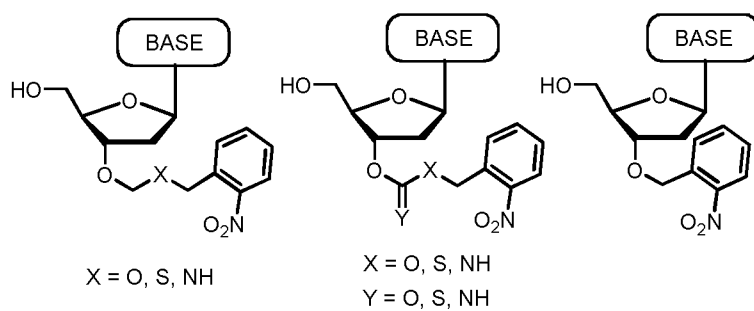
10



Cyanoethyl reversible terminators:

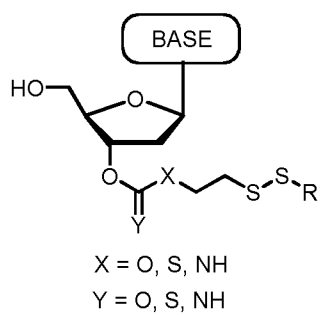


5 Nitrobenzyl reversible terminators:

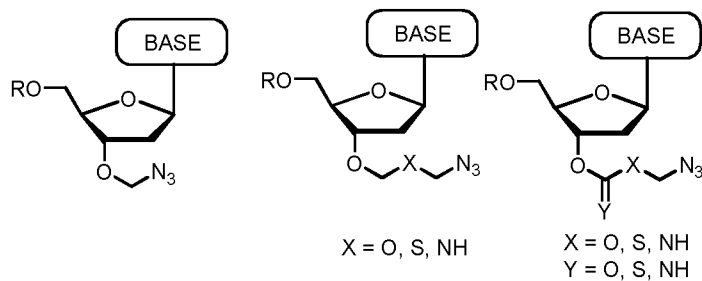


Disulfide reversible terminators:

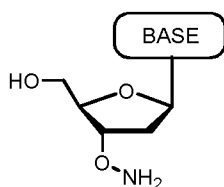
10



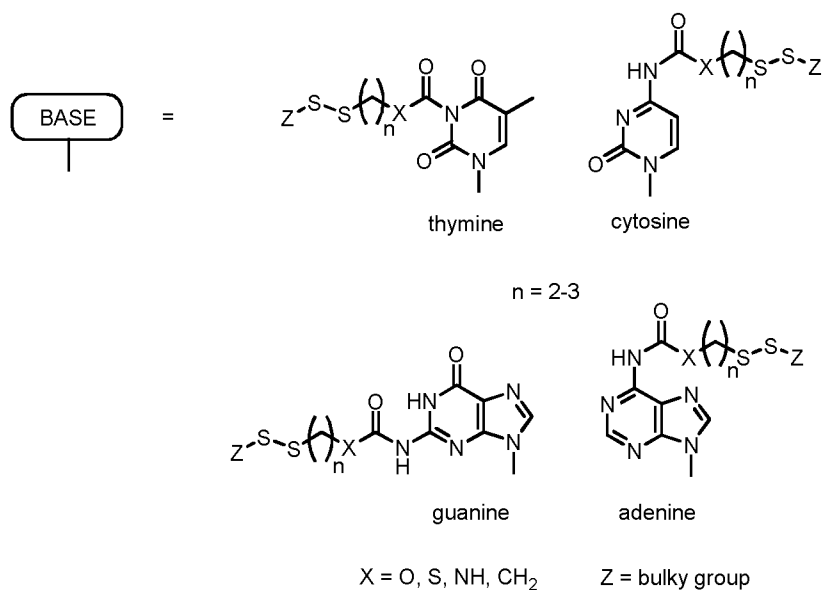
Azidomethyl reversible terminators:



5 Aminoalkoxy reversible terminators:



10 Nucleoside triphosphates with bulky groups attached to the base can serve as substitutes for a reversible terminator group on 3'-hydroxy group and can block further incorporation. This group can be deprotected by TCEP or DTT producing natural nucleotides.

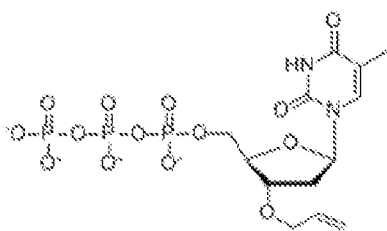


For synthesising DNA polynucleotides according to any of the methods of the invention preferred modified nucleosides are 3'-*O*-modified-2'-deoxyribonucleoside-5'-*O*-triphosphate. For synthesising RNA polynucleotides according to any of the methods of the invention preferred modified nucleosides are 3'-*O*-modified-ribonucleoside-5'-*O*-triphosphate.

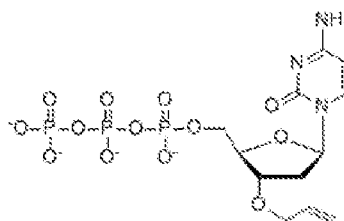
Preferred modified dNTPs are modified dNTPs which are 3'-*O*-allyl-dNTPs and 3'-*O*-azidomethyl-dNTPs.

10 3'-*O*-allyl-dNTPs are shown below.

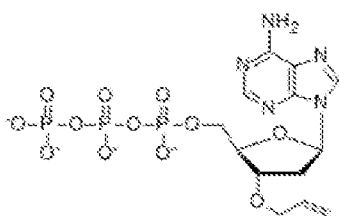
3'-*O*- allyl -dTTP:



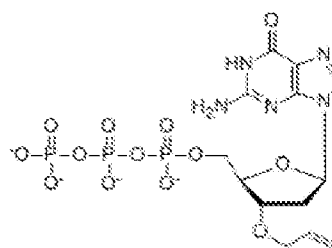
3'-*O*- allyl -dCTP:



3'-*O*- allyl -dATP:



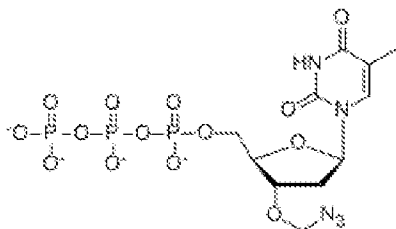
3'-*O*- allyl -dGTP:



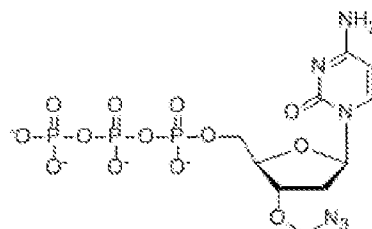
15

3'-*O*-azidomethyl-dNTPs are shown below.

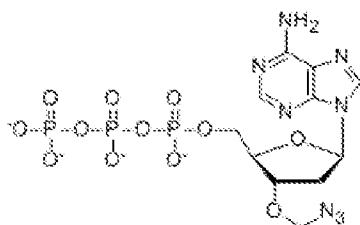
3'-O-azidomethyl-dTTP:



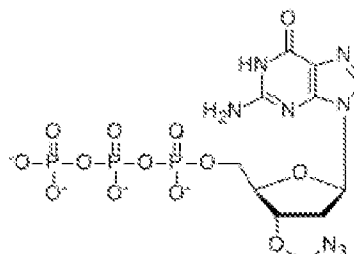
3'-O-azidomethyl-dCTP:



3'-O-azidomethyl-dATP:



3'-O-azidomethyl-dGTP:



5

Methods of the invention described and defined herein may refer to a deprotection or deblocking step. Such a step involves removal of the reversible blocking group (*e.g.* the reversible terminator group) by any suitable means, or otherwise reversing the functionality of the blocking/terminator group to inhibit further extension by the enzyme/polymerase.

Any suitable reagent may be used to remove the reversible terminator group at the deprotection step.

A preferred deprotecting reagent is tris(carboxyethyl)phosphine (TCEP). TCEP may be used to remove reversible terminator groups from 3'-O-allyl-nucleotides (in conjunction with Pd⁰) and 3'-O-azidomethyl- nucleotides following incorporation.

Examples of deprotecting reagents are provided below.

Propargyl reversible terminators:

Treatment by Pd catalysts – Na₂PdCl₄, PdCl₂.

Ligands can be used e. g.: Triphenylphosphine-3,3',3''- trisulfonic acid trisodium salt.

Allyl reversible terminators:

Treatment by Pd catalysts – Na_2PdCl_4 , PdCl_2 .

- 5 Ligands can be used e. g.: Triphenylphosphine-3,3',3''- trisulfonic acid trisodium salt.

Azidomethyl reversible terminators:

Treatment by thiol (mercaptoethanol or dithiothreitol), or Tris (2-carboxyethyl)phosphine – TCEP.

10

Cyanoethyl reversible terminators:

Treatment by fluoride – ammonium fluoride, tetrabutylammonium fluoride (TBAF).

Nitrobenzyl reversible terminators:

- 15 Exposure to UV light

Disulfide reversible terminators:

Treatment by thiol (mercaptoethanol or dithiothreitol), or Tris (2-carboxyethyl)phosphine – TCEP.

20

Aminoalkoxy reversible terminators:

Treatment by nitrite (NO_2^- , HNO_2) pH = 5.5

- 25 A reversible blocking group (*e.g.*, a reversible terminator group) can be removed by a step performed immediately after the incorporation step and before the cleavage step, provided that unwanted reagent from the incorporation step is removed to prevent further incorporation following removal of the reversible terminator group. A reversible blocking group (*e.g.*, a reversible terminator group) can be removed by a step performed immediately after the cleavage step and before the ligation step. A reversible blocking

group (*e.g.*, a reversible terminator group) can be removed by a step performed immediately after the ligation step.

Synthetic polynucleotide

5

The polynucleotide having a predefined sequence synthesised according to the methods described herein is double-stranded. The synthesised polynucleotide overall is double-stranded and wherein the first strand is attached to the second strand by hybridization. Mismatches and regions of non-hybridization may be tolerated, provided that overall the first strand is attached to the second strand by hybridization.

Hybridisation may be defined by moderately stringent or stringent hybridisation conditions. A moderately stringent hybridisation condition uses a prewashing solution containing 5x sodium chloride/sodium citrate (SSC), 0.5% SDS, 1.0 mM EDTA (pH 8.0), hybridisation buffer of about 50% formamide, 6xSSC, and a hybridisation temperature of 55° C (or other similar hybridisation solutions, such as one containing about 50% formamide, with a hybridisation temperature of 42° C), and washing conditions of 60° C, in 0.5xSSC, 0.1% SDS. A stringent hybridisation condition hybridises in 6xSSC at 45° C, followed by one or more washes in 0.1xSSC, 0.2% SDS at 68° C.

The double-stranded polynucleotide having a predefined sequence synthesised according to the methods described herein may be retained as a double-stranded polynucleotide. Alternatively the two strands of the double-stranded polynucleotide may be separated to provide a single-stranded polynucleotide having a predefined sequence. Conditions that permit separation of two strands of a double-stranded polynucleotide (melting) are well-known in the art (for example, Sambrook *et al.*, 2001, Molecular Cloning: a laboratory manual, 3rd edition, Cold Spring Harbour Laboratory Press; and Current Protocols in Molecular Biology, Greene Publishing and Wiley-Interscience, New York (1995)).

The double-stranded polynucleotide having a predefined sequence synthesised according to the methods described herein may be amplified following synthesis. Any region of the double-stranded polynucleotide may be amplified. The whole or any region

of the double-stranded polynucleotide may be amplified together with the whole or any region of the scaffold polynucleotide. Conditions that permit amplification of a double-stranded polynucleotide are well-known in the art (for example, Sambrook *et al.*, 2001, Molecular Cloning: a laboratory manual, 3rd edition, Cold Spring Harbour Laboratory Press; and Current Protocols in Molecular Biology, Greene Publishing and Wiley-Interscience, New York (1995)). Thus any of the synthesis methods described herein may further comprise an amplification step wherein the double-stranded polynucleotide having a predefined sequence, or any region thereof, is amplified as described above.

Amplification may be performed by any suitable method, such as polymerase chain reaction (PCR), polymerase spiral reaction (PSR), loop mediated isothermal amplification (LAMP), nucleic acid sequence based amplification (NASBA), self-sustained sequence replication (3SR), rolling circle amplification (RCA), strand displacement amplification (SDA), multiple displacement amplification (MDA), ligase chain reaction (LCR), helicase dependant amplification (HDA), ramification amplification method (RAM) etc.

Preferably, amplification is performed by polymerase chain reaction (PCR).

The double-stranded or single-stranded polynucleotide having a predefined sequence synthesised according to the methods described herein can be any length. For example, the polynucleotides can be at least 10, at least 50, at least 100, at least 150, at least 200, at least 250, at least 300, at least 350, at least 400, at least 450 or at least 500 nucleotides or nucleotide pairs in length. For example, the polynucleotides may be from about 10 to about 100 nucleotides or nucleotide pairs, about 10 to about 200 nucleotides or nucleotide pairs, about 10 to about 300 nucleotides or nucleotide pairs, about 10 to about 400 nucleotides or nucleotide pairs and about 10 to about 500 nucleotides or nucleotide pairs in length. The polynucleotides can be up to about 1000 or more nucleotides or nucleotide pairs, up to about 5000 or more nucleotides or nucleotide pairs in length or up to about 100000 or more nucleotides or nucleotide pairs in length.

Cleavage of scaffold polynucleotide

In methods requiring the presence of scaffold polynucleotides and steps of cleavage prior to ligation, the selection of the reagent to perform the cleavage step will depend upon the particular method employed. The cleavage site is defined by the specific position of the universal nucleotide in the support strand and the requirement for a blunt-ended scaffold polynucleotide once cleaved or the requirement for an overhang in the scaffold polynucleotide once cleaved. Configuration of the desired cleavage site and selection of the appropriate cleavage reagent will therefore depend upon the specific chemistry employed in the method, as will readily be apparent by reference to the exemplary methods described herein.

Some examples of DNA cleaving enzymes recognizing modified bases are shown in the Table below.

DNA glycosylase/ Endonuclease	Main substrate	Cleavage site	Termini created from the cleavage	
			5'-end	3'-end
APE1	AP site	1 st phosphodiester bond 5' to the lesion	Deoxyribose- 5'-phosphate	OH
Endonuclease III	AP site, thymine glycol	1 st phosphodiester bond 3' to the lesion	phosphate	3'-phospho- α , β -unsaturated aldehyde
Endonuclease IV	AP site	1 st phosphodiester bond 5' to the lesion	Deoxyribose- 5'-phosphate	OH
Endonuclease V	Inosine	2 nd phosphodiester	phosphate	OH

		bond 3' to the lesion		
Endonuclease VIII	AP site, thymine glycol	1 st phosphodiester bond 5' and 3' to the lesion	phosphate	phosphate
FpG	8-oxoguanine	1 st phosphodiester bond 5' and 3' to the lesion	phosphate	phosphate
hOGG1	8-oxoguanine	1 st phosphodiester bond 3' to the lesion	phosphate	3'-phospho- α , β -unsaturated aldehyde
hNeil1	Oxidized purines	1 st phosphodiester bond 5' and 3' to the lesion	phosphate	phosphate
ROS1	5-methylcytosine	1 st phosphodiester bond 5' and 3' to the lesion	phosphate	phosphate
Uracil DNA glycosylase	Uracil	<i>N</i> -glycosidic bond	AP site (no break)	
hSMUG	Uracil	<i>N</i> -glycosidic bond	AP site (no break)	
hAAG	Inosine	<i>N</i> -glycosidic bond	AP site (no break)	

Ligation polynucleotide

In methods requiring the presence of scaffold polynucleotides and steps of ligation following cleavage, the selection of the configuration and structure of the ligation polynucleotide will also depend upon the particular method employed. The ligation polynucleotide generally comprises a support strand as described herein and a helper strand as described herein. The support strand and the helper strand used in the ligation

polynucleotide can be the same or different from those used in the initial scaffold polynucleotide construct. For example, the requirement for a single- or double-nucleotide overhang in the support strand of the ligation end of the ligation polynucleotide will depend upon the method employed. The appropriate structure can readily be achieved by
5 reference to the exemplary methods described herein.

The complementary ligation end of the ligation polynucleotide is typically provided with a non-phosphorylated terminal nucleotide in the helper strand adjacent the overhang. This prevents ligation of the helper strand portion of the synthesis strand to the primer strand portion of the synthesis strand and thus maintains the single-strand break in the
10 synthesis strand. Alternative means for preventing ligation in the synthesis strands could be employed. For example blocking moieties could be attached to the terminal nucleotide in the helper strand. Moreover, the helper stand may be removed from the scaffold molecule, *e.g.* by denaturation, prior to cleavage and incorporation of the next predefined nucleotide in the next synthesis cycle, as described further herein.

15

Ligation

In methods of the invention which involve a ligation step, ligation may be achieved using any suitable means. Preferably, the ligation step will be performed by a ligase
20 enzyme. The ligase may be a modified ligase with enhanced activity for single-base overhang substrates. The ligase may be a T3 DNA ligase or a T4 DNA ligase. The ligase may be a blunt TA ligase. For example a blunt TA ligase is available from New England BioLabs (NEB). This is a ready-to-use master mix solution of T4 DNA Ligase, ligation enhancer, and optimized reaction buffer specifically formulated to improve ligation and
25 transformation of both blunt-end and single-base overhang substrates. Molecules, enzymes, chemicals and methods for ligating (joining) single- and double-stranded polynucleotides are well known to the skilled person.

Solid phase synthesis

Synthetic polynucleotides produced in accordance with the synthesis methods of the invention may preferably be synthesised using solid phase or reversible solid phase techniques. A variety of such techniques is known in the art and may be used. Before initiating synthesis of a new double-stranded polynucleotide of predefined sequence, scaffold polynucleotides may be immobilized to a surface *e.g.* a planar surface such as glass, a gel-based material, or the surface of a microparticle such as a bead or functionalised quantum dot. The material comprising the surface may itself be bound to a substrate. For example, scaffold polynucleotides may be immobilized to a gel-based material such as *e.g.* polyacrylamide, and wherein the a gel-based material is bound to a supporting substrate such as glass.

Polynucleotides may be immobilized or tethered to surfaces directly or indirectly. For example they may be attached directly to surfaces by chemical bonding. They may be indirectly tethered to surfaces via an intermediate surface, such as the surface of a microparticle or bead *e.g.* as in SPRI or as in electrowetting systems, as described below. Cycles of synthesis may then be initiated and completed whilst the scaffold polynucleotide incorporating the newly-synthesised polynucleotide is immobilized.

In such methods a double-stranded scaffold polynucleotide may be immobilized to a surface prior to the incorporation of the first nucleotide of the predefined sequence. Such an immobilized double-stranded scaffold polynucleotide may therefore act as an anchor to tether the double-stranded polynucleotide of the predefined sequence to the surface during and after synthesis.

Only one strand of such a double-stranded anchor/scaffold polynucleotide may be immobilized to the surface at the same end of the molecule. Alternatively both strands of a double-stranded anchor/scaffold polynucleotide may each be immobilized to the surface at the same end of the molecule. A double-stranded anchor/scaffold polynucleotide may be provided with each strand connected at adjacent ends, such as via a hairpin loop at the opposite end to the site of initiation of new synthesis, and connected ends may be immobilized on a surface (for example as depicted schematically in Figure 11).

In methods involving a scaffold polynucleotide, as described herein, the scaffold polynucleotide may be attached to a surface prior to the incorporation of the first nucleotide in the predefined sequence. Thus the synthesis strand comprising the primer strand portion and the portion of the support strand hybridized thereto may both be
5 separately attached to a surface, as depicted in Figure 11(a) and (c). The synthesis strand comprising the primer strand portion and the portion of the support strand hybridized thereto may be connected at adjacent ends, such as via a hairpin loop, *e.g.* at the opposite end to the site of initiation of new synthesis, and connected ends may be tethered to a surface, as depicted in Figure 11(b) and (d). One or other of the synthesis strand
10 comprising the primer strand portion and the portion of the support strand hybridized thereto may be attached separately to a surface, as depicted in Figure 11(e) to (h). Preferably the synthesis strand comprising the primer strand portion and the portion of the support strand hybridized thereto is attached to a surface.

15 Solid phase synthesis on planar surfaces

Before initiating synthesis of a new double-stranded polynucleotide of predefined sequence synthetic anchor/scaffold polynucleotides can be synthesised by methods known in the art, including those described herein, and tethered to a surface.

20 Pre-formed polynucleotides can be tethered to surfaces by methods commonly employed to create nucleic acid microarrays attached to planar surfaces. For example, anchor/scaffold polynucleotides may be created and then spotted or printed onto a planar surface. Anchor/scaffold polynucleotides may be deposited onto surfaces using contact printing techniques. For example, solid or hollow tips or pins may be dipped into solutions
25 comprising pre-formed scaffold polynucleotides and contacted with the planar surface. Alternatively, oligonucleotides may be adsorbed onto micro-stamps and then transferred to a planar surface by physical contact. Non-contact printing techniques include thermic printing or piezoelectric printing wherein sub-nanolitre size microdroplets comprising pre-formed scaffold polynucleotides may be ejected from a printing tip using methods similar
30 to those used in inkjet and bubblejet printing.

Single-stranded oligonucleotides may be synthesised directly on planar surfaces such as using so-called “on-chip” methods employed to create microarrays. Such single-stranded oligonucleotides may then act as attachment sites to immobilize pre-formed anchor/scaffold polynucleotides.

5 On-chip techniques for generating single-stranded oligonucleotides include photolithography which involves the use of UV light directed through a photolithographic mask to selectively activate a protected nucleotide allowing for the subsequent incorporation of a new protected nucleotide. Cycles of UV-mediated deprotection and coupling of pre-determined nucleotides allows the in situ generation of an oligonucleotide
10 having a desired sequence. As an alternative to the use of a photolithographic mask, oligonucleotides may be created on planar surfaces by the sequential deposition of nucleobases using inkjet printing technology and the use of cycles of coupling, oxidation and deprotection to generate an oligonucleotide having a desired sequence (for a review see Kosuri and Church, Nature Methods, 2014, 11, 499-507).

15 In any of the synthesis methods described herein, including methods involving reversible immobilisation as described below, surfaces can be made of any suitable material. Typically a surface may comprise silicon, glass or polymeric material. A surface may comprise a gel surface, such as a polyacrylamide surface, such as about 2% polyacrylamide, optionally a polyacrylamide surface derived using N- (5-
20 bromoacetamidylpentyl) acrylamide (BRAPA), preferably the polyacrylamide surface is coupled to a solid support, such as glass.

Reversible immobilization

25 Synthetic polynucleotides having a predefined sequence can be synthesised in accordance with the invention using binding surfaces and structures, such as microparticles and beads, which facilitate reversible immobilization. Solid phase reversible immobilization (SPRI) methods or modified methods are known in the art and may be employed (*e.g.* see DeAngelis M. M. et al. (1995) Solid-Phase Reversible Immobilization
30 for the Isolation of PCR Products, Nucleic Acids Research, 23(22): 4742–4743.).

Surfaces can be provided in the form of microparticles, such as paramagnetic beads. Paramagnetic beads can agglomerate under the influence of a magnetic field. For example, paramagnetic surfaces can be provided with chemical groups, *e.g.* carboxyl groups, which in appropriate attachment conditions will act as binding moieties for nucleic acids, as described in more detail below. Nucleic acids can be eluted from such surfaces in appropriate elution conditions. Surfaces of microparticles and beads can be provided with UV-sensitive polycarbonate. Nucleic acids can be bound to the activated surface in the presence of a suitable immobilization buffer.

Microparticles and beads may be allowed to move freely within a reaction solution and then reversibly immobilized, *e.g.* by holding the bead within a microwell or pit etched into a surface. A bead can be localised as part of an array *e.g.* by the use of a unique nucleic acid “barcode” attached to the bead or by the use of colour-coding.

Thus before initiating synthesis of a new double-stranded polynucleotide of predefined sequence, anchor/scaffold polynucleotides in accordance with the invention can be synthesised and then reversibly immobilized to such binding surfaces. Polynucleotides synthesised by methods of the invention can be synthesised whilst reversibly immobilized to such binding surfaces.

Microfluidic techniques and systems

20

The surface may be part of an electrowetting-on-dielectric system (EWOD). EWOD systems provide a dielectric-coated surface which facilitates microfluidic manipulation of very small liquid volumes in the form of microdroplets (*e.g.* see Chou, W-L., et al. (2015) Recent Advances in Applications of Droplet Microfluidics, Micromachines, 6: 1249-1271.). Droplet volumes can programmably be created, moved, partitioned and combined on-chip by electrowetting techniques. Thus electrowetting systems provide alternative means to reversibly immobilize polynucleotides during and after synthesis.

Polynucleotides having a predefined sequence may be synthesised in solid phase by methods described herein, wherein polynucleotides are immobilized on an EWOD surface

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and required steps in each cycle facilitated by electrowetting techniques. For example, in methods involving scaffold polynucleotides and requiring incorporation, cleavage, ligation and deprotection steps, reagents required for each step, as well as for any required washing steps to remove used and unwanted reagent, can be provided in the form of microdroplets
5 transported under the influence of an electric field via electrowetting techniques.

Other microfluidic platforms are available which may be used in the synthesis methods of the invention. For example, the emulsion-based microdroplet techniques which are commonly employed for nucleic acid manipulation can be used. In such systems microdroplets are formed in an emulsion created by the mixing of two immiscible
10 fluids, typically water and an oil. Emulsion microdroplets can be programmably be created, moved, partitioned and combined in microfluidic networks. Hydrogel systems are also available. In any of the synthesis methods described herein microdroplets may be manipulated in any suitable compatible system, such as EWOD systems described above and other microfluidic systems, *e.g.* microfluidic systems comprising architectures based
15 on components comprising elastomeric materials.

Microdroplets may be of any suitable size, provided that they are compatible with the synthesis methods herein. Microdroplet sizes will vary depending upon the particular system employed and the relevant architecture of the system. Sizes may thus be adapted as appropriate. In any of the synthesis methods described herein droplet diameters may be in
20 the range from about 150nm to about 5mm. Droplet diameters below 1µm may be verified by means known in the art, such as by techniques involving capillary jet methods, *e.g.* as described in Gañán-Calvo *et al.* (Nature Physics, 2007, 3, pp737–742)

Sequencing of intermediate or final synthesis products.

25

The intermediate products of synthesis or assembly, or the final polynucleotide synthesis products may be sequenced as a quality control check to determine whether the desired polynucleotide or polynucleotides have been correctly synthesised or assembled. The polynucleotide or polynucleotides of interest can be removed from the solid phase
30 synthesis platform and sequenced by any one of a number of known commercially

available sequencing techniques such as nanopore sequencing using a MinION™ device sold by Oxford Nanopore Technologies Ltd. In a particular example, the sequencing may be carried out on the solid phase platform itself, removing the need to transfer the polynucleotide to a separate synthesis device. Sequencing may be conveniently carried out
5 on the same electrowetting device, such as an EWOD device as used for synthesis whereby the synthesis device comprises one or more measurement electrode pairs. A droplet comprising the polynucleotide of interest can be contacted with one of the electrodes of the electrode pair, the droplet forming a droplet interface bilayer with a second droplet in contact with the second electrode of the electrode pair wherein the droplet bilayer interface
10 comprises a nanopore in an amphipathic membrane. The polynucleotide can be caused to translocate the nanopore for example under enzyme control and ion current flow through the nanopore can be measured under a potential difference between the electrode pair during passage of the polynucleotide through the nanopore. The ion current measurements over time can be recorded and used to determine the polynucleotide sequence. Prior to
15 sequencing, the polynucleotide may be subjected to one or more sample preparation steps in order to optimise it for sequencing such as disclosed in patent application no. PCT/GB2015/050140. Examples of enzymes, amphipathic membranes and nanopores which may be suitably employed are disclosed in patent application nos. PCT/GB2013/052767 and PCT/GB2014/052736. The necessary reagents for sample
20 preparation of the polynucleotide, nanopores, amphipathic membranes and so on may be supplied to the EWOD device via sample inlet ports. The sample inlet ports may be connected to reagent chambers.

Surface attachment chemistries

25

Although oligonucleotides will typically be attached chemically, they may also be attached to surfaces by indirect means such as via affinity interactions. For example, oligonucleotides may be functionalised with biotin and bound to surfaces coated with avidin or streptavidin.

For the immobilization of polynucleotides to surfaces (*e.g.* planar surfaces), microparticles and beads etc., a variety of surface attachment methods and chemistries are available. Surfaces may be functionalised or derivatized to facilitate attachment. Such functionalisations are known in the art. For example, a surface may be functionalised with a polyhistidine-tag (hexa histidine-tag, 6xHis-tag, His6 tag or His-tag®), Ni-NTA, streptavidin, biotin, an oligonucleotide, a polynucleotide (such as DNA, RNA, PNA, GNA, TNA or LNA), carboxyl groups, quaternary amine groups, thiol groups, azide groups, alkyne groups, DIBO, lipid, FLAG-tag (FLAG octapeptide), polynucleotide binding proteins, peptides, proteins, antibodies or antibody fragments. The surface may be functionalised with a molecule or group which specifically binds to the anchor/scaffold polynucleotide.

Some examples of chemistries suitable for attaching polynucleotides to surfaces are shown in Figure 11i and Figure 11j.

In any of the methods described herein the scaffold polynucleotide comprising the synthesis strand comprising the primer strand portion and the portion of the support strand hybridized thereto may be tethered to a common surface via one or more covalent bonds. The one or more covalent bonds may be formed between a functional group on the common surface and a functional group on the scaffold molecule. The functional group on the scaffold molecule may be *e.g.* an amine group, a thiol group, a thiophosphate group or a thioamide group. The functional group on the common surface may be a bromoacetyl group, optionally wherein the bromoacetyl group is provided on a polyacrylamide surface derived using N- (5- bromoacetamidylpentyl) acrylamide (BRAPA).

In any of the methods of the invention a scaffold polynucleotide may be attached to a surface, either directly or indirectly, via a linker. Any suitable linker which is biocompatible and hydrophilic in nature may be used.

A linker may be a linear linker or a branched linker.

A linker may comprise a hydrocarbon chain. A hydrocarbon chain may comprise from 2 to about 2000 or more carbon atoms. The hydrocarbon chain may comprise an alkylene group, *e.g.* C2 to about 2000 or more alkylene groups. The hydrocarbon chain may have a general formula of $-(CH_2)_n-$ wherein *n* is from 2 to about 2000 or more. The

hydrocarbon chain may be optionally interrupted by one or more ester groups (i.e. $-\text{C}(\text{O})-\text{O}-$) or one or more amide groups (i.e. $-\text{C}(\text{O})-\text{N}(\text{H})-$).

Any linker may be used selected from the group comprising PEG, polyacrylamide, poly(2-hydroxyethyl methacrylate), Poly-2-methyl-2-oxazoline (PMOXA), zwitterionic
 5 polymers, *e.g.* poly(carboxybetaine methacrylate) (PCBMA), poly[N -(3-sulfopropyl)- N -methacryloxyethyl- N , N dimethyl ammonium betaine] (PSBMA), glycopolymers, and polypeptides.

A linker may comprise a polyethylene glycol (PEG) having a general formula of $-(\text{CH}_2-\text{CH}_2-\text{O})_n-$, wherein n is from 1 to about 600 or more.

10 A linker may comprise oligoethylene glycol-phosphate units having a general formula of $-\text{[(CH}_2-\text{CH}_2-\text{O})_n-\text{PO}_2^--\text{O}]_m-$ where n is from 1 to about 600 or more and m could be 1-200 or more.

Any of the above-described linkers may be attached at one end of the linker to a scaffold molecule as described herein, and at the other end of the linker to a first functional
 15 group wherein the first functional group may provide a covalent attachment to a surface. The first functional group may be *e.g.* an amine group, a thiol group, a thiophosphate group or a thioamide group as further described herein. The surface may be functionalised with a further functional group to provide a covalent bond with the first functional group. The further functional group may be *e.g.* a 2-bromoacetamido group as further described
 20 herein. Optionally a bromoacetyl group is provided on a polyacrylamide surface derived using N- (5- bromoacetamidylpentyl) acrylamide (BRAPA). The further functional group on the surface may be a bromoacetyl group, optionally wherein the bromoacetyl group is provided on a polyacrylamide surface derived using N- (5- bromoacetamidylpentyl) acrylamide (BRAPA) and the first functional group may be *e.g.* an amine group, a thiol
 25 group, a thiophosphate group or a thioamide group as appropriate. The surface to which polynucleotides are attached may comprise a gel. The surface comprises a polyacrylamide surface, such as about 2% polyacrylamide, preferably the polyacrylamide surface is coupled to a solid support such as glass.

In any of the methods of the invention a scaffold polynucleotide may optionally be
 30 attached to a linker via a branching nucleotide incorporated into the scaffold

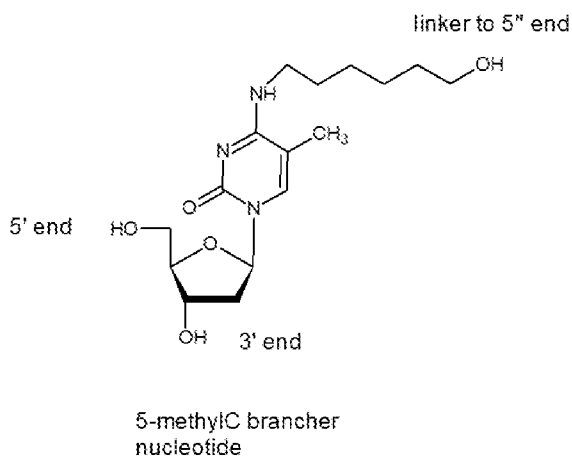
polynucleotide. Any suitable branching nucleotide may be used with any suitable compatible linker.

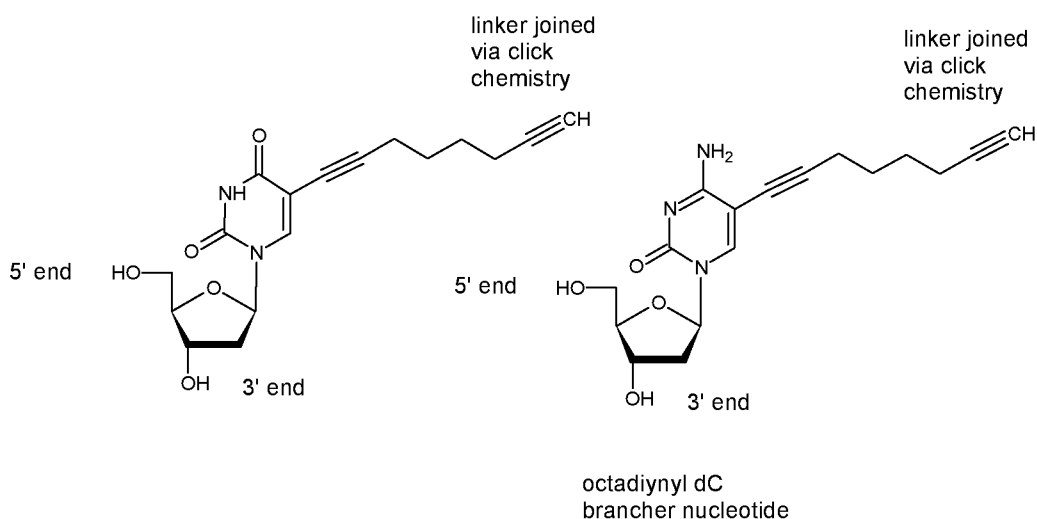
Prior to initiating synthesis cycles of the invention, scaffold polynucleotides may be synthesised with one or more branching nucleotides incorporated into the scaffold polynucleotide. The exact position at which the one or more branching nucleotides are incorporated into the scaffold polynucleotide, and thus where a linker may be attached, may vary and may be chosen as desired. The position may *e.g.* be at the terminal end of a support strand and/or a synthesis strand or *e.g.* in the loop region which connects the support strand to the synthesis strand in embodiments which comprise a hairpin loop.

During synthesis of the scaffold polynucleotide the one or more branching nucleotides may be incorporated into the scaffold polynucleotide with a blocking group which blocks a reactive group of the branching moiety. The blocking group may then be removed (deblocked) prior to the coupling to the branching moiety of the linker, or a first unit (molecule) of the linker if a linker comprises multiple units.

During synthesis of the scaffold polynucleotide the one or more branching nucleotides may be incorporated into the scaffold polynucleotide with a group suitable for use in a subsequent “click chemistry” reaction to couple to the branching moiety the linker, or a first unit of the linker if a linker comprises multiple units. An example of such a group is an acetylene group.

Some non-limiting exemplary branching nucleotides are shown below.

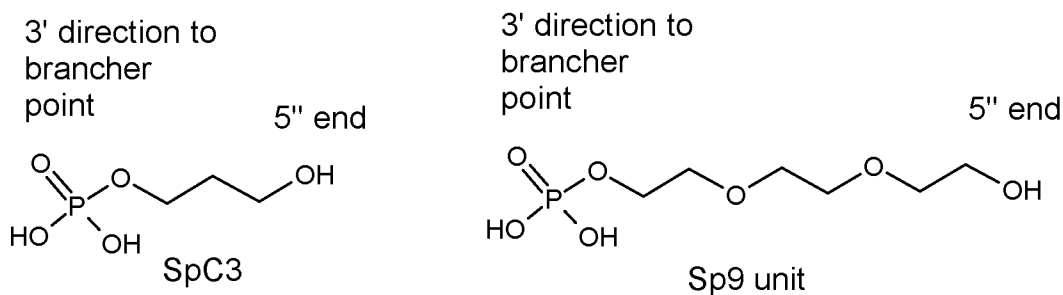


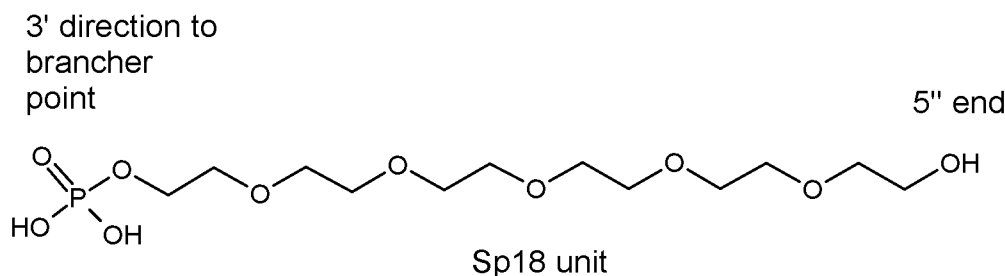


A linker may optionally comprise one or more spacer molecules (units), such as *e.g.* an Sp9 spacer, wherein the first spacer unit is attached to the branching nucleotide.

The linker may comprise one or more further spacer groups attached to the first spacer group. For example, the linker may comprise multiple *e.g.* Sp9 spacer groups. A first spacer group is attached to the branching moiety and then one or more further spacer groups are sequentially added to extend a spacer chain comprising multiple spacer units connected with phosphate groups therebetween.

Shown below are some non-limiting examples of spacer units (Sp3, Sp9 and Sp13) which could comprise the first spacer unit attached to a branching nucleotide, or a further spacer unit to be attached to an existing spacer unit already attached to the branching nucleotide.





A linker may comprise one or more ethylene glycol units.

A linker may comprise an oligonucleotide, wherein multiple units are nucleotides.

- 5 In the structures depicted above the term 5'' is used to differentiate from the 5' end of the nucleotide to which the branching moiety is attached, wherein 5' has its ordinary meaning in the art. By 5'' it is intended to mean a position on the nucleotide from which a linker can be extended. The 5'' position may vary. The 5'' position is typically a position in the nucleobase of the nucleotide. The 5'' position in the nucleobase may vary
- 10 depending on the nature of the desired branching moiety, as depicted in the structures above.

Microarrays

- 15 Any of the polynucleotide synthesis methods described herein may be used to manufacture a polynucleotide microarray (Trevino, V. et al., Mol. Med. 2007 13, pp527-541). Thus anchor or scaffold polynucleotides may be tethered to a plurality of individually addressable reaction sites on a surface and polynucleotides having a predefined sequence may be synthesised in situ on the microarray.

- 20 Following synthesis, at each reaction area the polynucleotide of predefined sequence may be provided with a unique sequence. The anchor or scaffold polynucleotides may be provided with barcode sequences to facilitate identification.

- Other than the method of synthesising the polynucleotides of predefined sequence, microarray manufacture may be performed using techniques commonly used in this
- 25 technical field, including techniques described herein. For example, anchor or scaffold

polynucleotides may be tethered to surfaces using known surface attachment methods and chemistries, including those described herein.

Following synthesis of the polynucleotides of predefined sequence, there may be provided a final cleavage step to remove any unwanted polynucleotide sequence from
5 untethered ends.

Polynucleotides of predefined sequence may be provided at reaction sites in double-stranded form. Alternatively, following synthesis double-stranded polynucleotides may be separated and one strand removed, leaving single-stranded polynucleotides at reaction sites. Selective tethering of strands may be provided to facilitate this process. For
10 example, in methods involving a scaffold polynucleotide the synthesis strand may be tethered to a surface and the support strand may be untethered, or vice versa. The synthesis strand may be provided with a non-cleavable linker and the support strand may be provided with a cleavable linker, or vice versa. Separation of strands may be performed by conventional methods, such as heat treatment.

15

Assembly of synthetic polynucleotides

A polynucleotide having a predefined sequence synthesised by methods described herein, and optionally amplified by methods described herein, may be joined to one or
20 more other such polynucleotides to create larger synthetic polynucleotides.

Joining of multiple polynucleotides can be achieved by techniques commonly known in the art. A first polynucleotide and one or more additional polynucleotides synthesised by methods described herein may be cleaved to create compatible termini and then polynucleotides joined together by ligation. Cleavage can be achieved by any suitable
25 means. Typically, restriction enzyme cleavage sites may be created in polynucleotides and then restriction enzymes used to perform the cleavage step, thus releasing the synthesised polynucleotides from any anchor/scaffold polynucleotide. Cleavage sites could be designed as part of the anchor/scaffold polynucleotides. Alternatively, cleavage sites could be created within the newly-synthesised polynucleotide as part of the predefined nucleotide
30 sequence.

Assembly of polynucleotides is preferably performed using solid phase methods. For example, following synthesis a first polynucleotide may be subject to a single cleavage at a suitable position distal to the site of surface immobilization. The first polynucleotide will thus remain immobilized to the surface, and the single cleavage will generate a
5 terminus compatible for joining to another polynucleotide. An additional polynucleotide may be subject to cleavage at two suitable positions to generate at each terminus a compatible end for joining to other polynucleotides, and at the same time releasing the additional polynucleotide from surface immobilization. The additional polynucleotide may be compatibly joined with the first polynucleotide thus creating a larger immobilized
10 polynucleotide having a predefined sequence and having a terminus compatible for joining to yet another additional polynucleotide. Thus iterative cycles of joining of preselected cleaved synthetic polynucleotides may create much longer synthetic polynucleotide molecules. The order of joining of the additional polynucleotides will be determined by the required predefined sequence.

15 Thus the assembly methods of the invention may allow the creation of synthetic polynucleotide molecules having lengths in the order of one or more Mb.

The assembly and/or synthesis methods of the invention may be performed using apparatuses known in the art. Techniques and apparatuses are available which allow very small volumes of reagents to be selectively moved, partitioned and combined with other
20 volumes in different locations of an array, typically in the form of droplets. Electrowetting techniques, such as electrowetting-on-dielectric (EWOD), may be employed, as described above. Suitable electrowetting techniques and systems that may be employed in the invention that are able to manipulate droplets are disclosed for example in US8653832, US8828336, US20140197028 and US20140202863.

25 Cleavage from the solid phase may be achieved by providing cleavable linkers in one or both the primer strand portion and the portion of the support strand hybridized thereto. The cleavable linker may be *e.g.* a UV cleavable linker.

Examples of cleavage methods involving enzymatic cleavage are shown in Figure 29. The schematic shows a scaffold polynucleotide attached to a surface (via black
30 diamond structures) and comprising a polynucleotide of predefined sequence. The scaffold

polynucleotide comprises top and bottom hairpins. In each case the top hairpin can be cleaved using a cleavage step utilizing the universal nucleotide to define a cleavage site. The bottom hairpin can be removed by a restriction endonuclease via a site that is engineered into the scaffold polynucleotide or engineered into the newly-synthesised polynucleotide of predefined sequence.

Thus polynucleotides having a predefined sequence may be synthesised whilst immobilized to an electrowetting surface, as described above. Synthesised polynucleotides may be cleaved from the electrowetting surface and moved under the influence of an electric field in the form of a droplet. Droplets may be combined at specific reaction sites on the surface where they may deliver cleaved synthesised polynucleotides for ligation with other cleaved synthesised polynucleotides. Polynucleotides can then be joined, for example by ligation. Using such techniques populations of different polynucleotides may be synthesised and attached in order according to the predefined sequence desired. Using such systems a fully automated polynucleotide synthesis and assembly system may be designed. The system may be programmed to receive a desired sequence, supply reagents, perform synthesis cycles and subsequently assemble desired polynucleotides according to the predefined sequence desired.

Systems and kits

20

The invention also provides polynucleotide synthesis systems for carrying out any of the synthesis methods described and defined herein, as well as any of the subsequent amplification and assembly steps described and defined herein.

Typically, synthesis cycle reactions will be carried out by incorporating nucleotides of predefined sequence into scaffold polynucleotide molecules which are tethered to a surface by means described and defined herein. The surface may be any suitable surface as described and defined herein.

In one embodiment, reactions to incorporate nucleotides of predefined sequence into a scaffold polynucleotide molecule involve performing any of the synthesis methods on a scaffold polynucleotide within a reaction area.

A reaction area is any area of a suitable substrate to which a scaffold polynucleotide molecule is attached and wherein reagents for performing the synthesis methods may be delivered.

In one embodiment a reaction area may be a single area of a surface comprising a
5 single scaffold polynucleotide molecule wherein the single scaffold polynucleotide molecule can be addressed with reagents.

In another embodiment a reaction area may be a single area of a surface comprising multiple scaffold polynucleotide molecules, wherein the scaffold polynucleotide molecules cannot be individually addressed with reagent in isolation from
10 each other. Thus in such an embodiment the multiple scaffold polynucleotide molecules in the reaction area are exposed to the same reagents and conditions and may thus give rise to synthetic polynucleotide molecules having the same or substantially the same nucleotide sequence.

In one embodiment a synthesis system for carrying out any of the synthesis
15 methods described and defined herein may comprise multiple reaction areas, wherein each reaction area comprises one or more attached scaffold polynucleotide molecules and wherein each reaction area may be individually addressed with reagent in isolation from each of the other reaction areas. Such a system may be configured *e.g.* in the form of an array, *e.g.* wherein reaction areas are formed upon a substrate, typically a planar substrate.

20 A system having a substrate comprising a single reaction area or comprising multiple reaction areas may be comprised within *e.g.* an EWOD system or a microfluidic system and the systems configured to deliver reagents to the reaction site. EWOD and microfluidic systems are described in more detail herein. For example an EWOD system may be configured to deliver reagents to the reaction site(s) under electrical control. A
25 microfluidic system, such as comprising microfabricated architecture *e.g.* as formed from elastomeric or similar material, may be configured to deliver reagents to the reaction site(s) under fluidic pressure and/or suction control or by mechanical means. Reagents may be delivered by any suitable means, for example via carbon nanotubes acting as conduits for reagent delivery. Any suitable system may be envisaged.

EWOD, microfluidic and other systems may be configured to deliver any other desired reagents to reaction sites, such as enzymes for cleaving a synthesised double-stranded polynucleotide from the scaffold polynucleotide following synthesis, and/or reagents for cleaving a linker to release an entire scaffold polynucleotide from the substrate and/or reagents for amplifying a polynucleotide molecule following synthesis or any region or portion thereof, and/or reagents for assembling larger polynucleotide molecules from smaller polynucleotide molecules which have been synthesised according to the synthesis methods of the invention.

The invention also provides kits for carrying out any of the synthesis methods described and defined herein. A kit may contain any desired combination of reagents for performing any of the synthesis and/or assembly methods of the invention described and defined herein. For example, a kit may comprise any one or more volume(s) of reaction reagents comprising scaffold polynucleotides, volume(s) of reaction reagents corresponding to any one or more steps of the synthesis cycles described and defined herein, volume(s) of reaction reagents comprising nucleotides comprising reversible blocking groups or reversible terminator groups, volume(s) of reaction reagents for amplifying one or more polynucleotide molecules following synthesis or any region or portion thereof, volume(s) of reaction reagents for assembling larger polynucleotide molecules from smaller polynucleotide molecules which have been synthesised according to the synthesis methods of the invention, volume(s) of reaction reagents for cleaving a synthesised double-stranded polynucleotide from the scaffold polynucleotide following synthesis, and volume(s) of reaction reagents for cleaving one or more linkers to release entire scaffold polynucleotides from a substrate.

Synthesis strand

In methods of synthesising a polynucleotide or oligonucleotide described herein including, but not limited to, synthesis method versions 1 to 5 of the invention as described in Figures 1 to 5 and further herein, the scaffold polynucleotide is provided with a synthesis strand. During cycles of synthesis each new nucleotide of the predefined

sequence is incorporated into the synthesis strand. An enzyme, such as a polymerase enzyme or enzyme having terminal transferase activity, can be used to catalyse incorporation/addition of each new nucleotide, nucleotide analogue/derivative or non-nucleotide. The synthesis strand comprises a primer strand portion and preferably
5 comprises a helper strand portion.

Helper strand

A helper strand may be provided in the scaffold polynucleotide to facilitate binding
10 of cleavage enzyme(s) at the cleavage step. A helper strand may be provided in the ligation polynucleotide to facilitate ligation of the ligation polynucleotide to the cleaved scaffold polynucleotide at the ligation step. The helper strand may be omitted, provided that alternative means are provided to ensure binding of cleavage enzyme(s) at the cleavage step and to ensure ligation at the ligation step, if necessary. In preferred methods
15 of the invention the synthesis strand is provided with a helper strand. Preferably, the ligation polynucleotide is provided with a helper strand and the helper strand is retained in the scaffold polynucleotide at the cleavage step, as show in in Figures 1 to 5.

There are no special requirements for the parameters of length, sequence and structure of the helper strand, provided that the helper strand is suitable to facilitate binding
20 of cleavage enzyme(s) at the cleavage step.

The helper strand may comprise nucleotides, nucleotide analogues/derivatives and/or non-nucleotides.

Preferably, within the region of sequence of the helper strand mismatches with the support strand should be avoided, GC- and AT-rich regions should be avoided, and in
25 addition regions of secondary structure such as hairpins or bulges should be avoided.

The length of the helper strand may be 10 bases or more. Optionally, the length of the helper strand may be 15 bases or more, preferably 30 bases or more. However, the length of the helper strand may be varied, provided that the helper strand is capable of facilitating cleavage and/or ligation.

The helper strand must be hybridized to the corresponding region of the support strand. It is not essential that the entirety of the helper strand is hybridized to the corresponding region of the support strand, provided that the helper strand can facilitate binding of cleavage enzyme(s) at the cleavage step and/or binding of ligase enzyme at the ligation step. Thus, mismatches between the helper strand and the corresponding region of the support strand can be tolerated. The helper strand may be longer than the corresponding region of the support strand. The support strand may extend beyond the region which corresponds with the helper strand in the direction distal to the primer strand. The helper strand may be connected to the corresponding region of the support strand, *e.g.* via a hairpin.

The helper strand is preferably hybridized to the support strand such that the terminal nucleotide of the helper strand at the site of the nick occupies the next sequential nucleotide position in the synthesis strand relative to the terminal nucleotide of the primer strand at the site of the nick. Thus in this configuration there are no nucleotide position gaps between the helper strand and the primer strand. The helper strand and primer strand will nevertheless be physically separated due to the presence of the single-stranded break or nick. Preferably, the terminal nucleobase of the helper strand at the site of the nick is hybridized to its partner nucleotide in the support strand.

The nucleotide in the helper strand which pairs with the universal nucleotide may be any suitable nucleotide. Preferably, pairings which are likely to distort the helical structure of the molecule should be avoided. Preferably cytosine acts as a partner for the universal nucleotide. In a particularly preferred embodiment the universal nucleotide is inosine, or an analogue, variant or derivative thereof, and the partner nucleotide for the universal nucleotide in the helper strand is cytosine.

25

Removal of helper strand

Although it is preferred that the helper strand is retained during steps of synthesis, in any of the synthesis methods of the invention described herein, including exemplary method versions 1 to 5, in step (1) (*i.e.* in the first cycle) of providing a scaffold

30

polynucleotide comprising a synthesis strand and a support strand hybridized thereto (101, 201, 301, 401, 501), the synthesis strand may be provided without a helper strand.

Furthermore, 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 cleavage of the scaffold polynucleotide (106, 206, 306, 406, 506), the helper strand portion of the synthesis strand may be removed from the scaffold polynucleotide.

The helper strand portion of the synthesis strand may be removed from the scaffold polynucleotide by any suitable means including, but not limited to: (i) heating the scaffold polynucleotide to a temperature of about 80°C to about 95°C and separating the helper strand portion from the scaffold polynucleotide, (ii) treating the scaffold polynucleotide with urea solution, such as 8M urea and separating the helper strand portion from the scaffold polynucleotide, (iii) treating the scaffold polynucleotide with formamide or formamide solution, such as 100% formamide and separating the helper strand portion from the scaffold polynucleotide, or (iv) contacting the scaffold polynucleotide with a single-stranded polynucleotide molecule which comprises a region of nucleotide sequence which is complementary with the sequence of the helper strand portion, thereby competitively inhibiting the hybridisation of the helper strand portion to the scaffold polynucleotide.

In methods wherein the helper strand portion is removed from the scaffold polynucleotide after the step of ligating the double-stranded ligation polynucleotide to the cleaved scaffold polynucleotide and before the step of cleavage of the scaffold polynucleotide, the cleavage step will comprise cleaving the support strand in the absence of a double-stranded region provided by the helper strand. Any suitable enzyme may be chosen for performing such a cleavage step, such as selected from any suitable enzyme disclosed herein.

Primer strand

The primer strand portion should be suitable to allow an enzyme, such as a polymerase enzyme or enzyme having terminal transferase activity, to initiate synthesis, *i.e.* catalyse
5 the addition of a new nucleotide at the terminal end of the primer strand at the site of the nick.

The primer strand may comprise a region of sequence which can act to prime new polynucleotide synthesis (*e.g.* as shown by the dotted line in the structures depicted in each of Figures 1 to 5). The primer strand may consist of a region of sequence which can act to
10 prime new polynucleotide synthesis, thus the entirety of the primer strand may be sequence which can act to prime new polynucleotide synthesis as described herein.

There are no special requirements for the parameters of length, sequence and structure of the primer strand, provided that the primer strand is suitable to prime new polynucleotide synthesis.

15 The primer strand may comprise nucleotides, nucleotide analogues/derivatives and/or non-nucleotides.

The skilled person is readily able to construct a primer strand which will be capable of priming new polynucleotide synthesis. Thus, within the region of sequence of the primer strand which can act to prime new polynucleotide synthesis mismatches with the
20 support strand should be avoided, GC- and AT-rich regions should be avoided, and in addition regions of secondary structure such as hairpins or bulges should be avoided.

The length of the region of sequence of the primer strand which can act to prime new polynucleotide synthesis can be chosen by the skilled person depending on preference and the polymerase enzyme to be used. The length of this region may be 7 bases or more,
25 8 bases or more, 9 bases or more or 10 bases or more. Optionally the length of this region will be 15 bases or more, preferably 30 bases or more.

The primer strand must be hybridized to the corresponding region of the support strand. It is not essential that the entirety of the primer strand is hybridized to the corresponding region of the support strand, provided that the primer strand is capable of
30 priming new polynucleotide synthesis. Thus, mismatches between the primer strand and

the corresponding region of the support strand can be tolerated to a degree. Preferably, the region of sequence of the primer strand which can act to prime new polynucleotide synthesis should comprise nucleobases which are complementary to corresponding nucleobases in the support strand.

- 5 The primer strand may be longer than the corresponding region of the support strand. The support strand may extend beyond the region which corresponds with the primer strand in the direction distal to the helper strand. The primer strand may be connected to the corresponding region of the support strand, *e.g.* via a hairpin.

10 **Support strand**

- In methods of the invention including, but not limited to, synthesis method versions of the invention 1 to 5, as described above, the scaffold polynucleotide is provided with a support strand. The support strand is hybridized to the synthesis strand. There are no
15 special requirements for the parameters of length, sequence and structure of the support strand, provided that the support strand is compatible with the primer strand portion and, if included, the helper strand portion of the synthesis strand, as described above.

RNA synthesis

20

- Methods described for DNA synthesis may be adapted for the synthesis of RNA. In one adaptation the synthesis steps described for synthesis method versions of the invention 1 to 5 may be adapted. Thus in each of synthesis method versions 1 to 5 the support strand of the scaffold polynucleotide is a DNA strand, as described above. The
25 primer strand portion of the synthesis strand of the scaffold polynucleotide is an RNA strand. The helper strand, if present, is preferably an RNA strand. The helper strand, if present, may be a DNA strand.

 Nucleotides may be incorporated from ribonucleoside-5'-*O*-triphosphates (NTPs) which may be modified to comprise a reversible terminator group, as described above.

Preferably 3'-*O*-modified-ribonucleoside-5'-*O*-triphosphates are used. Modified nucleotides are incorporated by the action of RNA polymerase.

Thus the descriptions relating to synthesis method versions of the invention 1 to 5 may be applied *mutatis mutandis* for RNA synthesis but adapted as described.

5 Figures 31 and 32 describe reaction schemes for RNA synthesis which are adaptations of DNA synthesis method versions 1, 2 of the Examples as depicted in Figures 6 and 7 respectively. Method versions of the invention 1 to 5, as depicted in Figures 1 to 5 respectively may be adapted in the same way.

10 In any of the adapted methods for RNA synthesis, the above descriptions of support strand, primer strand, helper strand, ligation polynucleotide and universal nucleotide may be applied *mutatis mutandis* but adapted as described. Cleavage steps and cleavage positions as previously described may be applied *mutatis mutandis* since the support strand which comprises the universal nucleotide is a DNA strand. In a preferred embodiment SplintR DNA ligase is used in the ligation step.

15

Exemplary methods

Exemplary methods of synthesising a polynucleotide or an oligonucleotide molecule according to the invention are described herein, including in the appended
20 claims.

25 In the following five exemplary methods of synthesising a polynucleotide or an oligonucleotide molecule according to the invention references to synthesis method versions 1, 2, 3, 4 and 5 are to be interpreted according to the reaction schematics set out respectively in Figures 1, 2, 3, 4 and 5 and not according to the reaction schematics set out in any of Figures 6 to 10 or descriptions of the same in the Examples section. The reaction schematics set out in any of Figures 6 to 10 and descriptions of the same in the Examples section below provide illustrative support for the methods of the invention based on reaction schemes which are modified in comparison with the methods of the present invention. Thus reference signs in the text below correspond with those in Figures 1 to 5.

In each exemplary method described below, the structures described in each step may be referred to by reference to specific figures with the aid of reference signs as appropriate. However, such references are not intended to be limited to the structures shown in the figures, and the description of the relevant structures correspond to the description thereof as provided herein in its entirety, including but not limited to those specifically illustrated.

Five non-limiting exemplary methods, method versions 1 to 5, are described below (see *e.g.* Figures 1 to 5 respectively). In step (1) of each of these exemplary methods a scaffold polynucleotide (see structure depicted in step 1 of each of Figures 1 to 5) is provided (101, 201, 301, 401, 501) comprising a synthesis strand (see strand labelled “b” in the structure depicted in step 1 of each of Figures 1 to 5) hybridized to a complementary support strand (see strand labelled “a” in structure depicted in step 1 of each of Figures 1 to 5).

The scaffold polynucleotide is double-stranded and provides a support structure to accommodate the region of synthetic polynucleotide as it is synthesised *de novo*. The scaffold polynucleotide comprises a synthesis strand comprising a primer strand portion (see dotted portion of strand labelled “b” in structure depicted in step 1 of each of Figures 1 to 5) and a helper strand portion (see dashed portion of strand labelled “b” in structure depicted in step 1 of each of Figures 1 to 5) separated by a single-strand break or “nick”. As described in more detail herein, in certain of the exemplary method versions 1 to 5 and variants thereof described herein the helper strand may be removed prior to the cleavage step (2), *e.g.* by denaturation.

Both the primer strand portion and the helper strand portion of the synthesis strand are provided hybridized to a complementary support strand.

In each of the five methods a universal nucleotide (labelled “Un” in the structures depicted in each of Figures 1 to 5) is provided in the support strand which facilitates cleavage of the scaffold polynucleotide (102, 202, 302, 402, 502). The role of the universal nucleotide will be apparent from the detailed description of each method below.

Cleavage of the scaffold polynucleotide (step 2) results in loss of the helper strand, if present immediately prior to cleavage, and loss of the support strand comprising the

universal nucleotide. Cleavage leaves in place a cleaved double-stranded scaffold polynucleotide comprising, at the site of cleavage, a cleaved terminal end of the support strand and the terminal end of the primer strand portion of the synthesis strand which comprised the nick site prior to cleavage.

5 The terminal end of the primer strand portion of the synthesis strand provides a primer site for use in the initiation of synthesis by an enzyme having transferase activity. Thus the enzyme will act to extend the terminal nucleotide of the primer strand portion. This terminal nucleotide will therefore typically define a 3' terminus of the primer strand portion, *e.g.* to allow extension by polymerase or transferase enzymes which catalyse
10 extension in a 5' to 3' direction. The opposite terminus of the synthesis strand comprising the primer strand portion will consequently typically define a 5' terminus of the synthesis strand, and the terminal nucleotide of the support strand adjacent the 5' terminus of the synthesis strand will consequently typically define the 3' terminus of the support strand.

 The terminal nucleotide of the helper strand portion of the synthesis strand, which
15 is positioned at the site of the single-strand break, will typically define a 5' terminus of the helper strand portion and consequently the opposite terminus of the helper strand portion of the synthesis strand will typically define the 3' terminus of the synthesis strand.

 The single-stranded break or "nick" between the helper strand portion and the primer strand portion of the synthesis strand is typically achieved by providing the (5')
20 terminal nucleotide of the helper strand in such a form that will prevent it from ligating to the primer strand at the ligation step. This can typically be achieved by providing the (5') terminal nucleotide of the helper strand without a phosphate group. The break is typically achieved by assembling the scaffold polynucleotide from separate components comprising:
(i) the support strand; (ii) the helper strand portion of the synthesis strand, typically having
25 a non-phosphorylated (5') terminal nucleotide; and (iii) the synthesis strand portion comprising the primer strand portion. Upon mixing of the components in suitable conditions the scaffold polynucleotide forms upon hybridization of the separate components.

In certain methods described herein the helper strand may optionally be removed from the scaffold polynucleotide prior to the step of cleavage, *e.g.* by denaturation and release from the support strand to which it was previously hybridized.

Cleavage results in removal of the support strand comprising the universal
5 nucleotide and the helper strand hybridised to the support strand, if present immediately prior to cleavage.

In step (3) of the methods a first nucleotide in the predefined nucleotide sequence is incorporated into the synthesis strand by the action of an enzyme having transferase activity such as polymerase or a terminal nucleotidyl transferase enzyme (102, 202, 302,
10 402, 502). The first nucleotide is provided with a reversible terminator group (depicted as the small triangle of the incorporated nucleotide in step 3 of each of Figures 1 to 5) which prevents further extension by the enzyme. Thus in step (3) only a single nucleotide is incorporated.

Nucleotides comprising any suitable reversible terminator group could be used.
15 Preferred nucleotides with reversible terminator groups are 3'-*O*-allyl-dNTPs and/or 3'-*O*-azidomethyl-dNTPs as described herein.

In step (4) of the methods a deprotection step is performed to remove the reversible terminator group from the incorporated first nucleotide of the predefined nucleotide sequence (104, 204, 304, 404, 504). The deprotection step may alternatively be performed
20 after the ligation step (5) described below.

In step (5) of the methods a ligation step is performed (105, 205, 305, 405, 505) wherein a ligation polynucleotide is ligated to the cleaved double-stranded scaffold polynucleotide comprising the first nucleotide of the predefined nucleotide sequence. The ligation polynucleotide comprises a support strand and a helper strand hybridized to the
25 support strand. The ligation polynucleotide comprises a complementary ligation end which is complementary with the cleaved end of the cleaved double-stranded scaffold polynucleotide comprising the first nucleotide. The support strand of the ligation polynucleotide comprises a partner nucleotide for the first nucleotide of the predefined nucleotide sequence at the complementary ligation end wherein upon ligation the partner
30 nucleotide is positioned opposite the first nucleotide of the predefined nucleotide sequence

to form a nucleotide pair. The support strand of the ligation polynucleotide also comprises a universal nucleotide at the complementary ligation end which will facilitate cleavage in the next cycle of synthesis. The terminal nucleotide of the helper strand of the ligation polynucleotide at the complementary ligation end is provided such that the helper strand cannot be ligated to the primer strand. This is typically achieved by providing the terminal nucleotide of the helper strand without a phosphate group. Thus upon ligation of the support strand of the ligation polynucleotide and the support strand of the cleaved double-stranded scaffold polynucleotide, a single-strand break or “nick” is provided in the synthesis strand between the primer strand portion and the helper strand.

Upon ligation of the ligation polynucleotide to the cleaved double-stranded scaffold polynucleotide an intact double-stranded scaffold polynucleotide is reformed comprising a newly incorporated nucleotide pair and a universal nucleotide for use in facilitating cleavage in the next cycle of synthesis.

Iterative cycles of synthesis comprising the same steps as described above are performed in order to generate the synthetic polynucleotide.

Specific methods are described in more detail below.

Synthesis Method Version 1

In a first exemplary version of the synthesis method of the invention a double-stranded scaffold polynucleotide is provided comprising a universal nucleotide positioned in the support strand (step 1 of Figure 1; 101). In each cycle of synthesis the scaffold polynucleotide is cleaved at a cleavage site defined by a sequence comprising the universal nucleotide (step 2 of Figure 1; 102, 107).

Cleavage of the scaffold polynucleotide (step 2) results in loss of the helper strand, if present immediately prior to cleavage, and loss of the support strand comprising the universal nucleotide. Cleavage leaves in place a cleaved double-stranded scaffold polynucleotide comprising, at the site of cleavage, a cleaved terminal end of the support strand and the terminal end of the primer strand portion of the synthesis strand which

comprised the nick site prior to cleavage. Cleavage results in a blunt-ended cleaved double-stranded scaffold polynucleotide at the site of cleavage, with no overhang.

In step (3) of the methods a first nucleotide in the predefined nucleotide sequence is added to the terminal end of the primer strand portion of the synthesis strand by the action of an enzyme having transferase activity such as polymerase or a terminal nucleotidyl transferase enzyme (103). The first nucleotide is provided with a reversible terminator group which prevents further extension by the enzyme. Thus in step (3) only a single nucleotide is incorporated.

In step (4) of the methods a deprotection step is performed to remove the terminator group from the newly-incorporated nucleotide. In principle the deprotection step may be performed after the ligation step (5). Performance of the deprotection step as step (4) is preferred.

In step (5) of the methods a ligation polynucleotide (see structure depicted at the top left of the upper part of Figure 1) is ligated to the cleaved scaffold polynucleotide. Ligation incorporates a partner nucleotide into the scaffold polynucleotide and allows the newly-incorporated nucleotide to pair with the partner nucleotide (step 5 of Figure 1; 105), thus completing a synthesis cycle.

In the first exemplary version of the synthesis method of the invention a scaffold polynucleotide is provided in step (1) (101) as described above. In this method the universal nucleotide in the support strand of the scaffold polynucleotide is positioned opposite the terminal nucleotide of the helper strand at the single-strand break site (labelled "X" in the structures of Figure 1), and is paired therewith (see structure depicted in step 1 of Figure 1).

In step (2) of the method the scaffold polynucleotide is cleaved (102) at a cleavage site. The cleavage site is defined by a sequence comprising the universal nucleotide in the support strand. Cleavage results in a double-stranded break in the scaffold polynucleotide. Cleavage of the scaffold polynucleotide (step 2) results in loss of the helper strand, if present immediately prior to cleavage, and loss of the support strand comprising the universal nucleotide. Cleavage of the scaffold polynucleotide leaves in place a cleaved double-stranded scaffold polynucleotide comprising, at the site of cleavage, a cleaved

terminal end of the support strand and the terminal end of the primer strand portion of the synthesis strand which comprised the nick site prior to cleavage.

The synthesis strand is already provided with a single-stranded break or “nick” in this exemplary method, thus only cleavage of the support strand is necessary to provide a
5 double-stranded break in the scaffold polynucleotide.

In this exemplary method version cleavage generates a blunt-ended cleaved double-stranded scaffold polynucleotide with no overhang in either the synthesis strand or the support strand.

In this method the universal nucleotide occupies position “n” in the support strand
10 prior to the cleavage step. To obtain such a blunt-ended cleaved double-stranded scaffold polynucleotide when the universal nucleotide occupies position n in the support strand, the support strand is cleaved at a specific position relative to the universal nucleotide. The support strand of the scaffold polynucleotide is cleaved between nucleotide positions n and n-1.

By “n” it is meant the nucleotide position in the support strand which is opposite
15 the position in the synthesis strand which will be occupied by the nucleotide of the predefined sequence upon its addition to the terminal end of the cleaved synthesis strand in that cycle of synthesis. By “n” it is also meant the nucleotide position in the synthesis strand which will be occupied by the nucleotide of the predefined sequence upon its
20 addition to the terminal end of the primer strand portion of the synthesis strand in that cycle of synthesis. Thus at the cleavage step, the universal nucleotide at position n in the support strand is opposite the position which will be occupied by the nucleotide of the predefined sequence incorporated in that cycle of synthesis. At steps (1) and (2) the position which will be occupied by the nucleotide of the predefined sequence incorporated
25 in that cycle of synthesis corresponds with the terminal nucleotide of the helper strand portion of the synthesis strand (shown as “X” in Figure 1).

By “n-1” it is meant the next nucleotide position in the support strand relative to the position which is occupied by, or has been occupied by, the universal nucleotide, in the direction distal to the helper strand/proximal to the primer strand (nucleotide labelled “H”
30 at position n-1, as shown schematically in steps (1) and (2) of Figure 1). n-1 may also refer

to the corresponding position in the synthesis strand. Thus at the cleavage step, position n-1 in the support strand is opposite the position occupied by the terminal nucleotide of the primer strand portion of the synthesis strand (*i.e.* the nucleotide labelled "I" at position n-1, as shown schematically in step (2) of Figure 1). In methods according to version 1, the
5 nucleotide pair which occupy positions H and I can be any nucleotide pair.

Upon cleavage of the support strand between nucleotide positions n and n-1, the universal nucleotide, helper strand, if present immediately prior to cleavage, and the portion of the support strand which is or was hybridized to the helper strand are removed from the remaining cleaved scaffold polynucleotide (see structure depicted at the top right
10 of the upper part of Figure 1).

A phosphate group should continue to be attached to the terminal nucleotide of the support strand of the cleaved scaffold polynucleotide at the cleavage site (as depicted in the structure shown in the middle of the lower part of Figure 1). This ensures that the support strand of the ligation polynucleotide can be ligated to the support strand of the cleaved
15 scaffold polynucleotide in the ligation step.

Thus in method version 1 the universal nucleotide occupies position n in the support strand at steps (1) and (2) and the support strand is cleaved between nucleotide positions n and n-1.

Preferably, the support strand is cleaved by cleavage of the phosphodiester bond
20 between nucleotide positions n and n-1 (the first phosphodiester bond of the support strand relative to the position of the universal nucleotide, in the direction distal to the helper strand/proximal to the primer strand).

The support strand may be cleaved by cleavage of one ester bond of the phosphodiester bond between nucleotide positions n and n-1.

25 Preferably the support strand is cleaved by cleavage of the first ester bond relative to nucleotide position n. This will have the effect of retaining a terminal phosphate group on the support strand of the cleaved scaffold polynucleotide at the cleavage position.

Any suitable mechanism may be employed to effect cleavage of the support strand between nucleotide positions n and n-1 when the universal nucleotide occupies position n.

Cleavage of the support strand between nucleotide positions n and n-1 as described above may be performed by the action of an enzyme.

Cleavage of the support strand between nucleotide positions n and n-1 as described above may be performed as a two-step cleavage process.

- 5 The first cleavage step of a two-step cleavage process may comprise removing the universal nucleotide from the support strand thus forming an abasic site at position n, and the second cleavage step may comprise cleaving the support strand at the abasic site, between positions n and n-1.

- 10 One mechanism of cleaving the support strand at a cleavage site defined by a sequence comprising a universal nucleotide which is occupying position n in the support strand is described in Example 2. The cleavage mechanism described in Example 2 is exemplary and other mechanisms could be employed, provided that the blunt-ended cleaved double-stranded scaffold polynucleotide described above is achieved.

- 15 In the first cleavage step of a two-step cleavage process the universal nucleotide is removed from the support strand whilst leaving the sugar-phosphate backbone intact. This can be achieved by the action of an enzyme which can specifically excise a single universal nucleotide from a double-stranded polynucleotide. In the exemplified cleavage methods the universal nucleotide is inosine and inosine is excised from the support strand by the action of an enzyme, thus forming an abasic site. In the exemplified cleavage
20 method the enzyme is a 3-methyladenine DNA glycosylase enzyme, specifically human alkyladenine DNA glycosylase (hAAG). Other enzymes, molecules or chemicals could be used provided that an abasic site is formed. The nucleotide-excising enzyme may be UDG DNA glycosylase.

- 25 In the second cleavage step of a two-step cleavage process the support strand is cleaved at the abasic site by making a single-strand break. In the exemplified methods the support strand is cleaved by the action of a chemical which is a base, such as NaOH. Alternatively, an organic chemical such as N,N'-dimethylethylenediamine may be used. Alternatively, enzymes having abasic site lyase activity, such as AP Endonuclease 1, Endonuclease III (Nth), or Endonuclease VIII, may be used. Other enzymes, molecules or

chemicals could be used provided that the support strand is cleaved at the abasic site as described.

Thus in embodiments wherein the universal nucleotide is at position n of the support strand at steps (1) and (2) and the support strand is cleaved between positions n and n-1, a first cleavage step may be performed with a nucleotide-excising enzyme. An example of such an enzyme is a 3-methyladenine DNA glycosylase enzyme, such as human alkyladenine DNA glycosylase (hAAG). The second cleavage step may be performed with a chemical which is a base, such as NaOH. The second step may be performed with an organic chemical having abasic site cleavage activity such as N,N'-dimethylethylenediamine. The second step may performed with an enzyme having abasic site lyase activity such as Endonuclease VIII.

Cleavage of the support strand between nucleotide positions n and n-1 as described above may also be performed as a one-step cleavage process. Examples of enzymes which may be used in any such process include Endonuclease III, Endonuclease VIII, formamidopyrimidine DNA glycosylase (Fpg) and 8-oxoguanine DNA glycosylase (hOGG1).

In this exemplary method version of the invention, as well as with all versions, to allow the next nucleotide to be incorporated in the next synthesis cycle, the reversible terminator group must be removed from the first nucleotide (deprotection step; 104). This can be performed at various stages of the first cycle. Typically, and preferably, it will be performed as step (4) of the method, before ligation step (5), as shown in step 4 of Figure 1 (104). However, the deprotection step could be performed at any step after incorporation of the new nucleotide, such as after the ligation step (5). Regardless of which stage the deprotection step is performed, enzyme and residual unincorporated first nucleotides should first be removed in order to prevent multiple incorporation of first nucleotides. Enzyme and unincorporated first nucleotides are preferably removed prior to the ligation step (step (5)).

Removal of the reversible terminator group from the first nucleotide can be performed by any suitable means. For example, removal can be performed by the use of a chemical, such as tris(carboxyethyl)phosphine (TCEP).

In step (5) of the method a double-stranded ligation polynucleotide is ligated (105) to the cleaved scaffold polynucleotide. The ligation polynucleotide comprises a support strand and a helper strand. The ligation polynucleotide further comprises a complementary ligation end comprising in the support strand a universal nucleotide and a single
5 overhanging nucleotide which overhangs the terminal nucleotide of the helper strand and which is the partner nucleotide for the first nucleotide of the predefined sequence. The ligation polynucleotide further comprises in the helper strand adjacent the overhang a terminal nucleotide which is configured such that it cannot be ligated to another polynucleotide strand (position labelled “X” in the structure depicted at the top left of the
10 upper part of Figure 1). Typically this terminal nucleotide will lack a phosphate group. Typically, as described above, this terminal nucleotide of the helper strand will define the 5’ terminus of the helper strand.

The complementary ligation end is configured so that it will compatibly join with the overhanging end of the cleaved scaffold polynucleotide product following
15 incorporation step (3) when subjected to suitable ligation conditions. Upon ligation of the support strands, the first nucleotide becomes paired with its partner nucleotide.

Ligation of the support strands may be performed by any suitable means. Ligation will result in the joining of the support strands only, with the maintenance of a single-stranded break between the first nucleotide in the synthesis strand, *i.e.* in the primer strand
20 portion, and the terminal nucleotide of the helper strand adjacent the first nucleotide.

Ligation may typically be performed by enzymes having ligase activity. For example, ligation may be performed with T3 DNA ligase or T4 DNA ligase or functional variants or equivalents thereof. The use of such enzymes will result in the maintenance of the single-stranded break in the synthesis strand, since the terminal nucleotide of the helper
25 strand is provided such that it cannot act as a substrate for ligase, *e.g.* due to the absence of a terminal phosphate group.

Ligation of the ligation polynucleotide to the cleaved scaffold polynucleotide completes a first synthesis cycle whereupon the scaffold polynucleotide of step (1) is effectively re-constituted except that the first nucleotide of the predefined nucleotide
30 sequence is incorporated into the scaffold polynucleotide opposite its partner nucleotide.

In step (5) of this exemplary method (105), in the complementary ligation end of the ligation polynucleotide the universal nucleotide in the support strand is positioned opposite the terminal nucleotide of the helper strand and is paired therewith. It will be noted that in the context of the ligation polynucleotide the universal nucleotide is

5 positioned at position $n+1$, and at a position next to the terminal nucleotide of the support strand (position n). Position n in the context of the ligation polynucleotide has the same meaning as described above in the context of the scaffold polynucleotide at steps (1) and (2). Thus in step (5) by “ n ” it is meant the nucleotide position in the support strand which, following ligation, is opposite the position in the synthesis strand which is now occupied

10 by the nucleotide of the predefined sequence following its addition to the terminal end of the primer strand portion of the synthesis strand in that cycle of synthesis. By “ n ” it is also meant the nucleotide position in the synthesis strand which, following ligation, is now occupied by the nucleotide of the predefined sequence following its addition to the terminal end of the primer strand portion of the synthesis strand in that cycle of synthesis.

15 It will be noted that the universal nucleotide occupies position n in the scaffold polynucleotide during steps (1) and (2) (101 and 102 of Figure 1), whereas the universal nucleotide occupies position $n+1$ in the ligation polynucleotide in the same synthesis cycle (105 of Figure 1). This is because at step (5) the cleaved double-stranded scaffold polynucleotide is now provided with the nucleotide of the predefined sequence following

20 its addition in that cycle of synthesis and because the ligation polynucleotide is provided with the nucleotide to partner the nucleotide of the predefined sequence; and furthermore because as defined herein “ n ” always refers to the nucleotide position in the synthesis strand which is occupied by (or will be occupied by) the nucleotide of the predefined sequence incorporated in that cycle or refers to the nucleotide position opposite thereto in

25 the support strand. Thus at the end of any given cycle of synthesis, immediately after ligation step (5), the position occupied by a universal nucleotide will be $n+1$ and will have moved from n , the position a universal nucleotide will have occupied at step (1). The product of the ligation step (5) (structure labelled “ligation product” in Figure 1) will be the scaffold polynucleotide for use in the next cycle of synthesis. When the ligation

30 product is considered as the scaffold polynucleotide for use in the next cycle of synthesis

(106) it is to be understood that the position occupied by the universal nucleotide is now to be once again referred to as position n (106), rather than $n+1$ (105), since the position occupied by the universal nucleotide in the next cycle of synthesis (106) will be 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 addition to the terminal end of the cleaved synthesis strand in the next cycle (108). Thus in method version 1 the position occupied by the universal nucleotide at the beginning of any given cycle of synthesis (101, 106 etc.) is always referred to as position n , and the nucleotide which is newly incorporated in that cycle always occupies a position referred to as n .

Following completion of the first synthesis cycle, second and subsequent cycles are performed using the same method steps.

As in step (2) of the first synthesis cycle of method version 1, in step (6) the scaffold polynucleotide is cleaved (107) at a cleavage site. The cleavage site is defined by a sequence comprising the universal nucleotide in the support strand. Cleavage results in a double-stranded break in the scaffold polynucleotide. Cleavage of the scaffold polynucleotide leaves in place a cleaved double-stranded scaffold polynucleotide comprising, at the site of cleavage, a cleaved terminal end of the support strand and the terminal end of the primer strand portion of the synthesis strand which comprised the nick site prior to cleavage. Cleavage results in removal of the support strand comprising the universal nucleotide and the helper strand hybridised to the support strand, if present immediately prior to cleavage.

The cleavage steps may be performed as described above for step (2) of the first cycle.

In step (7) the next nucleotide is added to the terminal end of the cleaved primer strand portion of the synthesis strand as in step (3) of the first cycle.

In step (8) the reversible terminator group is removed from the next nucleotide (deprotection step; 109). As described above for first cycle, this can be performed at various stages. Typically, and preferably, it will be performed as step (8) of the method,

before ligation step (9). However, the deprotection step could be performed at any step after incorporation of the new nucleotide, such as after the ligation step (9).

Deprotection of the reversible terminator group in the next cycle (109) and subsequent cycles may be performed as described above with respect to the first synthesis
5 cycle.

In step (9) of the next cycle a double-stranded ligation polynucleotide is ligated (110) to the cleaved scaffold polynucleotide. The ligation polynucleotide of step (9) of the next and subsequent synthesis cycles may be configured, and the ligation step may be performed, as described above for step (5) of the first synthesis cycle.

10 Synthesis cycles are repeated as described above for as many times as necessary to synthesise the double-stranded polynucleotide having the predefined nucleotide sequence.

Synthesis Method Version 2

15 A second exemplary version of the synthesis method of the invention is performed in an analogous matter to the first version except with modified structural arrangements for the positioning of the universal nucleotide relative to the position to be occupied by the new nucleotide to be incorporated in a given synthesis cycle and relative to the nick and cleavage sites.

20 Thus as with version 1, in version 2 cleavage of the scaffold polynucleotide (step 2, 202) results in loss of the helper strand, if present immediately prior to cleavage, and loss of the support strand comprising the universal nucleotide. Cleavage leaves in place a cleaved double-stranded scaffold polynucleotide comprising, at the site of cleavage, a
25 cleaved terminal end of the support strand and the terminal end of the primer strand portion of the synthesis strand which comprised the nick site prior to cleavage. Cleavage results in a blunt-ended cleaved double-stranded scaffold polynucleotide at the site of cleavage, with no overhang.

In step (3) of the methods a first nucleotide in the predefined nucleotide sequence is added to the terminal end of the primer strand portion of the synthesis strand by the action
30 of an enzyme having transferase activity such as polymerase or a terminal nucleotidyl

transferase enzyme (203). The first nucleotide is provided with a reversible terminator group which prevents further extension by the enzyme. Thus in step (3) only a single nucleotide is incorporated.

5 In step (4) of the methods a deprotection step is performed (204) to remove the terminator group from the newly-incorporated nucleotide. In principle the deprotection step may be performed after the ligation step (5). Performance of the deprotection step as step (4) is preferred.

In step (5) of the methods a ligation polynucleotide (see structure depicted at the top left of the upper part of Figure 2) is ligated to the cleaved scaffold polynucleotide.
10 Ligation incorporates a partner nucleotide into the scaffold polynucleotide and allows the newly-incorporated nucleotide to pair with the partner nucleotide (step 5 of Figure 2; 205), thus completing a synthesis cycle.

In the second exemplary version of the synthesis method of the invention a scaffold polynucleotide is provided in step (1) (201) as described above. In this method the
15 universal nucleotide in the support strand of the scaffold polynucleotide is positioned opposite the penultimate nucleotide of the helper strand adjacent the single-strand break site (labelled “X” in the structures of Figure 2), and is paired therewith (see structure depicted in step 1 of Figure 2).

In step (2) of the method the scaffold polynucleotide is cleaved (202) at a cleavage
20 site. The cleavage site is defined by a sequence comprising the universal nucleotide in the support strand. Cleavage results in a double-stranded break in the scaffold polynucleotide. Cleavage of the scaffold polynucleotide (step 2) results in loss of the helper strand, if present immediately prior to cleavage, and loss of the support strand comprising the universal nucleotide. Cleavage of the scaffold polynucleotide leaves in place a cleaved
25 double-stranded scaffold polynucleotide comprising, at the site of cleavage, a cleaved terminal end of the support strand and the terminal end of the primer strand portion of the synthesis strand which comprised the nick site prior to cleavage.

The synthesis strand is already provided with a single-stranded break or “nick” in this exemplary method, thus only cleavage of the support strand is necessary to provide a
30 double-stranded break in the scaffold polynucleotide.

In this exemplary method version cleavage generates a blunt-ended cleaved double-stranded scaffold polynucleotide with no overhang in either the synthesis strand or the support strand.

5 In this method the universal nucleotide occupies position “n+1” in the support strand prior to the cleavage step. To obtain such a blunt-ended cleaved double-stranded scaffold polynucleotide when the universal nucleotide occupies position n+1 in the support strand, the support strand is cleaved at a specific position relative to the universal nucleotide. The support strand of the scaffold polynucleotide is cleaved between nucleotide positions n and n-1.

10 By “n” it is meant 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 addition to the terminal end of the cleaved synthesis strand in that cycle of synthesis. By “n” it is also meant the nucleotide position in the synthesis strand which will be occupied by the nucleotide of the predefined sequence upon its
15 addition to the terminal end of the primer strand portion of the synthesis strand in that cycle of synthesis. Thus at the cleavage step, the universal nucleotide is at position n+1 in the support strand, which is opposite the position occupied by the penultimate nucleotide of the helper strand adjacent the nick site (shown at position “X” in Figure 2).

At steps (1) and (2) the position which will be occupied by the nucleotide of the
20 predefined sequence incorporated in that cycle of synthesis corresponds with the terminal nucleotide of the helper strand portion of the synthesis strand (shown at position “I” in Figure 2).

By “n+1” it is meant 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
25 (nucleotide labelled “Un” at position n+1, which occupies the position next to the position labelled “H” at position n in the direction distal to the primer strand, as shown schematically in steps (1) and (2) of Figure 2). n+1 may also refer to the corresponding position in the synthesis strand (labelled “X” at position n+1 in steps (1) and (2) of Figure 2). Thus at the cleavage step, position n+1 in the support strand is opposite the position
30 occupied by the penultimate nucleotide of the helper strand adjacent the nick site.

By “n-1” it is meant 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 (position labelled “J” at position n-1, as shown schematically in steps (1) and (2) of Figure 2). n-1 may also refer to the corresponding position in the synthesis strand (*i.e.* the position labelled “K” at position n-1, as shown schematically in step (2) of Figure 2). Thus at the cleavage step, position n-1 in the support strand is opposite the position occupied by the terminal nucleotide of the primer strand portion of the synthesis strand.

In methods according to version 2, the nucleotide pair which occupy positions H and I, and J and K, can be any nucleotide pair.

Upon cleavage of the support strand between nucleotide positions n and n-1, the universal nucleotide, helper strand (if present) and the portion of the support strand which is or was hybridized to the helper strand are removed from the remaining cleaved scaffold polynucleotide (see structure depicted at the top right of the upper part of Figure 2).

A phosphate group should continue to be attached to the terminal nucleotide of the support strand of the cleaved scaffold polynucleotide at the cleavage site (as depicted in the structure shown in the middle of the lower part of Figure 2). This ensures that the support strand of the ligation polynucleotide can be ligated to the support strand of the cleaved scaffold polynucleotide in the ligation step.

Thus in method version 2 the universal nucleotide occupies position n+1 in the support strand at steps (1) and (2) and the support strand is cleaved between nucleotide positions n and n-1.

Preferably, the support strand is cleaved by cleavage of the phosphodiester bond between nucleotide positions n and n-1 (the second phosphodiester bond of the support strand relative to the position of the universal nucleotide, in the direction distal to the helper strand/proximal to the primer strand).

The support strand may be cleaved by cleavage of one ester bond of the phosphodiester bond between nucleotide positions n and n-1.

Preferably the support strand is cleaved by cleavage of the first ester bond relative to nucleotide position n. This will have the effect of retaining a terminal phosphate group on the support strand of the cleaved scaffold polynucleotide at the cleavage position.

Any suitable mechanism may be employed to effect cleavage of the support strand between nucleotide positions n and $n-1$ when the universal nucleotide occupies position $n+1$.

Cleavage of the support strand between nucleotide positions n and $n-1$ as described
5 above may be performed by the action of an enzyme.

Cleavage of the support strand between nucleotide positions n and $n-1$ when the universal nucleotide occupies position $n+1$ in the support strand, as described above, may be performed by the action of an enzyme such as Endonuclease V.

One mechanism of cleaving the support strand between nucleotide positions n and
10 $n-1$ at a cleavage site defined by a sequence comprising a universal nucleotide which is occupying position $n+1$ in the support strand is described in analogous fashion in Example 3. The mechanism described is exemplary and other mechanisms could be employed, provided that the cleavage arrangement described above is achieved.

In this exemplary mechanism an endonuclease enzyme is employed. In the
15 exemplified method the enzyme is Endonuclease V. Other enzymes, molecules or chemicals could be used provided that the support strand is cleaved between nucleotide positions n and $n-1$ when the universal nucleotide occupies position $n+1$ in the support strand.

In this exemplary method version of the invention, as well as with all versions, to
20 allow the next nucleotide to be incorporated in the next synthesis cycle, the reversible terminator group must be removed from the first nucleotide (deprotection step; 204). This can be performed at various stages of the first cycle. Typically, and preferably, it will be performed as step (4) of the method, before ligation step (5), as shown in step 4 of Figure 2 (204). However, the deprotection step could be performed at any step after incorporation
25 of the new nucleotide, such as after the ligation step (5). Regardless of which stage the deprotection step is performed, enzyme and residual unincorporated first nucleotides should first be removed in order to prevent multiple incorporation of first nucleotides. Enzyme and unincorporated first nucleotides are preferably removed prior to the ligation step (step (5)).

Removal of the reversible terminator group from the first nucleotide can be performed by any suitable means. For example, removal can be performed by the use of a chemical, such as tris(carboxyethyl)phosphine (TCEP).

In step (5) of the method a double-stranded ligation polynucleotide is ligated (205)
5 to the cleaved scaffold polynucleotide. The ligation polynucleotide comprises a support strand and a helper strand. The ligation polynucleotide further comprises a complementary ligation end comprising in the support strand a universal nucleotide and a single overhanging nucleotide which overhangs the terminal nucleotide of the helper strand and which is the partner nucleotide for the first nucleotide of the predefined sequence. The
10 ligation polynucleotide further comprises in the helper strand adjacent the overhang a terminal nucleotide which is configured such that it cannot be ligated to another polynucleotide strand (position labelled "I" in the structure depicted at the top left of the upper part of Figure 2). Typically this terminal nucleotide will lack a phosphate group. Typically, as described above, this terminal nucleotide of the helper strand will define the
15 5' terminus of the helper strand.

In the complementary ligation end of the ligation polynucleotide the universal nucleotide in the support strand is positioned opposite the penultimate nucleotide of the helper strand (position labelled "X" in step (5) of Figure 2) and is paired therewith. The terminal nucleotide of the helper strand (position labelled "I" in step (5) of Figure 2 at
20 position n+1) is paired with a nucleotide in the support strand which occupies a position (position labelled "H" in step (5) of Figure 2 at position n+1) which is between the universal nucleotide at position n+2 and the partner nucleotide at position n.

The complementary ligation end is configured so that it will compatibly join with the overhanging end of the cleaved scaffold polynucleotide product following
25 incorporation step (3) when subjected to suitable ligation conditions. Upon ligation of the support strands, the first nucleotide becomes paired with its partner nucleotide.

Ligation of the support strands may be performed by any suitable means. Ligation will result in the joining of the support strands only, with the maintenance of a single-stranded break between the first nucleotide in the synthesis strand, *i.e.* in the primer strand
30 portion, and the terminal nucleotide of the helper strand adjacent the first nucleotide.

Ligation may typically be performed by enzymes having ligase activity. For example, ligation may be performed with T3 DNA ligase or T4 DNA ligase or functional variants or equivalents thereof. The use of such enzymes will result in the maintenance of the single-stranded break in the synthesis strand, since the terminal nucleotide of the helper strand is provided such that it cannot act as a substrate for ligase, *e.g.* due to the absence of a terminal phosphate group.

Ligation of the ligation polynucleotide to the cleaved scaffold polynucleotide completes a first synthesis cycle whereupon the scaffold polynucleotide of step (1) is effectively re-constituted except that the first nucleotide of the predefined nucleotide sequence is incorporated into the scaffold polynucleotide opposite its partner nucleotide.

In step (5) of this exemplary method (205), in the complementary ligation end of the ligation polynucleotide the universal nucleotide in the support strand is positioned opposite the penultimate nucleotide of the helper strand (position labelled “X”) and is paired therewith. It will be noted that in the context of the ligation polynucleotide the universal nucleotide is positioned at position $n+2$, *i.e.* two positions removed from the terminal nucleotide of the support strand (position n). Position n in the context of the ligation polynucleotide has the same meaning as described above in the context of the scaffold polynucleotide at steps (1) and (2). Thus in step (5) by “ n ” it is meant the nucleotide position in the support strand which, following ligation, is opposite the position in the synthesis strand which is now occupied by the nucleotide of the predefined sequence following its addition to the terminal end of the primer strand portion of the synthesis strand in that cycle of synthesis. By “ n ” it is also meant the nucleotide position in the synthesis strand following ligation which is now occupied by the nucleotide of the predefined sequence following its addition to the terminal end of the primer strand portion of the synthesis strand in that cycle of synthesis.

It will be noted that the universal nucleotide occupies position $n+1$ in the scaffold polynucleotide during steps (1) and (2) (201 and 202 of Figure 2), whereas the universal nucleotide occupies position $n+2$ in the ligation polynucleotide in the same synthesis cycle (205 of Figure 2). This is because at step (5) the cleaved double-stranded scaffold polynucleotide is now provided with the nucleotide of the predefined sequence following

its addition in that cycle of synthesis and because the ligation polynucleotide is provided with the nucleotide to partner the nucleotide of the predefined sequence; and furthermore because as defined herein “n” always refers to the nucleotide position in the synthesis strand which is occupied by (or will be occupied by) the nucleotide of the predefined sequence incorporated in that cycle or refers to the nucleotide position opposite thereto in the support strand. Thus at the end of any given cycle of synthesis, immediately after ligation step (5), the position occupied by a universal nucleotide will be n+2 and will have moved from n+1, the position a universal nucleotide will have occupied at step (1). The product of the ligation step (5) (structure labelled “ligation product” in Figure 2) will be the scaffold polynucleotide for use in the next cycle of synthesis. When the ligation product is considered as the scaffold polynucleotide for use in the next cycle of synthesis (206) it is to be understood that the position occupied by the universal nucleotide is now to be once again referred to as position n+1 (206), rather than n+2 (205). Thus in method version 2 the position occupied by the universal nucleotide at the beginning of any given cycle of synthesis (201, 206 etc.) is always referred to as position n+1, and the nucleotide which is newly incorporated in that cycle always occupies a position referred to as n.

Following completion of the first synthesis cycle, second and subsequent cycles are performed using the same method steps.

As in step (2) of the first synthesis cycle of method version 2, in step (6) the scaffold polynucleotide is cleaved (207) at a cleavage site. The cleavage site is defined by a sequence comprising the universal nucleotide in the support strand. Cleavage results in a double-stranded break in the scaffold polynucleotide. Cleavage of the scaffold polynucleotide leaves in place a cleaved double-stranded scaffold polynucleotide comprising, at the site of cleavage, a cleaved terminal end of the support strand and the terminal end of the primer strand portion of the synthesis strand which comprised the nick site prior to cleavage. Cleavage results in removal of the support strand comprising the universal nucleotide and the helper strand hybridised to the support strand, if present immediately prior to cleavage.

The cleavage steps may be performed as described above for step (2) of the first cycle.

In step (7) the next nucleotide is added to the terminal end of the cleaved primer strand portion of the synthesis strand as in step (3) of the first cycle.

In step (8) the reversible terminator group is removed from the next nucleotide (deprotection step; 209). As described above for first cycle, this can be performed at various stages. Typically, and preferably, it will be performed as step (8) of the method, before ligation step (9). However, the deprotection step could be performed at any step after incorporation of the new nucleotide, such as after the ligation step (9).

Deprotection of the reversible terminator group in the next cycle (209) and subsequent cycles may be performed as described above with respect to the first synthesis cycle.

In step (9) of the next cycle a double-stranded ligation polynucleotide is ligated (210) to the cleaved scaffold polynucleotide. The ligation polynucleotide of step (9) of the next and subsequent synthesis cycles may be configured, and the ligation step may be performed, as described above for step (5) of the first synthesis cycle.

Synthesis cycles are repeated as described above for as many times as necessary to synthesise the double-stranded polynucleotide having the predefined nucleotide sequence.

Synthesis Method Version 3

A third exemplary version of the synthesis method of the invention is performed in an analogous matter to the first version except with modified structural arrangements for the positioning of the universal nucleotide relative to the cleavage site.

Thus as with version 1, in version 3 cleavage of the scaffold polynucleotide (step 2, 302) results in loss of the helper strand, if present immediately prior to cleavage, and loss of the support strand comprising the universal nucleotide. Cleavage leaves in place a cleaved double-stranded scaffold polynucleotide comprising, at the site of cleavage, a cleaved terminal end of the support strand and the terminal end of the primer strand portion of the synthesis strand which comprised the nick site prior to cleavage. Cleavage results in a cleaved double-stranded scaffold polynucleotide with a single nucleotide overhang at the site of cleavage.

In step (3) of the methods a first nucleotide in the predefined nucleotide sequence is added to the terminal end of the primer strand portion of the synthesis strand by the action of an enzyme having transferase activity such as polymerase or a terminal nucleotidyl transferase enzyme (303). The first nucleotide is provided with a reversible terminator
5 group which prevents further extension by the enzyme. Thus in step (3) only a single nucleotide is incorporated.

In step (4) of the methods a deprotection step is performed (304) to remove the terminator group from the newly-incorporated nucleotide. In principle the deprotection step may be performed after the ligation step (5). Performance of the deprotection step as
10 step (4) is preferred.

In step (5) of the methods a ligation polynucleotide (see structure depicted at the top left of the upper part of Figure 3) is ligated to the cleaved scaffold polynucleotide. Ligation incorporates a partner nucleotide into the scaffold polynucleotide and allows the newly-incorporated nucleotide to pair with the partner nucleotide (step 5 of Figure 3; 305),
15 thus completing a synthesis cycle.

In the third exemplary version of the synthesis method of the invention a scaffold polynucleotide is provided in step (1) (301) as described above. In this method 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 site
20 (labelled "X" in the structures of Figure 3), and is paired therewith (see structure depicted in step 1 of Figure 3).

In step (2) of the method the scaffold polynucleotide is cleaved (302) at a cleavage site. The cleavage site is defined by a sequence comprising the universal nucleotide in the support strand. Cleavage results in a double-stranded break in the scaffold polynucleotide.
25 Cleavage of the scaffold polynucleotide (step 2) results in loss of the helper strand, if present immediately prior to cleavage, and loss of the support strand comprising the universal nucleotide. Cleavage of the scaffold polynucleotide leaves in place a cleaved double-stranded scaffold polynucleotide comprising, at the site of cleavage, a cleaved terminal end of the support strand and the terminal end of the primer strand portion of the
30 synthesis strand which comprised the nick site prior to cleavage.

The synthesis strand is already provided with a single-stranded break or “nick” in this exemplary method, thus only cleavage of the support strand is necessary to provide a double-stranded break in the scaffold polynucleotide.

In this exemplary method version cleavage generates a cleaved double-stranded scaffold polynucleotide with a single nucleotide overhang. Thus the terminal nucleotide of the primer strand portion of the synthesis strand overhangs the terminal nucleotide of the support strand.

In this method the universal nucleotide occupies position “n” in the support strand prior to the cleavage step. To obtain such a cleaved double-stranded scaffold polynucleotide wherein the terminal nucleotide of the primer strand portion of the synthesis strand overhangs the terminal nucleotide of the support strand when the universal nucleotide occupies position n in the support strand, the support strand is cleaved at a specific position relative to the universal nucleotide and relative to the nick site. The support strand of the scaffold polynucleotide is cleaved between nucleotide positions n-1 and n-2.

By “n” it is meant 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 addition to the terminal end of the cleaved synthesis strand in that cycle of synthesis. By “n” it is also meant the nucleotide position in the synthesis strand which will be occupied by the nucleotide of the predefined sequence upon its addition to the terminal end of the primer strand portion of the synthesis strand in that cycle of synthesis. Thus at the cleavage step, the universal nucleotide is at position n in the support strand, which is opposite the position occupied by the terminal nucleotide of the helper strand adjacent the nick site (shown at position “X” in Figure 3).

At steps (1) and (2) the position which will be occupied by the nucleotide of the predefined sequence incorporated in that cycle of synthesis corresponds with the terminal nucleotide of the helper strand portion of the synthesis strand (shown at position “X” in Figure 3).

By “n-1” it is meant 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 (position

labelled “H” at position n-1, as shown schematically in steps (1) and (2) of Figure 3). n-1 may also refer to the corresponding position in the synthesis strand (*i.e.* the position labelled “I” at position n-1, as shown schematically in step (2) of Figure 3). Thus at the cleavage step, position n-1 in the support strand is opposite the position occupied by the terminal nucleotide of the primer strand portion of the synthesis strand. In steps (1) and (2) of Figure 3 the nucleotides at position “J” and “K” thus occupy position n-2.

In methods according to version 3, the nucleotide pair which occupy positions H and I, and J and K, can be any nucleotide pair.

Upon cleavage of the support strand between nucleotide positions n-1 and n-2, the universal nucleotide, helper strand (if present) and the portion of the support strand which is or was hybridized to the helper strand are removed from the remaining cleaved scaffold polynucleotide (see structure depicted at the top right of the upper part of Figure 3).

A phosphate group should continue to be attached to the terminal nucleotide of the support strand of the cleaved scaffold polynucleotide at the cleavage site (as depicted in the structure shown in the middle of the lower part of Figure 3). This ensures that the support strand of the ligation polynucleotide can be ligated to the support strand of the cleaved scaffold polynucleotide in the ligation step.

Thus in method version 3 the universal nucleotide occupies position n in the support strand at steps (1) and (2) and the support strand is cleaved between nucleotide positions n-1 and n-2.

Preferably, the support strand is cleaved by cleavage of the phosphodiester bond between nucleotide positions n-1 and n-2 (the second phosphodiester bond of the support strand relative to the position of the universal nucleotide, in the direction distal to the helper strand/proximal to the primer strand).

The support strand may be cleaved by cleavage of one ester bond of the phosphodiester bond between nucleotide positions n-1 and n-2.

Preferably the support strand is cleaved by cleavage of the first ester bond relative to nucleotide position n-1. This will have the effect of retaining a terminal phosphate group on the support strand of the cleaved scaffold polynucleotide at the cleavage position.

Any suitable mechanism may be employed to effect cleavage of the support strand between nucleotide positions n-1 and n-2 when the universal nucleotide occupies position n.

Cleavage of the support strand between nucleotide positions n-1 and n-2 as
5 described above may be performed by the action of an enzyme.

Cleavage of the support strand between nucleotide positions n-1 and n-2 when the universal nucleotide occupies position n in the support strand, as described above, may be performed by the action of an enzyme such as Endonuclease V.

One mechanism of cleaving the support strand between nucleotide positions n-1
10 and n-2 at a cleavage site defined by a sequence comprising a universal nucleotide which is occupying position n in the support strand is described in analogous fashion in Example 3. The mechanism described is exemplary and other mechanisms could be employed, provided that the cleavage arrangement and single-nucleotide overhang described above is achieved.

15 In this exemplary mechanism an endonuclease enzyme is employed. In the exemplified method the enzyme is Endonuclease V. Other enzymes, molecules or chemicals could be used provided that the support strand is cleaved between nucleotide positions n-1 and n-2 when the universal nucleotide occupies position n in the support strand.

20 In this exemplary method version of the invention, as well as with all versions, to allow the next nucleotide to be incorporated in the next synthesis cycle, the reversible terminator group must be removed from the first nucleotide (deprotection step; 304). This can be performed at various stages of the first cycle. Typically, and preferably, it will be performed as step (4) of the method, before ligation step (5), as shown in step 4 of Figure 3
25 (304). However, the deprotection step could be performed at any step after incorporation of the new nucleotide, such as after the ligation step (5). Regardless of which stage the deprotection step is performed, enzyme and residual unincorporated first nucleotides should first be removed in order to prevent multiple incorporation of first nucleotides. Enzyme and unincorporated first nucleotides are preferably removed prior to the ligation
30 step (step (5)).

Removal of the reversible terminator group from the first nucleotide can be performed by any suitable means. For example, removal can be performed by the use of a chemical, such as tris(carboxyethyl)phosphine (TCEP).

In step (5) of the method a double-stranded ligation polynucleotide is ligated (305) to the cleaved scaffold polynucleotide. The ligation polynucleotide comprises a support strand and a helper strand. The ligation polynucleotide further comprises a complementary ligation end comprising in the support strand a universal nucleotide and two overhanging nucleotides which overhang the terminal nucleotide of the helper strand. The penultimate nucleotide of the support strand of the ligation polynucleotide at the complementary ligation end is the partner nucleotide for the first nucleotide of the predefined sequence. The ligation polynucleotide further comprises in the helper strand adjacent the overhang a terminal nucleotide which is configured such that it cannot be ligated to another polynucleotide strand (position labelled "X" in the structure depicted at the top left of the upper part of Figure 3). Typically this terminal nucleotide will lack a phosphate group. Typically, as described above, this terminal nucleotide of the helper strand will define the 5' terminus of the helper strand.

In the complementary ligation end of the ligation polynucleotide the universal nucleotide in the support strand is positioned (n+1) opposite the terminal nucleotide of the helper strand (position labelled "X" in step (5) of Figure 3) and is paired therewith. The partner nucleotide for the first nucleotide of the predefined sequence occupies the next nucleotide position relative to the universal nucleotide in the direction distal to the helper strand (position n) and is positioned between the universal nucleotide and the terminal nucleotide of the support strand of the ligation polynucleotide (position labelled "H" in step (5) of Figure 3 at position n-1).

The complementary ligation end is configured so that it will compatibly join with the overhanging end of the cleaved scaffold polynucleotide product following incorporation step (3) when subjected to suitable ligation conditions. Upon ligation of the support strands, the first nucleotide becomes paired with its partner nucleotide.

Ligation of the support strands may be performed by any suitable means. Ligation will result in the joining of the support strands only, with the maintenance of a single-

stranded break between the first nucleotide in the synthesis strand, *i.e.* in the primer strand portion, and the terminal nucleotide of the helper strand adjacent the first nucleotide.

Ligation may typically be performed by enzymes having ligase activity. For example, ligation may be performed with T3 DNA ligase or T4 DNA ligase or functional
5 variants or equivalents thereof. The use of such enzymes will result in the maintenance of the single-stranded break in the synthesis strand, since the terminal nucleotide of the helper strand is provided such that it cannot act as a substrate for ligase, *e.g.* due to the absence of a terminal phosphate group.

Ligation of the ligation polynucleotide to the cleaved scaffold polynucleotide
10 completes a first synthesis cycle whereupon the scaffold polynucleotide of step (1) is effectively re-constituted except that the first nucleotide of the predefined nucleotide sequence is incorporated into the scaffold polynucleotide opposite its partner nucleotide.

In step (5) of this exemplary method (305), in the complementary ligation end of the ligation polynucleotide the universal nucleotide in the support strand is positioned
15 opposite the terminal nucleotide of the helper strand (position labelled “X”) and is paired therewith. It will be noted that in the context of the ligation polynucleotide the universal nucleotide is positioned at position $n+1$ and two positions removed from the terminal nucleotide of the support strand (position $n-1$). Position n in the context of the ligation polynucleotide has the same meaning as described above in the context of the scaffold
20 polynucleotide at steps (1) and (2). Thus in step (5) by “ n ” it is meant the nucleotide position in the support strand which, following ligation, is opposite the position in the synthesis strand which is now occupied by the nucleotide of the predefined sequence following its addition to the terminal end of the primer strand portion of the synthesis strand in that cycle of synthesis. By “ n ” it is also meant the nucleotide position in the
25 synthesis strand following ligation which is now occupied by the nucleotide of the predefined sequence following its addition to the terminal end of the primer strand portion of the synthesis strand in that cycle of synthesis.

It will be noted that the universal nucleotide occupies position n in the scaffold polynucleotide during steps (1) and (2) (301 and 302 of Figure 3), whereas the universal
30 nucleotide occupies position $n+1$ in the ligation polynucleotide in the same synthesis cycle

(305 of Figure 3). This is because at step (5) the cleaved double-stranded scaffold polynucleotide is now provided with the nucleotide of the predefined sequence following its addition in that cycle of synthesis and because the ligation polynucleotide is provided with the nucleotide to partner the nucleotide of the predefined sequence; and furthermore because as defined herein “n” always refers to the nucleotide position in the synthesis strand which is occupied by (or will be occupied by) the nucleotide of the predefined sequence incorporated in that cycle or refers to the nucleotide position opposite thereto in the support strand. Thus at the end of any given cycle of synthesis, immediately after ligation step (5), the position occupied by a universal nucleotide will be $n+1$ and will have moved from n , the position a universal nucleotide will have occupied at step (1). The product of the ligation step (5) (structure labelled “ligation product” in Figure 3) will be the scaffold polynucleotide for use in the next cycle of synthesis. When the ligation product is considered as the scaffold polynucleotide for use in the next cycle of synthesis (306) it is to be understood that the position occupied by the universal nucleotide is now to be once again referred to as position n (306), rather than $n+1$ (305). Thus in method version 3 the position occupied by the universal nucleotide at the beginning of any given cycle of synthesis (301, 306 etc.) is always referred to as position n , and the nucleotide which is newly incorporated in that cycle always occupies a position referred to as n .

Following completion of the first synthesis cycle, second and subsequent cycles are performed using the same method steps.

As in step (2) of the first synthesis cycle of method version 3, in step (6) the scaffold polynucleotide is cleaved (307) at a cleavage site. The cleavage site is defined by a sequence comprising the universal nucleotide in the support strand. Cleavage results in a double-stranded break in the scaffold polynucleotide. Cleavage of the scaffold polynucleotide leaves in place a cleaved double-stranded scaffold polynucleotide comprising, at the site of cleavage, a cleaved terminal end of the support strand and the terminal end of the primer strand portion of the synthesis strand which comprised the nick site prior to cleavage. Cleavage results in removal of the support strand comprising the universal nucleotide and the helper strand hybridised to the support strand, if present immediately prior to cleavage.

The cleavage steps may be performed as described above for step (2) of the first cycle.

In step (7) the next nucleotide is added to the terminal end of the cleaved primer strand portion of the synthesis strand as in step (3) of the first cycle.

5 In step (8) the reversible terminator group is removed from the next nucleotide (deprotection step; 309). As described above for first cycle, this can be performed at various stages. Typically, and preferably, it will be performed as step (8) of the method, before ligation step (9). However, the deprotection step could be performed at any step after incorporation of the new nucleotide, such as after the ligation step (9).

10 Deprotection of the reversible terminator group in the next cycle (309) and subsequent cycles may be performed as described above with respect to the first synthesis cycle.

In step (9) of the next cycle a double-stranded ligation polynucleotide is ligated (310) to the cleaved scaffold polynucleotide. The ligation polynucleotide of step (9) of the next and subsequent synthesis cycles may be configured, and the ligation step may be performed, as described above for step (5) of the first synthesis cycle.

Synthesis cycles are repeated as described above for as many times as necessary to synthesise the double-stranded polynucleotide having the predefined nucleotide sequence.

20 Synthesis Method Version 4

A fourth exemplary version of the synthesis method of the invention is performed in an analogous matter to the first version except with modified structural arrangements for the positioning of the universal nucleotide relative to the position to be occupied by the new nucleotide to be incorporated in a given synthesis cycle and relative to the nick and cleavage sites.

25 Thus as with version 1, in version 4 cleavage of the scaffold polynucleotide (step 2, 402) results in loss of the helper strand, if present immediately prior to cleavage, and loss of the support strand comprising the universal nucleotide. Cleavage leaves in place a
30 cleaved double-stranded scaffold polynucleotide comprising, at the site of cleavage, a

cleaved terminal end of the support strand and the terminal end of the primer strand portion of the synthesis strand which comprised the nick site prior to cleavage. Cleavage results in a blunt-ended cleaved double-stranded scaffold polynucleotide at the site of cleavage, with no overhang.

5 In step (3) of the methods a first nucleotide in the predefined nucleotide sequence is added to the terminal end of the primer strand portion of the synthesis strand by the action of an enzyme having transferase activity such as polymerase or a terminal nucleotidyl transferase enzyme (403). The first nucleotide is provided with a reversible terminator group which prevents further extension by the enzyme. Thus in step (3) only a single
10 nucleotide is incorporated.

 In step (4) of the methods a deprotection step is performed (404) to remove the terminator group from the newly-incorporated nucleotide. In principle the deprotection step may be performed after the ligation step (5). Performance of the deprotection step as step (4) is preferred.

15 In step (5) of the methods a ligation polynucleotide (see structure depicted at the top left of the upper part of Figure 4) is ligated to the cleaved scaffold polynucleotide. Ligation incorporates a partner nucleotide into the scaffold polynucleotide and allows the newly-incorporated nucleotide to pair with the partner nucleotide (step 5 of Figure 4; 405), thus completing a synthesis cycle.

20 In the fourth exemplary version of the synthesis method of the invention a scaffold polynucleotide is provided in step (1) (401) as described above. In this method the universal nucleotide in the support strand of the scaffold polynucleotide is positioned opposite and paired with a nucleotide (labelled “X” in the structures of Figure 4) which is two positions removed from the terminal nucleotide of the helper strand (labelled “K” in
25 the structure depicted in step (1) of Figure 4) adjacent the single-strand break site.

 In step (2) of the method the scaffold polynucleotide is cleaved (402) at a cleavage site. The cleavage site is defined by a sequence comprising the universal nucleotide in the support strand. Cleavage results in a double-stranded break in the scaffold polynucleotide. Cleavage of the scaffold polynucleotide (step 2) results in loss of the helper strand, if
30 present immediately prior to cleavage, and loss of the support strand comprising the

universal nucleotide. Cleavage of the scaffold polynucleotide leaves in place a cleaved double-stranded scaffold polynucleotide comprising, at the site of cleavage, a cleaved terminal end of the support strand and the terminal end of the primer strand portion of the synthesis strand which comprised the nick site prior to cleavage.

5 The synthesis strand is already provided with a single-stranded break or “nick” in this exemplary method, thus only cleavage of the support strand is necessary to provide a double-stranded break in the scaffold polynucleotide.

In this exemplary method version cleavage generates a blunt-ended cleaved double-stranded scaffold polynucleotide with no overhang.

10 In this method the universal nucleotide occupies position “n+2” in the support strand prior to the cleavage step. To obtain such a blunt-ended cleaved double-stranded scaffold polynucleotide when the universal nucleotide occupies position n+2 in the support strand, the support strand is cleaved at a specific position relative to the universal nucleotide and relative to the nick site. The support strand of the scaffold polynucleotide is
15 cleaved between nucleotide positions n and n-1.

By “n” it is meant 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 addition to the terminal end of the cleaved synthesis strand in that cycle of synthesis. By “n” it is also meant the nucleotide position in the synthesis
20 strand which will be occupied by the nucleotide of the predefined sequence upon its addition to the terminal end of the primer strand portion of the synthesis strand in that cycle of synthesis. Thus at the cleavage step, the universal nucleotide is at position n+2 in the support strand, which is opposite the nucleotide which is two positions removed from the terminal nucleotide of the helper strand adjacent the single-strand break site (shown at
25 position “X” in step (2) in Figure 4).

At steps (1) and (2) the position which will be occupied by the nucleotide of the predefined sequence incorporated in that cycle of synthesis corresponds with the nucleotide which is two positions removed from the terminal nucleotide of the helper strand adjacent the single-strand break site (shown at position “X” in step (2) of Figure 4).

By “n+1” it is meant 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 (nucleotide labelled “H” at position n+1, which occupies the position next to the position labelled “J” at position n in the direction distal to the primer strand, as shown

5 schematically in steps (1) and (2) of Figure 4). n+1 may also refer to the corresponding position in the synthesis strand (labelled “I” at position n+1 in steps (1) and (2) of Figure 4). Thus at the cleavage step, position n+1 in the support strand is opposite the position occupied by the penultimate nucleotide of the helper strand adjacent the nick site.

By “n-1” it is meant the next nucleotide position in the support strand relative to
10 position n in the direction distal to the helper strand/proximal to the primer strand (position labelled “L” at position n-1, as shown schematically in steps (1) and (2) of Figure 4). n-1 may also refer to the corresponding position in the synthesis strand (*i.e.* the position labelled “M” at position n-1, as shown schematically in steps (1) and (2) of Figure 4). Thus at the cleavage step, position n-1 in the support strand is opposite the position
15 occupied by the terminal nucleotide of the primer strand portion of the synthesis strand. In steps (1) and (2) of Figure 4 the nucleotides at position “J” and “K” thus occupy position n.

In methods according to version 4, the nucleotide pair which occupy positions H and I; J and K; and L and M can be any nucleotide pair.

Upon cleavage of the support strand between nucleotide positions n and n-1, the
20 universal nucleotide, helper strand (if present) and the portion of the support strand which is or was hybridized to the helper strand are removed from the remaining cleaved scaffold polynucleotide (see structure depicted at the top right of the upper part of Figure 4).

A phosphate group should continue to be attached to the terminal nucleotide of the support strand of the cleaved scaffold polynucleotide at the cleavage site (as depicted in the
25 structure shown in the middle of the lower part of Figure 4). This ensures that the support strand of the ligation polynucleotide can be ligated to the support strand of the cleaved scaffold polynucleotide in the ligation step.

Thus in method version 4 the universal nucleotide occupies position n+2 in the support strand at steps (1) and (2) and the support strand is cleaved between nucleotide
30 positions n and n-1.

Preferably, the support strand is cleaved by cleavage of the phosphodiester bond between nucleotide positions n and $n-1$ (the third phosphodiester bond of the support strand relative to the position of the universal nucleotide, in the direction distal to the helper strand/proximal to the primer strand).

5 The support strand may be cleaved by cleavage of one ester bond of the phosphodiester bond between nucleotide positions n and $n-1$.

Preferably the support strand is cleaved by cleavage of the first ester bond relative to nucleotide position n . This will have the effect of retaining a terminal phosphate group on the support strand of the cleaved scaffold polynucleotide at the cleavage position.

10 Any suitable mechanism may be employed to effect cleavage of the support strand between nucleotide positions n and $n-1$ when the universal nucleotide occupies position $n+2$.

Cleavage of the support strand between nucleotide positions n and $n-1$ as described above may be performed by the action of an enzyme.

15 In this exemplary method version of the invention, as well as with all versions, to allow the next nucleotide to be incorporated in the next synthesis cycle, the reversible terminator group must be removed from the first nucleotide (deprotection step; 404). This can be performed at various stages of the first cycle. Typically, and preferably, it will be performed as step (4) of the method, before ligation step (5), as shown in step 4 of Figure 4
20 (404). However, the deprotection step could be performed at any step after incorporation of the new nucleotide, such as after the ligation step (5). Regardless of which stage the deprotection step is performed, enzyme and residual unincorporated first nucleotides should first be removed in order to prevent multiple incorporation of first nucleotides. Enzyme and unincorporated first nucleotides are preferably removed prior to the ligation
25 step (step (5)).

Removal of the reversible terminator group from the first nucleotide can be performed by any suitable means. For example, removal can be performed by the use of a chemical, such as tris(carboxyethyl)phosphine (TCEP).

30 In step (5) of the method a double-stranded ligation polynucleotide is ligated (405) to the cleaved scaffold polynucleotide. The ligation polynucleotide comprises a support

strand and a helper strand. The ligation polynucleotide further comprises a complementary ligation end comprising in the support strand a universal nucleotide and a single overhanging nucleotide which overhang the terminal nucleotide of the helper strand. The overhanging nucleotide is the partner nucleotide for the first nucleotide of the predefined sequence. The ligation polynucleotide further comprises in the helper strand adjacent the overhang a terminal nucleotide which is configured such that it cannot be ligated to another polynucleotide strand (position labelled “K” in the structure depicted at the top left of the upper part of Figure 4). Typically this terminal nucleotide will lack a phosphate group. Typically, as described above, this terminal nucleotide of the helper strand will define the 5’ terminus of the helper strand.

In the complementary ligation end of the ligation polynucleotide the universal nucleotide in the support strand is positioned (n+3) opposite a nucleotide which is two positions removed from the terminal nucleotide of the helper strand (position labelled “X” in step (5) of Figure 4) and is paired therewith. The partner nucleotide for the first nucleotide of the predefined sequence occupies a position as the terminal nucleotide of the support strand of the ligation polynucleotide (position n). The nucleotide which is one position removed from the terminal nucleotide of the support strand of the ligation polynucleotide is labelled “J” in step (5) of Figure 4 at position n+1. The nucleotide which is two positions removed from the terminal nucleotide of the support strand of the ligation polynucleotide is labelled “H” in step (5) of Figure 4 at position n+2.

The complementary ligation end is configured so that it will compatibly join with the overhanging end of the cleaved scaffold polynucleotide product following incorporation step (3) when subjected to suitable ligation conditions. Upon ligation of the support strands, the first nucleotide becomes paired with its partner nucleotide.

Ligation of the support strands may be performed by any suitable means. Ligation will result in the joining of the support strands only, with the maintenance of a single-stranded break between the first nucleotide in the synthesis strand, *i.e.* in the primer strand portion, and the terminal nucleotide of the helper strand adjacent the first nucleotide.

Ligation may typically be performed by enzymes having ligase activity. For example, ligation may be performed with T3 DNA ligase or T4 DNA ligase or functional

variants or equivalents thereof. The use of such enzymes will result in the maintenance of the single-stranded break in the synthesis strand, since the terminal nucleotide of the helper strand is provided such that it cannot act as a substrate for ligase, *e.g.* due to the absence of a terminal phosphate group.

5 Ligation of the ligation polynucleotide to the cleaved scaffold polynucleotide completes a first synthesis cycle whereupon the scaffold polynucleotide of step (1) is effectively re-constituted except that the first nucleotide of the predefined nucleotide sequence is incorporated into the scaffold polynucleotide opposite its partner nucleotide.

10 In step (5) of this exemplary method (305), in the complementary ligation end of the ligation polynucleotide the universal nucleotide in the support strand is positioned opposite the nucleotide which is two positions removed from the terminal nucleotide of the helper strand adjacent the single-strand break site (shown at position “X” in step (5) of Figure 4). It will be noted that in the context of the ligation polynucleotide the universal nucleotide is positioned at position $n+3$ and three positions removed from the terminal nucleotide of the support strand (position n). Position n in the context of the ligation polynucleotide has the same meaning as described above in the context of the scaffold polynucleotide at steps (1) and (2). Thus in step (5) by “ n ” it is meant the nucleotide position in the support strand which, following ligation, is opposite the position in the synthesis strand which is now occupied by the nucleotide of the predefined sequence following its addition to the terminal end of the primer strand portion of the synthesis strand in that cycle of synthesis. By “ n ” it is also meant the nucleotide position in the synthesis strand following ligation which is now occupied by the nucleotide of the predefined sequence following its addition to the terminal end of the primer strand portion of the synthesis strand in that cycle of synthesis.

25 It will be noted that the universal nucleotide occupies position $n+2$ in the scaffold polynucleotide during steps (1) and (2) (401 and 402 of Figure 4), whereas the universal nucleotide occupies position $n+3$ in the ligation polynucleotide in the same synthesis cycle (405 of Figure 4). This is because at step (5) the cleaved double-stranded scaffold polynucleotide is now provided with the nucleotide of the predefined sequence following its addition in that cycle of synthesis and because the ligation polynucleotide is provided

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with the nucleotide to partner the nucleotide of the predefined sequence; and furthermore because as defined herein “n” always refers to the nucleotide position in the synthesis strand which is occupied by (or will be occupied by) the nucleotide of the predefined sequence incorporated in that cycle or refers to the nucleotide position opposite thereto in the support strand. Thus at the end of any given cycle of synthesis, immediately after ligation step (5), the position occupied by a universal nucleotide will be $n+3$ and will have moved from $n+2$, the position a universal nucleotide will have occupied at step (1). The product of the ligation step (5) (structure labelled “ligation product” in Figure 4) will be the scaffold polynucleotide for use in the next cycle of synthesis. When the ligation product is considered as the scaffold polynucleotide for use in the next cycle of synthesis (406) it is to be understood that the position occupied by the universal nucleotide is now to be once again referred to as position $n+2$ (406), rather than $n+3$ (405). Thus in method version 4 the position occupied by the universal nucleotide at the beginning of any given cycle of synthesis (401, 406 etc.) is always referred to as position $n+2$, and the nucleotide which is newly incorporated in that cycle always occupies a position referred to as n .

Following completion of the first synthesis cycle, second and subsequent cycles are performed using the same method steps.

As in step (2) of the first synthesis cycle of method version 4, in step (6) the scaffold polynucleotide is cleaved (407) at a cleavage site. The cleavage site is defined by a sequence comprising the universal nucleotide in the support strand. Cleavage results in a double-stranded break in the scaffold polynucleotide. Cleavage of the scaffold polynucleotide leaves in place a cleaved double-stranded scaffold polynucleotide comprising, at the site of cleavage, a cleaved terminal end of the support strand and the terminal end of the primer strand portion of the synthesis strand which comprised the nick site prior to cleavage. Cleavage results in removal of the support strand comprising the universal nucleotide and the helper strand hybridised to the support strand, if present immediately prior to cleavage.

The cleavage steps may be performed as described above for step (2) of the first cycle.

In step (7) the next nucleotide is added to the terminal end of the cleaved primer strand portion of the synthesis strand as in step (3) of the first cycle.

In step (8) the reversible terminator group is removed from the next nucleotide (deprotection step; 409). As described above for first cycle, this can be performed at various stages. Typically, and preferably, it will be performed as step (8) of the method, before ligation step (9). However, the deprotection step could be performed at any step after incorporation of the new nucleotide, such as after the ligation step (9).

Deprotection of the reversible terminator group in the next cycle (409) and subsequent cycles may be performed as described above with respect to the first synthesis cycle.

In step (9) of the next cycle a double-stranded ligation polynucleotide is ligated (410) to the cleaved scaffold polynucleotide. The ligation polynucleotide of step (9) of the next and subsequent synthesis cycles may be configured, and the ligation step may be performed, as described above for step (5) of the first synthesis cycle.

Synthesis cycles are repeated as described above for as many times as necessary to synthesise the double-stranded polynucleotide having the predefined nucleotide sequence.

Variation Methods Based on Synthesis Method Version 4

It will be appreciated that synthesis method version 4 is a variation of synthesis method version 2. Both methods require cleavage of the support strand of the scaffold polynucleotide between positions n and $n-1$. In version 2 prior to and at the cleavage step of the first and second cycles (steps 2 and 6) the universal nucleotide occupies position $n+1$ in the support strand of the scaffold polynucleotide. In contrast in version 4 prior to and at the cleavage step of the first and second cycles (steps 2 and 6) the universal nucleotide occupies position $n+2$ in the support strand of the scaffold polynucleotide. Thus synthesis method version 4 is the same as synthesis method version 2 except that in synthesis method version 4 the universal nucleotide occupies one position further removed from position n in the direction distal to primer strand portion.

Further variations of synthesis method version 4 are envisaged wherein the support strand of the scaffold polynucleotide is cleaved between positions n and $n-1$ and wherein in each further variant method the universal nucleotide occupies incrementally one position further removed from position n in the direction distal to primer strand portion, starting at position $n+3$ and increasing incrementally according to the formula $n+3+x$ wherein x is a whole number between 1 to 10 or more.

Thus an “ $n+3$ ” variation of synthesis method version 4 is provided wherein the method is performed in the same way as synthesis method version 4 described above except for the following variations.

Prior to and at the cleavage step of the first cycle (steps 1 and 2) the universal nucleotide instead occupies position $n+3$ in the support strand of the scaffold polynucleotide, wherein $n+3$ is the third nucleotide position in the support strand relative to position n in the direction proximal to the helper strand/distal to the primer strand portion; and the support strand of the scaffold polynucleotide is cleaved between positions n and $n-1$.

In the ligation step of the first cycle (step 5) the complementary ligation end of the ligation polynucleotide is structured such that the universal nucleotide instead occupies position $n+4$ in the support strand and is paired with the nucleotide which is 3 positions removed from the terminal nucleotide of the helper strand at the complementary ligation end; wherein position $n+4$ is position 4 in the support strand relative to position n in the direction proximal to the helper strand/distal to the primer strand portion.

Prior to and at the cleavage step of the second cycle (steps 5 and 6) and in cleavage steps of all subsequent cycles the universal nucleotide occupies position $n+3$ in the support strand of the scaffold polynucleotide, and the support strand of the scaffold polynucleotide is cleaved between positions n and $n-1$.

Finally, in the ligation step of the second cycle (step 9) and in ligation steps of all subsequent cycles the complementary ligation end of the ligation polynucleotide is structured such that the universal nucleotide occupies position $n+4$ in the support strand and is paired with the nucleotide which is 2 positions removed from the terminal nucleotide of the helper strand at the complementary ligation end.

In addition to the above-described “n+3” method, “n+3+x” variations of synthesis method version 4 are provided wherein the method is performed in the same way as synthesis method version 4 described above except for the following variations.

5 Prior to and at the cleavage step of the first cycle (steps 1 and 2) the universal nucleotide instead occupies position n+3+x in the support strand of the scaffold polynucleotide, wherein n+3 is the third nucleotide position in the support strand relative to position n in the direction proximal to the helper strand/distal to the primer strand portion; and the support strand of the scaffold polynucleotide is cleaved between positions n and n-1.

10 In the ligation step of the first cycle (step 5) the complementary ligation end of the ligation polynucleotide is structured such that the universal nucleotide instead occupies position n+4+x in the support strand and is paired with the nucleotide which is 3+x positions removed from the terminal nucleotide of the helper strand at the complementary ligation end; wherein position n+4 is position 4 in the support strand relative to position n

15 in the direction proximal to the helper strand/distal to the primer strand portion.

Prior to and at the cleavage step of the second cycle (step 6) and in cleavage steps of all subsequent cycles the universal nucleotide occupies position n+3+x in the support strand of the scaffold polynucleotide and the support strand of the scaffold polynucleotide is cleaved between positions n and n-1.

20 Finally, in the ligation step of the second cycle (step 9) and in ligation steps of all subsequent cycles the complementary ligation end of the ligation polynucleotide is structured such that the universal nucleotide occupies position n+4+x in the support strand and is paired with the nucleotide which is 2+x positions removed from the terminal nucleotide of the helper strand at the complementary ligation end.

25 In all of these methods, x is a whole number between 1 to 10 or more, and wherein x is the same whole number in steps (1), (2), (5), (6) and (9).

As with synthesis method version 4, with variant methods based on version 4 it will be noted that the universal nucleotide occupies position n+3 (or n+3+x, depending upon the particular variant method) in the scaffold polynucleotide during steps (1) and (2),

30 whereas the universal nucleotide occupies position n+4 (or n+4+x, depending upon the

particular variant method) in the ligation polynucleotide in the same synthesis cycle. This is because at step (5) the cleaved double-stranded scaffold polynucleotide is now provided with the nucleotide of the predefined sequence following its addition in that cycle of synthesis and because the ligation polynucleotide is provided with the nucleotide to partner the nucleotide of the predefined sequence; and furthermore because as defined herein “n” always refers to the nucleotide position in the synthesis strand which is occupied by (or will be occupied by) the nucleotide of the predefined sequence incorporated in that cycle or refers to the nucleotide position opposite thereto in the support strand. Thus at the end of any given cycle of synthesis, immediately after ligation step (5), the position occupied by a universal nucleotide will be $n+4$ (or $n+4+x$) and will have moved from $n+3$ (or correspondingly from $n+3+x$), the position a universal nucleotide will have occupied at step (1). The product of the ligation step (5) (corresponding to the structure labelled “ligation product” in Figure 4) will be the scaffold polynucleotide for use in the next cycle of synthesis. When the ligation product is considered as the scaffold polynucleotide for use in the next cycle of synthesis it is to be understood that the position occupied by the universal nucleotide is now to be once again referred to as position $n+3$ (or $n+3+x$) (c.f. 406), rather than $n+4$ (or $n+4+x$) (c.f. 405). Thus in variants of synthesis method version 4 the position occupied by the universal nucleotide at the beginning of any given cycle of synthesis (c.f. 401, 406 etc.) is always referred to as position $n+3$ (or $n+3+x$ depending upon the particular variant method, wherein x is a whole number between 1 to 10 or more), and the nucleotide which is newly incorporated in that cycle always occupies a position referred to as n .

Synthesis Method Version 5

A fifth exemplary version of the synthesis method of the invention is performed in an analogous matter to the first version except with modified structural arrangements for the positioning of the universal nucleotide relative to the position to be occupied by the new nucleotide to be incorporated in a given synthesis cycle and relative to the nick and cleavage sites.

Thus as with version 1, in version 5 cleavage of the scaffold polynucleotide (step 2, 502) results in loss of the helper strand, if present immediately prior to cleavage, and loss of the support strand comprising the universal nucleotide. Cleavage leaves in place a cleaved double-stranded scaffold polynucleotide comprising, at the site of cleavage, a cleaved terminal end of the support strand and the terminal end of the primer strand portion of the synthesis strand which comprised the nick site prior to cleavage. Cleavage results in a cleaved double-stranded scaffold polynucleotide with a single nucleotide overhang at the site of cleavage.

In step (3) of the methods a first nucleotide in the predefined nucleotide sequence is added to the terminal end of the primer strand portion of the synthesis strand by the action of an enzyme having transferase activity such as polymerase or a terminal nucleotidyl transferase enzyme (503). The first nucleotide is provided with a reversible terminator group which prevents further extension by the enzyme. Thus in step (3) only a single nucleotide is incorporated.

In step (4) of the methods a deprotection step is performed (504) to remove the terminator group from the newly-incorporated nucleotide. In principle the deprotection step may be performed after the ligation step (5). Performance of the deprotection step as step (4) is preferred.

In step (5) of the methods a ligation polynucleotide (see structure depicted at the top left of the upper part of Figure 5) is ligated to the cleaved scaffold polynucleotide. Ligation incorporates a partner nucleotide into the scaffold polynucleotide and allows the newly-incorporated nucleotide to pair with the partner nucleotide (step 5 of Figure 5; 505), thus completing a synthesis cycle.

In the fifth exemplary version of the synthesis method of the invention a scaffold polynucleotide is provided in step (1) (501) as described above. In this method the universal nucleotide in the support strand of the scaffold polynucleotide is positioned opposite and paired with a nucleotide (labelled “X” in the structure of step (1) of Figure 5) which is the penultimate nucleotide of the helper strand adjacent the single-strand break site.

In step (2) of the method the scaffold polynucleotide is cleaved (502) at a cleavage site. The cleavage site is defined by a sequence comprising the universal nucleotide in the support strand. Cleavage results in a double-stranded break in the scaffold polynucleotide. Cleavage of the scaffold polynucleotide (step 2) results in loss of the helper strand, if present immediately prior to cleavage, and loss of the support strand comprising the universal nucleotide. Cleavage of the scaffold polynucleotide leaves in place a cleaved double-stranded scaffold polynucleotide comprising, at the site of cleavage, a cleaved terminal end of the support strand and the terminal end of the primer strand portion of the synthesis strand which comprised the nick site prior to cleavage.

The synthesis strand is already provided with a single-stranded break or “nick” in this exemplary method, thus only cleavage of the support strand is necessary to provide a double-stranded break in the scaffold polynucleotide.

In this exemplary method version cleavage generates a cleaved double-stranded scaffold polynucleotide with a single nucleotide overhang. Thus the terminal nucleotide of the primer strand portion of the synthesis strand overhangs the terminal nucleotide of the support strand.

In this method the universal nucleotide occupies position “n+1” in the support strand prior to the cleavage step. To obtain such a cleaved double-stranded scaffold polynucleotide wherein the terminal nucleotide of the primer strand portion of the synthesis strand overhangs the terminal nucleotide of the support strand when the universal nucleotide occupies position n+1 in the support strand, the support strand is cleaved at a specific position relative to the universal nucleotide and relative to the nick site. The support strand of the scaffold polynucleotide is cleaved between nucleotide positions n-1 and n-2.

By “n” it is meant 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 addition to the terminal end of the cleaved synthesis strand in that cycle of synthesis. By “n” it is also meant the nucleotide position in the synthesis strand which will be occupied by the nucleotide of the predefined sequence upon its addition to the terminal end of the primer strand portion of the synthesis strand in that cycle of synthesis. Thus at the cleavage step, the universal nucleotide is at position n+1 in the support strand, which is opposite the nucleotide which the penultimate nucleotide of the helper strand adjacent the single-strand break site (shown at position “X” in step (2) in Figure 5).

At steps (1) and (2) the position which will be occupied by the nucleotide of the predefined sequence incorporated in that cycle of synthesis corresponds with the nucleotide which is the penultimate nucleotide of the helper strand adjacent the single-strand break site (shown at position “X” in step (2) of Figure 5).

By “n+1” it is meant 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 (nucleotide labelled “Un” at position n+1, which occupies the position next to the position labelled “H” at position n in the direction distal to the primer strand, as shown schematically in steps (1) and (2) of Figure 5). n+1 may also refer to the corresponding position in the synthesis strand (labelled “X” at position n+1 in steps (1) and (2) of Figure 5). Thus at the cleavage step, position n+1 in the support strand is opposite the position occupied by the penultimate nucleotide of the helper strand adjacent the nick site.

By “n-1” it is meant 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 (position labelled “J” at position n-1, as shown schematically in steps (1) and (2) of Figure 5). n-1 may also refer to the corresponding position in the synthesis strand (*i.e.* the position labelled “K” at position n-1, as shown schematically in steps (1) and (2) of Figure 5). Thus at the cleavage step, position n-1 in the support strand is opposite the position occupied by the terminal nucleotide of the primer strand portion of the synthesis strand. In steps (1) and (2) of Figure 5 the nucleotides at position “L” and “M” thus occupy position n-2.

In methods according to version 5, the nucleotide pair which occupy positions H and I; J and K; and L and M can be any nucleotide pair.

Upon cleavage of the support strand between nucleotide positions n-1 and n-2, the universal nucleotide, helper strand (if present) and the portion of the support strand which
5 is or was hybridized to the helper strand are removed from the remaining cleaved scaffold polynucleotide (see structure depicted at the top right of the upper part of Figure 5).

A phosphate group should continue to be attached to the terminal nucleotide of the support strand of the cleaved scaffold polynucleotide at the cleavage site (as depicted in the structure shown in the middle of the lower part of Figure 5). This ensures that the support
10 strand of the ligation polynucleotide can be ligated to the support strand of the cleaved scaffold polynucleotide in the ligation step.

Thus in method version 5 the universal nucleotide occupies position n+1 in the support strand at steps (1) and (2) and the support strand is cleaved between nucleotide positions n-1 and n-2.

15 Preferably, the support strand is cleaved by cleavage of the phosphodiester bond between nucleotide positions n-1 and n-2 (the third phosphodiester bond of the support strand relative to the position of the universal nucleotide, in the direction distal to the helper strand/proximal to the primer strand).

The support strand may be cleaved by cleavage of one ester bond of the
20 phosphodiester bond between nucleotide positions n-1 and n-2.

Preferably the support strand is cleaved by cleavage of the first ester bond relative to nucleotide position n-1. This will have the effect of retaining a terminal phosphate group on the support strand of the cleaved scaffold polynucleotide at the cleavage position.

Any suitable mechanism may be employed to effect cleavage of the support strand
25 between nucleotide positions n-1 and n-2 when the universal nucleotide occupies position n+1.

Cleavage of the support strand between nucleotide positions n-1 and n-2 as described above may be performed by the action of an enzyme.

In this exemplary method version of the invention, as well as with all versions, to
30 allow the next nucleotide to be incorporated in the next synthesis cycle, the reversible

terminator group must be removed from the first nucleotide (deprotection step; 504). This can be performed at various stages of the first cycle. Typically, and preferably, it will be performed as step (4) of the method, before ligation step (5), as shown in step 4 of Figure 5 (504). However, the deprotection step could be performed at any step after incorporation of the new nucleotide, such as after the ligation step (5). Regardless of which stage the deprotection step is performed, enzyme and residual unincorporated first nucleotides should first be removed in order to prevent multiple incorporation of first nucleotides. Enzyme and unincorporated first nucleotides are preferably removed prior to the ligation step (step (5)).

Removal of the reversible terminator group from the first nucleotide can be performed by any suitable means. For example, removal can be performed by the use of a chemical, such as tris(carboxyethyl)phosphine (TCEP).

In step (5) of the method a double-stranded ligation polynucleotide is ligated (505) to the cleaved scaffold polynucleotide. The ligation polynucleotide comprises a support strand and a helper strand. The ligation polynucleotide further comprises a complementary ligation end comprising in the support strand a universal nucleotide and two overhanging nucleotides which overhang the terminal nucleotide of the helper strand. The penultimate nucleotide of the support strand of the ligation polynucleotide at the complementary ligation end is the partner nucleotide for the first nucleotide of the predefined sequence.

The ligation polynucleotide further comprises in the helper strand adjacent the overhang a terminal nucleotide which is configured such that it cannot be ligated to another polynucleotide strand (position labelled "I" in the structure depicted at the top left of the upper part of Figure 5). Typically this terminal nucleotide will lack a phosphate group. Typically, as described above, this terminal nucleotide of the helper strand will define the 5' terminus of the helper strand.

In the complementary ligation end of the ligation polynucleotide the universal nucleotide in the support strand is positioned (n+2) opposite a nucleotide which is the penultimate nucleotide of the helper strand (position labelled "X" in step (5) of Figure 5) and is paired therewith. The terminal nucleotide of the helper strand (position labelled "I" in step (5) of Figure 5) occupies position n+1 and is shown paired with a nucleotide at a

position labelled “H” in the support strand and which is also at position $n+1$. The partner nucleotide for the first nucleotide of the predefined sequence occupies a position as the penultimate nucleotide of the support strand of the ligation polynucleotide at position n . Finally, the terminal nucleotide of the support strand of the ligation polynucleotide is
5 labelled “J” in step (5) of Figure 5 at position $n-1$.

The complementary ligation end is configured so that it will compatibly join with the overhanging end of the cleaved scaffold polynucleotide product following incorporation step (3) when subjected to suitable ligation conditions. Upon ligation of the support strands, the first nucleotide becomes paired with its partner nucleotide.

10 Ligation of the support strands may be performed by any suitable means. Ligation will result in the joining of the support strands only, with the maintenance of a single-stranded break between the first nucleotide in the synthesis strand, *i.e.* in the primer strand portion, and the terminal nucleotide of the helper strand adjacent the first nucleotide.

Ligation may typically be performed by enzymes having ligase activity. For
15 example, ligation may be performed with T3 DNA ligase or T4 DNA ligase or functional variants or equivalents thereof. The use of such enzymes will result in the maintenance of the single-stranded break in the synthesis strand, since the terminal nucleotide of the helper strand is provided such that it cannot act as a substrate for ligase, *e.g.* due to the absence of a terminal phosphate group.

20 Ligation of the ligation polynucleotide to the cleaved scaffold polynucleotide completes a first synthesis cycle whereupon the scaffold polynucleotide of step (1) is effectively re-constituted except that the first nucleotide of the predefined nucleotide sequence is incorporated into the scaffold polynucleotide opposite its partner nucleotide.

In step (5) of this exemplary method (505), in the complementary ligation end of
25 the ligation polynucleotide the universal nucleotide in the support strand is positioned opposite the nucleotide which is the penultimate nucleotide of the helper strand adjacent the single-strand break site (shown at position “X” in step (5) of Figure 5). It will be noted that in the context of the ligation polynucleotide the universal nucleotide is positioned at position $n+2$ and three positions removed from the terminal nucleotide of the support
30 strand (position $n-1$). Position n in the context of the ligation polynucleotide has the same

meaning as described above in the context of the scaffold polynucleotide at steps (1) and (2). Thus in step (5) by “n” it is meant the nucleotide position in the support strand which, following ligation, is opposite the position in the synthesis strand which is now occupied by the nucleotide of the predefined sequence following its addition to the terminal end of the primer strand portion of the synthesis strand in that cycle of synthesis. By “n” it is also meant the nucleotide position in the synthesis strand following ligation which is now occupied by the nucleotide of the predefined sequence following its addition to the terminal end of the primer strand portion of the synthesis strand in that cycle of synthesis.

It will be noted that the universal nucleotide occupies position $n+1$ in the scaffold polynucleotide during steps (1) and (2) (501 and 502 of Figure 5), whereas the universal nucleotide occupies position $n+2$ in the ligation polynucleotide in the same synthesis cycle (505 of Figure 5). This is because at step (5) the cleaved double-stranded scaffold polynucleotide is now provided with the nucleotide of the predefined sequence following its addition in that cycle of synthesis and because the ligation polynucleotide is provided with the nucleotide to partner the nucleotide of the predefined sequence; and furthermore because as defined herein “n” always refers to the nucleotide position in the synthesis strand which is occupied by (or will be occupied by) the nucleotide of the predefined sequence incorporated in that cycle or refers to the nucleotide position opposite thereto in the support strand. Thus at the end of any given cycle of synthesis, immediately after ligation step (5), the position occupied by a universal nucleotide will be $n+2$ and will have moved from $n+1$, the position a universal nucleotide will have occupied at step (1). The product of the ligation step (5) (structure labelled “ligation product” in Figure 5) will be the scaffold polynucleotide for use in the next cycle of synthesis. When the ligation product is considered as the scaffold polynucleotide for use in the next cycle of synthesis (506) it is to be understood that the position occupied by the universal nucleotide is now to be once again referred to as position $n+1$ (506), rather than $n+2$ (505). Thus in method version 5 the position occupied by the universal nucleotide at the beginning of any given cycle of synthesis (501, 506 etc.) is always referred to as position $n+1$, and the nucleotide which is newly incorporated in that cycle always occupies a position referred to as n .

Following completion of the first synthesis cycle, second and subsequent cycles are performed using the same method steps.

As in step (2) of the first synthesis cycle of method version 5, in step (6) the scaffold polynucleotide is cleaved (507) at a cleavage site. The cleavage site is defined by a sequence comprising the universal nucleotide in the support strand. Cleavage results in a double-stranded break in the scaffold polynucleotide. Cleavage of the scaffold polynucleotide leaves in place a cleaved double-stranded scaffold polynucleotide comprising, at the site of cleavage, a cleaved terminal end of the support strand and the terminal end of the primer strand portion of the synthesis strand which comprised the nick site prior to cleavage. Cleavage results in removal of the support strand comprising the universal nucleotide and the helper strand hybridised to the support strand, if present immediately prior to cleavage.

The cleavage steps may be performed as described above for step (2) of the first cycle.

In step (7) the next nucleotide is added to the terminal end of the cleaved primer strand portion of the synthesis strand as in step (3) of the first cycle.

In step (8) the reversible terminator group is removed from the next nucleotide (deprotection step; 509). As described above for first cycle, this can be performed at various stages. Typically, and preferably, it will be performed as step (8) of the method, before ligation step (9). However, the deprotection step could be performed at any step after incorporation of the new nucleotide, such as after the ligation step (9).

Deprotection of the reversible terminator group in the next cycle (509) and subsequent cycles may be performed as described above with respect to the first synthesis cycle.

In step (9) of the next cycle a double-stranded ligation polynucleotide is ligated (510) to the cleaved scaffold polynucleotide. The ligation polynucleotide of step (9) of the next and subsequent synthesis cycles may be configured, and the ligation step may be performed, as described above for step (5) of the first synthesis cycle.

Synthesis cycles are repeated as described above for as many times as necessary to synthesise the double-stranded polynucleotide having the predefined nucleotide sequence.

Variation Methods Based on Synthesis Method Version 5

It will be appreciated that synthesis method version 5 is a variation of synthesis method version 3. Both methods require cleavage of the support strand of the scaffold polynucleotide between positions n-1 and n-2. In version 3 prior to and at the cleavage step of the first and second cycles (steps 2 and 6) the universal nucleotide occupies position n in the support strand of the scaffold polynucleotide. In contrast in version 5 prior to and at the cleavage step of the first and second cycles (steps 2 and 6) the universal nucleotide occupies position n+1 in the support strand of the scaffold polynucleotide. Thus synthesis method version 5 is the same as synthesis method version 3 except that in synthesis method version 5 the universal nucleotide occupies one position further removed from position n in the direction distal to primer strand portion.

Further variations of synthesis method version 5 are envisaged wherein the support strand of the scaffold polynucleotide is cleaved between positions n-1 and n-2 and wherein in each further variant method the universal nucleotide occupies incrementally one position further removed from position n in the direction distal to primer strand portion, starting at position n+2 and increasing incrementally according to the formula $n+2+x$ wherein x is a whole number between 1 to 10 or more.

Thus an “n+2” variation of synthesis method version 5 is provided wherein the method is performed in the same way as synthesis method version 5 described above except for the following variations.

In the cleavage step of the first cycle (step 2) the universal nucleotide instead occupies position n+2 in the support strand of the scaffold polynucleotide, wherein n+2 is the second nucleotide position in the support strand relative to position n in the direction proximal to the helper strand/distal to the primer strand portion; and the support strand of the scaffold polynucleotide is cleaved between positions n and n-1.

In the ligation step of the first cycle (step 5) the complementary ligation end of the ligation polynucleotide is structured such that the universal nucleotide instead occupies position n+3 in the support strand and is paired with the nucleotide which is 2 positions

removed from the terminal nucleotide of the helper strand at the complementary ligation end; wherein position $n+3$ is position 3 in the support strand relative to position n in the direction proximal to the helper strand/distal to the primer strand portion.

In the cleavage step of the second cycle (step 6) and in cleavage steps of all subsequent cycles the universal nucleotide occupies position $n+2$ in the support strand of the scaffold polynucleotide, and the support strand of the scaffold polynucleotide is cleaved between positions n and $n-1$.

Finally, in the ligation step of the second cycle (step 9) and in ligation steps of all subsequent cycles the complementary ligation end of the ligation polynucleotide is structured such that the universal nucleotide occupies position $n+3$ in the support strand and is paired with the nucleotide which is 2 positions removed from the terminal nucleotide of the helper strand at the complementary ligation end.

In addition to the above-described method, “ $n+2+x$ ” variations of synthesis method version 5 are provided wherein the method is performed in the same way as synthesis method version 5 described above except for the following variations.

In the cleavage step of the first cycle (step 2) the universal nucleotide instead occupies position $n+2+x$ in the support strand of the scaffold polynucleotide, wherein $n+2$ is the second nucleotide position in the support strand relative to position n in the direction proximal to the helper strand/distal to the primer strand portion; and the support strand of the scaffold polynucleotide is cleaved between positions n and $n-1$.

In the ligation step of the first cycle (step 5) the complementary ligation end of the ligation polynucleotide is structured such that the universal nucleotide instead occupies position $n+3+x$ in the support strand and is paired with the nucleotide which is $2+x$ positions removed from the terminal nucleotide of the helper strand at the complementary ligation end; wherein position $n+3$ is position 3 in the support strand relative to position n in the direction proximal to the helper strand/distal to the primer strand portion.

In the cleavage step of the second cycle (step 6) and in cleavage steps of all subsequent cycles the universal nucleotide occupies position $n+2+x$ in the support strand of the scaffold polynucleotide and the support strand of the scaffold polynucleotide is cleaved between positions n and $n-1$.

Finally, in the ligation step of the second cycle (step 9) and in ligation steps of all subsequent cycles the complementary ligation end of the ligation polynucleotide is structured such that the universal nucleotide occupies position $n+3+x$ in the support strand and is paired with the nucleotide which is $2+x$ positions removed from the terminal nucleotide of the helper strand at the complementary ligation end.

In all of these methods, x is a whole number between 1 to 10 or more, and wherein x is the same whole number in steps (2), (5), (6) and (9).

As with synthesis method version 5, with variant methods based on version 5 it will be noted that the universal nucleotide occupies position $n+2$ (or $n+2+x$, depending upon the particular variant method) in the scaffold polynucleotide during steps (1) and (2), whereas the universal nucleotide occupies position $n+3$ (or $n+3+x$, depending upon the particular variant method) in the ligation polynucleotide in the same synthesis cycle. This is because at step (5) the cleaved double-stranded scaffold polynucleotide is now provided with the nucleotide of the predefined sequence following its addition in that cycle of synthesis and because the ligation polynucleotide is provided with the nucleotide to partner the nucleotide of the predefined sequence; and furthermore because as defined herein “ n ” always refers to the nucleotide position in the synthesis strand which is occupied by (or will be occupied by) the nucleotide of the predefined sequence incorporated in that cycle or refers to the nucleotide position opposite thereto in the support strand. Thus at the end of any given cycle of synthesis, immediately after ligation step (5), the position occupied by a universal nucleotide will be $n+3$ (or $n+3+x$) and will have moved from $n+2$ (or correspondingly from $n+2+x$), the position a universal nucleotide will have occupied at step (1). The product of the ligation step (5) (corresponding to the structure labelled “ligation product” in Figure 5) will be the scaffold polynucleotide for use in the next cycle of synthesis. When the ligation product is considered as the scaffold polynucleotide for use in the next cycle of synthesis it is to be understood that the position occupied by the universal nucleotide is now to be once again referred to as position $n+2$ (or $n+2+x$) (c.f. 506), rather than $n+3$ (or $n+3+x$) (c.f. 505). Thus in variants of synthesis method version 5 the position occupied by the universal nucleotide at the beginning of any given cycle of synthesis (c.f. 501, 506 etc.) is always referred to as position $n+2$ (or $n+2+x$ depending

upon the particular variant method, wherein x is a whole number between 1 to 10 or more), and the nucleotide which is newly incorporated in that cycle always occupies a position referred to as n.

5 **EXAMPLES**

The following Examples provide support for the methods for synthesising a polynucleotide or oligonucleotide according to the invention, as well as exemplary constructs used in the methods. The Examples do not limit the invention.

10 Other than Example 13, the following Examples describe synthesis methods according to reaction schemes which are related to but which are not within the scope of the synthesis methods according to the invention.

The Examples demonstrate the ability to perform synthesis reactions which involve steps of addition of a nucleotide of a predefined sequence to the synthesis strand of a
15 scaffold polynucleotide, cleavage of the scaffold polynucleotide at a cleavage site defined by a universal nucleotide and ligation of a ligation polynucleotide which comprises a partner nucleotide for the added nucleotide of the predefined sequence as well as a new universal nucleotide for use in creating a cleavage site for use in the next cycle of synthesis. The methods of the present invention incorporate these steps in a modified
20 manner. Thus other than Example 13 the following Examples provide illustrative support for the methods of the invention defined herein. Example 13 provides data relating to incorporation of 3'-O-modified-dNTPs by Terminator X DNA polymerase using an incorporation step according to methods of the invention, *e.g.* synthesis method versions of the invention 1, 2 and 4 (Figures 1, 2 and 4 respectively).

25 In the following Examples, and in corresponding Figures 12 to 50, references to synthesis method "versions 1, 2 and 3" or "version 1, 2 or 3 chemistry" etc. are to be interpreted according to the reaction schematics set out respectively in Figures 6, 7 and 8 and not according to the reaction schematics set out in any of Figures 1 to 5 or descriptions of the same herein. Example 13 and Figure 51 are to be interpreted according to synthesis
30 methods the invention. In particular according to synthesis methods of the invention 1, 2

and 4 and associated reaction schematics set out in Figures 1, 2 and 4, and more particularly incorporation step 3 of such methods.

Example 1. Synthesis in the Absence of a Helper Strand.

5

This example describes the synthesis of polynucleotides using 4 steps: incorporation of 3'-*O*-modified dNTPs on partial double-stranded DNA, cleavage, ligation and deprotection, with the first step taking place opposite a universal nucleotide, in this particular case inosine.

10

Step1: Incorporation

The first step describes controlled addition of a 3'-*O*-protected single nucleotide to an oligonucleotide by enzymatic incorporation by DNA polymerase (Figure 12a).

15

Materials and Methods

Materials

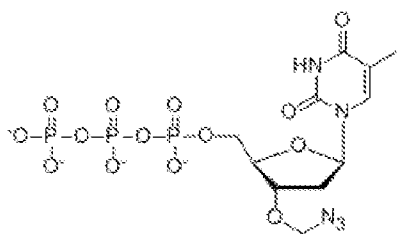
- 20 1. 3'-*O*-modified dNTPs were synthesised in-house according to the protocol described in PhD thesis Jian Wu: Molecular Engineering of Novel Nucleotide Analogues for DNA Sequencing by Synthesis, Columbia University, 2008. The protocol for synthesis is also described in the patent application publication: J. William Efcavitch, Julieta E. Sylvester, Modified Template-Independent Enzymes for Polydeoxynucleotide Synthesis,
25 Molecular Assemblies US2016/0108382A1.

2. Oligonucleotides were designed in house and obtained from Sigma-Aldrich (Figure 12h). The stock solutions were prepared at a concentration of 100 μ M.

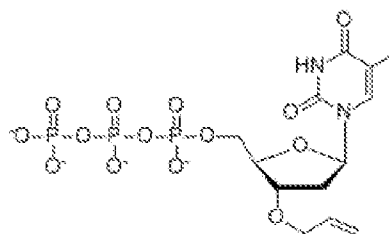
3. Terminator IX DNA polymerase was used that has been engineered by New England BioLabs with enhanced ability to incorporate 3'-O-modified dNTPs. However, any DNA polymerase that could incorporate modified dNTPs could be used.

5 Two types of reversible terminators were tested:

3'-O-azidomethyl-dTTP:



3'-O-allyl-dTTP:



Methods

10

1. 2 µl of 10x Thermopol® buffer (20 mM Tris-HCl, 10 mM (NH₄)₂SO₄, 10 mM KCl, 2 mM MgSO₄, 0.1% Triton® X-100, pH 8.8, New England BioLabs) was mixed with 12.25 µl of sterile deionized water (ELGA VEOLIA) in 1.5ml Eppendorf tube.

15

2. 0.5 µl of 10 µM primer (synthesised strand) (5 pmol, 1 equiv) (SEQ ID NO: 1, Figure 12h) and 0.75 µl of 10 µM template (support strand) (6 pmol, 1.5equiv) (SEQ ID NO: 2, Figure 12h) were added to the reaction mixture.

20

3. 3'-O-modified-dTTP (2 µl of 100 µM) and MnCl₂ (1 µl of 40 mM) were added.

4. 1.5 µl of Terminator IX DNA polymerase (15 U, New England BioLabs) was then added. However, any DNA polymerase that could incorporate modified dNTPs could be used.

25

5. The reaction was incubated for 20 minutes at 65°C.

6. The reaction was stopped by addition of TBE-Urea sample buffer (Novex).

7. The reaction was separated on polyacrylamide gel (15%) with TBE buffer and visualized by ChemiDoc MP imaging system (BioRad).

5

Gel Electrophoresis and DNA Visualization:

1. 5 µl of reaction mixture was added to 5 µl of TBE-Urea sample buffer (Novex) in a sterile 1.5ml Eppendorf tube and heated to 95°C for 5 minutes using a heat ThermoMixer (Eppendorf).

10

2. 5 µl of the sample was then loaded into the wells of a 15% TBE-Urea gel 1.0 mm x 10 well (Invitrogen) which contained preheated 1X TBE buffer Thermo Scientific (89mM Tris, 89mM boric acid and 2mM EDTA).

15

3. X-cell sure lock module (Novex) was fastened in place and electrophoresis performed at the following conditions; 260V, 90 Amps for 40 minutes at room temperature.

20 4. The gel was visualized by ChemiDoc MP (BioRad) using Cy3 LEDS. Visualization and analysis was carried out on the Image lab 2.0 platform.

Results

Customised engineered Terminator IX DNA polymerase from New England BioLabs is an efficient DNA polymerase able to incorporate 3'-*O*-modified-dNTPs opposite a
5 universal nucleotide *e.g.* inosine (Figure 12b-c).

Efficient incorporation opposite inosine occurred at a temperature of 65°C (Figure 12d-e).

Incorporation of 3'-*O*-modified-dTTPs opposite inosine requires the presence of Mn²⁺ ions
10 (Figure 12f-g). Successful conversion is marked in bold in Figures 12 c, e, g and h.

Conclusion

Incorporation of 3'-*O*-modified-dTTPs opposite inosine can be achieved with particularly
15 high efficiency using customized engineered Terminator IX DNA polymerase from New England BioLabs, in the presence of Mn²⁺ ions and at a temperature at 65°C.

Step2: Cleavage

20 The second step describes a two-step cleavage of polynucleotides with either hAAG/Endo VIII or hAAG/chemical base (Figure 13a).

Materials and Methods

25 Materials

1. Oligonucleotides utilized in Example 1 were designed in-house and synthesised by Sigma Aldrich (see table in Figure 13(e) for sequences).

2. The oligonucleotides were diluted to a stock concentration of 100uM using sterile distilled water (ELGA VEOLIA).

Methods

5

A cleavage reaction on oligonucleotides was carried out using the procedure below:

1. A pipette (Gilson) was used to transfer 41µl sterile distilled water (ELGA VEOLIA) into a 1.5ml Eppendorf tube.
- 10 2. 5µl of 10X ThermoPol® reaction buffer NEB (20 mM Tris-HCl, 10 mM (NH₄)₂SO₄, 10 mM KCl, 2 mM MgSO₄, 0.1% Triton® X-100, pH 8.8) were then added into the same Eppendorf tube.
- 15 3. 1µl each of oligonucleotides (Figure 13e); template (SEQ ID NO: 3) or any fluorescently tagged long oligo strand, primer with T (SEQ ID NO: 4) and control (SEQ ID NO: 5) all at 5pmols were added into the same tube.
- 20 4. 1µl of Human Alkyladenine DNA Glycosylase (hAAG) NEB (10units/µl) was added into the same tube.
5. Reaction mixture was then gently mixed by resuspension with a pipette, centrifuged at 13,000rpm for 5 seconds and incubated at 37°C for 1 hour.
- 25 6. Typically after incubation time had elapsed, the reaction was terminated by enzymatic heat inactivation (*i.e.* 65°C for 20 minutes).

Purification under ambient conditions. The sample mixture was purified using the protocol outlined below:

30

1. 500 µl of buffer PNI QIAGEN (5M guanidinium chloride) was added to the sample and mixed by gentle resuspension with a pipette.
2. The mixture was transferred into a QIAquick spin column (QIAGEN) and
5 centrifuged for 1 min at 6000 rpm.
3. After centrifugation, flow-through was discarded and 750 µl of buffer PE QIAGEN (10mM Tris-HCl pH 7.5 and 80% ethanol) was added into the spin column and centrifuged for 1min at 6000 rpm.
10
4. The flow-through was discarded and the spin column was centrifuged for an additional 1 min at 13000 rpm to remove residual PE buffer.
5. The spin column was then placed in a sterile 1.5ml Eppendorf tube.
15
6. For DNA elution, 50 µl of Buffer EB QIAGEN (10mM Tris.CL, pH 8.5) was added to the centre of the column membrane and left to stand for 1 min at room temperature.
7. The tube was then centrifuged at 13000 rpm for 1 minutes. Eluted DNA concentration was measured and stored at -20°C for subsequent use.
20

Measurement of purified DNA concentration was determined using the protocol below:

- 25 1. NanoDrop one (Thermo Scientific) was equilibrated by adding 2 µl of sterile distilled water (ELGA VEOLIA) onto the pedestal.
2. After equilibration, the water was gently wiped off using a lint-free lens cleaning tissue (Whatman).

30

3. NanoDrop one was blanked by adding 2 μ l of Buffer EB QIAGEN (10mM Tris.CL, pH 8.5). Then step 2 was repeated after blanking.

4. DNA concentration was measured by adding 2 μ l of the sample onto the pedestal
5 and selecting the measure icon on the touch screen.

Cleavage of the generated abasic site was carried out using the procedure below:

1. 2 μ l (10-100ng/ μ l) DNA was added into a sterile 1.5ml Eppendorf tube.
10

2. 40 μ l (0.2M) NaOH or 1.5 μ l Endo VIII NEB (10units/ μ l) and 5 μ l 10X Reaction Buffer NEB (10 mM Tris-HCl, 75 mM NaCl, 1 mM EDTA , pH 8 @ 25°C) was also added into the same tube and gently mixed by resuspension and centrifugation at 13000 rpm for 5 sec.
15

3. The resulting mixture was incubated at room temperature for 5 minutes for the NaOH treated sample while Endo VIII reaction mixture was incubated at 37°C for 1hr.

4. After incubation time had elapsed, the reaction mixture was purified using steps 1-7
20 of purification protocol as outlined above.

Gel Electrophoresis and DNA Visualization:

1. 5 μ l of DNA and TBE-Urea sample buffer (Novex) was added into a sterile 1.5 ml
25 Eppendorf tube and heated to 95°C for 2 minutes using a heat thermoblock (Eppendorf).

2. The DNA mixtures were then loaded into the wells of a 15% TBE-Urea gel 1.0mm x 10 well (Invitrogen) which contained preheated 1X TBE buffer Thermo Scientific (89mM Tris, 89mM boric acid and 2mM EDTA).
30

3. X-cell sure lock module (Novex) was fastened in place and electrophoresis performed at the following conditions; 260V, 90 Amps for 40 minutes at room temperature.

5 4. Detection and visualization of DNA in the gel was carried out with ChemiDoc MP (BioRad) using Cy3 LEDS. Visualization and analysis was carried out on the Image lab 2.0 platform.

Results and Conclusion

10

The cleavage reaction without a helper strand showed a low percentage yield of cleaved to uncleaved DNA ratio of ~7% : 93% (Figure 13b-d).

15 Cleavage results showed that in this specific example, and based on the specific reagents used, a low yield of cleaved DNA is obtained in the absence of a helper strand in comparison to the positive control. In addition the use of a chemical base for cleavage of the abasic site was less time-consuming compared to EndoVIII cleavage.

Step 3: Ligation

20

The third step describes ligation of polynucleotides with DNA ligase in the absence of a helper strand. A diagrammatic illustration is shown in Figure 14.

Materials and Methods

25

Materials

1. Oligonucleotides utilized in Example 1 were designed in-house and synthesised by Sigma Aldrich (see table in Figure 14c for sequences).

30

2. The oligonucleotides were diluted to a stock concentration of 100uM using sterile distilled water (ELGA VEOLIA).

Methods

5

Ligation reaction on oligonucleotides was carried out using the procedure below:

1. A pipette (Gilson) was used to transfer 16µl sterile distilled water (ELGA VEOLIA) into a 1.5ml Eppendorf tube.
- 10 2. 10µl of 2X Quick Ligation Reaction buffer NEB (132 mM Tris-HCl, 20mM MgCl₂, 2mM dithiothreitol, 2mM ATP, 15% Polyethylene glycol (PEG6000) and pH 7.6 at 25°C) was then added into the same Eppendorf tube.
- 15 3. 1µl each of oligonucleotides (Figure 14c); TAMRA or any fluorescently tagged phosphate strand (SEQ ID NO: 7), primer with T (SEQ ID NO: 8) and inosine strand (SEQ ID NO: 9), all at 5 pmols, was added into the same tube.
- 20 4. 1µl of Quick T4 DNA Ligase NEB (400units/µl) was added into the same tube.
5. The reaction mixture was then gently mixed by resuspension with a pipette, centrifuged at 13,000rpm for 5 seconds and incubated at room temperature for 20 minutes.
- 25 6. Typically after incubation time had elapsed, reaction was terminated with the addition of TBE-Urea sample Buffer (Novex).
7. The reaction mixture was purified using the protocol outlined in purification steps 1-7 as described above.
- 30 Measurement of purified DNA concentration was determined using the protocol below:

1. NanoDrop one (Thermo Scientific) was equilibrated by adding 2 µl of sterile distilled water (ELGA VEOLIA) onto the pedestal.
- 5 2. After equilibration, the water was gently wiped off using a lint-free lens cleaning tissue (Whatman).
3. NanoDrop one was blanked by adding 2 µl of Buffer EB QIAGEN (10mM Tris.CL, pH 8.5), then step 2 was repeated after blanking.
- 10 4. DNA concentration was measured by adding 2 µl of the sample onto the pedestal and selecting the measure icon on the touch screen.
5. Purified DNA was run on a polyacrylamide gel and visualized in accordance with
15 the procedure in steps 5-8 described above. No change in conditions or reagents was introduced.

Results and Conclusion

- 20 In this specific example, and based on the specific reagents used, ligation of oligonucleotides with DNA ligase, in this particular case quick T4 DNA ligase, at room temperature (24°C) in the absence of a helper strand results in a reduced amount of ligation product (Figure 14b).

25 **Example 2. Version 1 Chemistry with a Helper Strand.**

This example describes the synthesis of polynucleotides using 4 steps: incorporation of 3'-O-modified dNTPs from a nick site, cleavage, ligation and deprotection, with the first step taking place opposite a universal nucleotide, in this particular case inosine. The

method uses a helper strand which improves the efficiency of the ligation and cleavage steps.

Step1: Incorporation

5

The first step describes controlled addition of 3'-*O*-protected single nucleotide to oligonucleotide by enzymatic incorporation using DNA polymerase (Figure 15a).

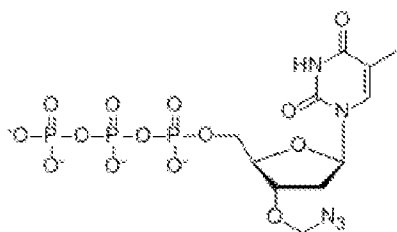
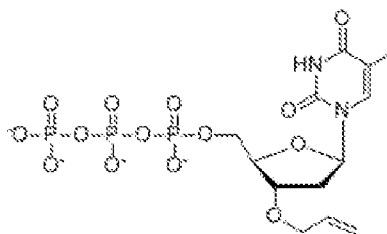
Materials and Methods

10

Materials

1. 3'-*O*-modified dNTPs were synthesised in-house according to the protocol described in PhD thesis Jian Wu: Molecular Engineering of Novel Nucleotide Analogues for DNA Sequencing by Synthesis. Columbia University, 2008. The protocol for synthesis is also described in the patent application publication: J. William Efcavitch, Julieta E. Sylvester, Modified Template-Independent Enzymes for Polydeoxynucleotide Synthesis, Molecular Assemblies US2016/0108382A1.
- 20 2. Oligonucleotides were designed in house and obtained from Sigma-Aldrich. The stock solutions were prepared at a concentration of 100 μ M. Oligonucleotides are shown in Figure 15b.
3. Terminator IX DNA polymerase was used that has been engineered by New England BioLabs with enhanced ability to incorporate 3'-*O*-modified dNTPs.
- 25

Two types of reversible terminators were tested:

3'-*O*-azidomethyl-dTTP:3'-*O*-allyl-dTTP:

Methods

5

1. 2 μ l of 10x Thermopol® buffer (20 mM Tris-HCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 10 mM KCl, 2 mM MgSO_4 , 0.1% Triton® X-100, pH 8.8, New England BioLabs) was mixed with 10.25 μ l of sterile deionized water (ELGA VEOLIA) in 1.5ml Eppendorf tube.
- 10 2. 0.5 μ l of 10 μ M primer (5 pmol, 1 equiv) (SEQ ID NO: 10, Table in Figure 15(b)), 0.75 μ l of 10 μ M template (6 pmol, 1.5equiv) (SEQ ID NO: 11, Table in Figure 15(b)), 2 μ l of 10 μ M of helper strand (SEQ ID NO: 12, Table in Figure 15(b)) were added to the reaction mixture.
- 15 3. 3'-*O*-modified-dTTP (2 μ l of 100 μ M) and MnCl_2 (1 μ l of 40 mM) were added.
4. 1.5 μ l of Therminator IX DNA polymerase (15 U, New England BioLabs) was then added.
- 20 5. The reaction was incubated for 20 minutes at 65°C.
6. The reaction was stopped by addition of TBE-Urea sample buffer (Novex).
7. The reaction was separated on polyacrylamide gel (15%) TBE buffer and
- 25 visualized by ChemiDoc MP imaging system (BioRad).

Gel Electrophoresis and DNA Visualization:

1. 5 µl of reaction mixture was added to 5 µl of TBE-Urea sample buffer (Novex) in a sterile 1.5 ml Eppendorf tube and heated to 95°C for 5 minutes using a heat ThermoMixer (Eppendorf).
5
2. 5 µl of the sample were then loaded into the wells of a 15% TBE-Urea gel 1.0mm x 10 well (Invitrogen) which contained preheated 1X TBE buffer Thermo Scientific (89mM Tris, 89mM Boric acid and 2mM EDTA).
10
3. X-cell sure lock module (Novex) was fastened in place and electrophoresis performed at the following conditions; 260V, 90 Amps for 40 minutes at room temperature.
- 15 4. The gel was visualized by ChemiDoc MP (BioRad) using Cy3 LEDS. Visualization and analysis was carried out on the Image lab 2.0 platform.

The incorporation step can be studied according to the protocol described above.

20 Step 2: Cleavage

The second step describes a two-step cleavage of polynucleotides with either hAAG/Endo VIII or hAAG/chemical base (x2) (Figure 16a).

25 Materials and Methods

Materials

1. Oligonucleotides utilized in Example 2 were designed in-house and synthesised by
30 Sigma Aldrich (see Figure 16f for sequences).

2. The oligonucleotides were diluted to a stock concentration of 100uM using sterile distilled water (ELGA VEOLIA).

5 Methods

Cleavage reaction on oligonucleotides was carried out using the procedure below:

1. A pipette (Gilson) was used to transfer 41µl sterile distilled water (ELGA
10 VEOLIA) into a 1.5 ml Eppendorf tube.
2. 5µl of 10X ThermoPol® Reaction buffer NEB (20 mM Tris-HCl, 10 mM
(NH₄)₂SO₄, 10 mM KCl, 2 mM MgSO₄, 0.1% Triton® X-100, pH 8.8) was then added
into the same Eppendorf tube.
15
3. 1µl each of oligonucleotides (Figure 16f); template (SEQ ID NO: 13) or any
fluorescently tagged long oligo strand, primer with T (SEQ ID NO: 14), control (SEQ ID
NO: 15) and helper strand (SEQ ID NO: 16), all at 5 pmols, were added into the same tube.
- 20 4. 1µl of Human Alkyladenine DNA Glycosylase (hAAG) NEB (10 units/µl) was
added into the same tube.
5. In the reaction using alternative base, 1µl of Human Alkyladenine DNA
Glycosylase (hAAG) NEB (100 units/µl) was added.
25
6. Reaction mixture was then gently mixed by resuspension with a pipette, centrifuged
at 13,000 rpm for 5 seconds and incubated at 37°C for 1 hour.
7. Typically after incubation time had elapsed, the reaction was terminated by
30 enzymatic heat inactivation (*i.e.* 65°C for 20 minutes).

Purification under ambient conditions. The sample mixture was purified using the protocol outlined below:

- 5 1. 500 µl of buffer PNI QIAGEN (5M guanidinium chloride) was added to the sample and mixed by gentle resuspension with a pipette.
2. The mixture was transferred into a QIAquick spin column (QIAGEN) and centrifuged for 1 min at 6000 rpm.
- 10 3. After centrifugation, flow-through was discarded and 750 µl of buffer PE QIAGEN (10mM Tris-HCl pH 7.5 and 80% ethanol) was added into the spin column and centrifuged for 1 min at 6000 rpm.
- 15 4. The flow-through was discarded and the spin column was centrifuged for an additional 1 min at 13000 rpm to remove residual PE buffer.
5. The spin column was then placed in a sterile 1.5 ml Eppendorf tube.
- 20 6. For DNA elution, 50 µl of Buffer EB QIAGEN (10mM Tris.CL, pH 8.5) was added to the centre of the column membrane and left to stand for 1min at room temperature.
7. The tube was then centrifuged at 13000 rpm for 1 minute. Eluted DNA
25 concentration was measured and stored at -20°C for subsequent use.

Measurement of purified DNA concentration was determined using the protocol below:

- 30 1. NanoDrop one (Thermo Scientific) was equilibrated by adding 2 µl of sterile distilled water (ELGA VEOLIA) onto the pedestal.

2. After equilibration, the water was gently wiped off using a lint-free lens cleaning tissue (Whatman).

5 3. NanoDrop one was blanked by adding 2 μ l of Buffer EB QIAGEN (10mM Tris.CL, pH 8.5). Then step 2 was repeated after blanking.

4. DNA concentration was measured by adding 2 μ l of the sample onto the pedestal and selecting the measure icon on the touch screen.

10

Cleavage of generated abasic site was carried out using the procedure below:

1. 2 μ l (10-100ng/ μ l) DNA was added into a sterile 1.5ml Eppendorf tube.

15 2. 40 μ l (0.2M) NaOH or 1.5 μ l Endo VIII NEB (10units/ μ l) and 5 μ l 10X Reaction Buffer NEB (10 mM Tris-HCl, 75 mM NaCl, 1 mM EDTA , pH 8 @ 25°C) was also added into the same tube and gently mixed by resuspension and centrifugation at 13000 rpm for 5 sec.

20 3. The resulting mixture was incubated at room temperature for 5 minutes for the 0.2 M NaOH treated sample while Endo VIII reaction mixture was incubated at 37°C for 1hr.

4. After incubation time had elapsed, the reaction mixture was purified using steps 1-7 of purification protocol as stated above.

25

Cleavage of generated abasic site using alternative basic chemical was carried out using the procedure below:

30 1. 1 μ l (10-100 ng/ μ l) DNA was added into a sterile 1.5 ml Eppendorf tube. 2 μ l of N,N' dimethylethylenediamine Sigma (100mM) which had been buffered at room

temperature with acetic acid solution sigma (99.8%) to pH 7.4 was then added into the same tube.

2. 20 µl of sterile distilled water (ELGA VEOLIA) was added into the tube and
5 gently mixed by resuspension and centrifugation at 13000 rpm for 5 sec.
3. The resulting mixture was incubated at 37°C for 20 minutes.
4. After incubation time had elapsed, the reaction mixture was purified using steps 1-7
10 of the purification protocol stated above.

Gel Electrophoresis and DNA Visualization:

1. 5 µl of DNA and TBE-Urea sample buffer (Novex) was added into a sterile 1.5 ml
15 Eppendorf tube and heated to 95°C for 2 minutes using a heat thermoblock (Eppendorf).
2. The DNA mixtures were then loaded into the wells of a 15% TBE-Urea gel 1.0mm
x 10 well (Invitrogen) which contained preheated 1X TBE buffer Thermo Scientific
(89mM Tris, 89mM boric acid and 2mM EDTA).
20
3. X-cell sure lock module (Novex) was fastened in place and electrophoresis
performed at the following conditions; 260V, 90 Amps for 40 minutes at room
temperature.
- 25 4. Detection and visualization of DNA in the gel was carried out with ChemiDoc MP
(BioRad) using Cy3 LEDS. Visualization and analysis was carried out on the Image lab
2.0 platform.

Results

Cleavage efficiency at a cleavage site comprising a universal nucleotide, in this particular case inosine, by hAAG DNA glycosylase was significantly increased from 10% in absence
5 of helper strand to 50% in presence of helper strand (Figure 16b). hAAG and Endonuclease VIII cleave inosine with lower efficiency (10%) than hAAG and NaOH (50%). Chemical cleavage using 0.2M NaOH was shown to be preferable for cleavage of AP sites than Endonuclease VIII in the described system using nicked DNA (Figure 16c). Mild N,N'-dimethylethylenediamine at neutral pH has high efficiency to cleave abasic
10 sites as 0.2M NaOH, and therefore it is preferable compared with Endonuclease VIII and NaOH (Figures 16d-e).

Conclusion

15 Three methods were evaluated for cleavage of DNA containing inosine. One full enzymatic method - hAAG/Endonuclease VIII, and two methods combining chemical and enzymatic cleavage - hAAG/NaOH and hAAG/dimethylethylamine were studied for DNA cleavage in Example 2.

20 hAAG/NaOH results showed a much higher yield of cleaved DNA (50%) in the presence of a helper strand in comparison to the absence of a helper strand (10%). In these specific examples, and based on the specific reagents used, helper strands increase yield of DNA cleavage.

25 Enzymatic cleavage using Endonuclease VIII as a substitute for NaOH was less efficient (10%) compared to NaOH (50%) in the presence of a helper strand.

The inclusion of an alternative mild chemical base N,N'-dimethylethylenediamine led to high cleavage efficiency of AP sites, as efficient as for NaOH, and, together with addition
30 of 10x hAAG enzyme, had a significant increase on cleaved DNA (see Figure 16e).

Step 3: Ligation

The third step describes ligation of polynucleotides with DNA ligase in the presence of a
5 helper strand. A diagrammatic illustration is shown in Figure 17a.

Materials and Methods

Materials

10

1. Oligonucleotides were designed in-house and synthesised by Sigma Aldrich (see Figure 17d for sequences).
2. The oligonucleotides were diluted to a stock concentration of 100uM using sterile
15 distilled water (ELGA VEOLIA).

Methods

Ligation reaction on oligonucleotides was carried out using the procedure below:
20

1. A pipette (Gilson) was used to transfer 16µl sterile distilled water (ELGA VEOLIA) into a 1.5 ml Eppendorf tube.
2. 10µl of 2X Quick Ligation Reaction buffer NEB (132 mM Tris-HCl, 20mM MgCl₂,
25 2mM dithiothreitol, 2mM ATP, 15% Polyethylene glycol (PEG6000) and pH 7.6 at 25°C) was then added into the same Eppendorf tube.
3. 1µl each of oligonucleotides (Figure 17d); TAMRA or any fluorescently tagged phosphate strand (SEQ ID NO: 18), primer with T (SEQ ID NO: 19) and inosine strand (SEQ

ID NO: 20) and helper strand (SEQ ID NO: 21), all at of 5 pmols, was added into the same tube.

4. 1µl of Quick T4 DNA Ligase NEB (400 units/µl) was added into the same tube.

5

5. Reaction mixture was then gently mixed by resuspension with a pipette, centrifuged at 13,000 rpm for 5seconds and incubated at room temperature for 20 minutes.

6. Typically after incubation time had elapsed, reaction was terminated with the addition of TBE-Urea sample Buffer (Novex).

10

7. The reaction mixture was purified using the protocol outlined in purification steps 1-7 as described above.

15 Measurement of purified DNA concentration was determined using the protocol below:

1. NanoDrop one (Thermo Scientific) was equilibrated by adding 2 µl of sterile distilled water (ELGA VEOLIA) onto the pedestal.

20 2. After equilibration, the water was gently wiped off using a lint-free lens cleaning tissue (Whatman).

3. NanoDrop one was blanked by adding 2 µl of Buffer EB QIAGEN (10mM Tris.CL, pH 8.5). Then step 2 was repeated after blanking.

25

4. DNA concentration was measured by adding 2 µl of the sample onto the pedestal and selecting the measure icon on the touch screen.

5. Purified DNA was run on a polyacrylamide gel and visualized in accordance with the procedure in steps 5-8 above. No change in conditions or reagents was introduced.

30

Results and Conclusion

In this specific example, and based on the specific reagents used, reduced ligation activity is
5 observed in the absence of a helper strand (Figure 17b), whereas ligation proceeds with high efficiency in presence of a helper strand (Figure 17c) and the product is formed in high yield.

Example 3. Version 2 Chemistry with a Helper Strand.

10 This example describes the synthesis of polynucleotides using 4 steps: incorporation of 3'-*O*- modified dNTPs on partial double-stranded DNA; cleavage, ligation and deprotection with the first step of incorporation taking place opposite a naturally complementary nucleotide which is positioned in the support strand adjacent to a universal nucleotide, in this particular case inosine.

15

Step1: Incorporation

Materials and Methods

20 Materials

The first step describes controlled addition of 3'-*O*-protected single nucleotide to oligonucleotide by enzymatic incorporation by DNA polymerase (Figure 18a).

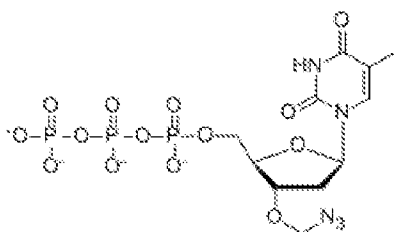
- 25 1. 3'-*O*-modified dNTPs were synthesised in-house according to the protocol described in PhD thesis Jian Wu: Molecular Engineering of Novel Nucleotide Analogues for DNA Sequencing by Synthesis. Columbia University, 2008. The protocol for synthesis is also described in the patent application publication: J. William Efcavitch, Julieta E. Sylvester, Modified Template-Independent Enzymes for Polydeoxynucleotide Synthesis,
30 Molecular Assemblies US2016/0108382A1.

2. Oligonucleotides were designed in house and obtained from Sigma-Aldrich (Figure 18j). The stock solutions are prepared in concentration of 100 μ M.

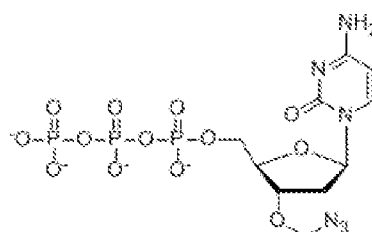
3. Therminator IX DNA polymerase was used that has been engineered by New England BioLabs with enhanced ability to incorporate 3'-O-modified dNTPs.

3'-O-azidomethyl reversible terminators of all dNTPs were tested independently for incorporation:

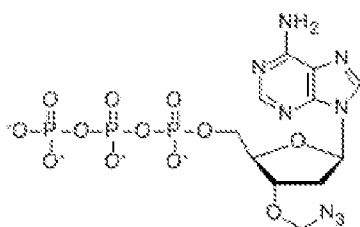
10 3'-O-azidomethyl-dTTP:



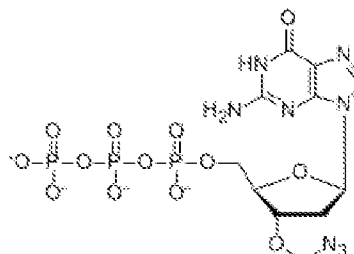
3'-O-azidomethyl-dCTP:



3'-O-azidomethyl-dATP:



3'-O-azidomethyl-dGTP:



15 Methods

1. 2 μ l of 10x Thermopol® buffer (20 mM Tris-HCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 10 mM KCl, 2 mM MgSO_4 , 0.1% Triton® X-100, pH 8.8, New England BioLabs) was mixed with 12.25 μ l of sterile deionized water (ELGA VEOLIA) in 1.5ml Eppendorf tube.

20

2. 0.5 μ l of 10 μ M primer (5 pmol, 1 equiv) (SEQ ID NO: 22, Figure 18j) and 0.75 μ l of 10 μ M template-A/G/T/C (6 pmol, 1.5equiv) (SEQ ID NOS: 23 to 26, Figure 18j) and 1 μ l of 10 μ M helper strand-T/C/A/G (10 pmol, 2 equiv) (SEQ ID NOS: 27 to 30, Figure 18j) were added to the reaction mixture.

5

3. 3'-O-modified-dTTP/dCTP/ dATP/dGTP (2 μ l of 100 μ M) and MnCl₂ (1 μ l of 40 mM) were added.

4. 1.5 μ l of Terminator IX DNA polymerase (15 U, New England BioLabs) was then added.

10

5. The reaction was incubated for 20 minutes at 65°C.

6. The reaction was stopped by addition of TBE-Urea sample buffer (Novex).

15

7. The reaction was separated on polyacrylamide gel (15%) TBE buffer and visualized by ChemiDoc MP imaging system (BioRad).

Gel Electrophoresis and DNA Visualization:

20

1. 5 μ l of reaction mixture was added to 5 μ l of TBE-Urea sample buffer (Novex) in a sterile 1.5 ml Eppendorf tube and heated to 95°C for 5 minutes using a heat ThermoMixer (Eppendorf).

25 2. 5 μ l of the sample were then loaded into the wells of a 15% TBE-Urea gel 1.0mm x 10 well (Invitrogen) which contained preheated 1X TBE buffer Thermo Scientific (89mM Tris, 89mM boric acid and 2mM EDTA).

3. X-cell sure lock module (Novex) was fastened in place and electrophoresis performed at the following conditions; 260V, 90 Amps for 40 minutes at room temperature.

- 5 4. The gel was visualized by ChemiDoc MP (BioRad) using Cy3 LEDS. Visualization and analysis was carried out on the Image lab 2.0 platform.

Results and Conclusions

- 10 Regarding the evaluation of the temperature on the incorporation of 3'-O-azidomethyl-dTTP using Therminator IX DNA polymerase, the results indicate that incorporation of 3'-O-azidomethyl-dTTP in the presence of a helper strand for ligation goes to 90% after 5 minutes. 10 % of primer remains unextended after 20 minutes at 37°C and 47°C.
- 15 Therminator IX DNA polymerase at 2mM Mn²⁺ ions and a temperature of 37°C provide good conditions for incorporation of 3'-O-modified-dNTPs opposite a complementary base in DNA with high efficiency in the presence of the helper strand (from the ligation step from the previous cycle).

20 Step 2: Cleavage

The second step describes a one-step cleavage of polynucleotides with Endonuclease V (Figure 19a).

25 Materials and Methods

Materials

1. Oligonucleotides utilized in Example 3 were designed in-house and synthesised by
30 Sigma Aldrich (see table in Figure 19d for sequences).

2. The oligonucleotides were diluted to a stock concentration of 100uM using sterile distilled water (ELGA VEOLIA).

5 Methods

Cleavage reaction on oligonucleotides was carried out using the procedure below:

1. A pipette (Gilson) was used to transfer 41 µl sterile distilled water (ELGA VEOLIA)
10 into a 1.5 ml Eppendorf tube.
2. 5µl of 10X Reaction buffer® NEB (50 mM Potassium Acetate, 20 mM Tris-acetate, 10 mM Magnesium Acetate, 1 mM DTT, pH 7.9 @ 25°C) was then added into the same Eppendorf tube.
- 15 3. 1µl each of oligonucleotides (Figure 19d); Template (SEQ ID NO: 31) or any fluorescently tagged long oligo strand, Primer with T (SEQ ID NO: 32) and control (SEQ ID NO: 33) and helper strand (SEQ ID NO: 34), all at 5pmols, were added into the same tube.
- 20 4. 1µl of Human Endonuclease V (Endo V) NEB (10 units/µl) was added into the same tube.
5. Reaction mixture was then gently mixed by resuspension with a pipette, centrifuged
25 at 13,000 rpm for 5 seconds and incubated at 37°C for 1 hour.
6. Typically after incubation time had elapsed, reaction was terminated by enzymatic heat inactivation (*i.e.* 65°C for 20 minutes).
- 30 The sample mixture was purified using the protocol outlined below:

1. 500 µl of buffer PNI QIAGEN (5M guanidinium chloride) was added to the sample and mixed by gentle resuspension with a pipette.
- 5 2. The mixture was transferred into a QIAquick spin column (QIAGEN) and centrifuged for 1 min at 6000 rpm.
3. After centrifugation, flow-through was discarded and 750 µl of buffer PE QIAGEN (10mM Tris-HCl pH 7.5 and 80% ethanol) was added into the spin column and centrifuged
10 for 1 min at 6000 rpm.
4. The flow-through was discarded and the spin column was centrifuged for an additional 1min at 13000 rpm to remove residual PE buffer.
- 15 5. The spin column was then placed in a sterile 1.5 ml Eppendorf tube.
6. For DNA elution, 50 µl of Buffer EB QIAGEN (10mM Tris.CL, pH 8.5) was added to the centre of the column membrane and left to stand for 1min at room temperature.
- 20 7. The tube was then centrifuged at 13000 rpm for 1 minutes. Eluted DNA concentration was measured and stored at -20°C for subsequent use.

Measurement of purified DNA concentration was determined using the protocol below:

- 25 1. NanoDrop one (Thermo Scientific) was equilibrated by adding 2 µl of sterile distilled water (ELGA VEOLIA) onto the pedestal.
2. After equilibration, the water was gently wiped off using a lint-free lens cleaning tissue (Whatman).

3. NanoDrop one was blanked by adding 2 μ l of Buffer EB QIAGEN (10mM Tris.CL, pH 8.5). Then step 2 was repeated after blanking.

4. DNA concentration was measured by adding 2 μ l of the sample onto the pedestal
5 and selecting the measure icon on the touch screen.

Gel Electrophoresis and DNA Visualization:

1. 5 μ l of DNA and TBE-Urea sample buffer (Novex) was added into a sterile 1.5ml
10 Eppendorf tube and heated to 95°C for 2 minutes using a heat thermoblock (Eppendorf).

2. The DNA mixtures were then loaded into the wells of a 15% TBE-Urea gel 1.0mm
x 10 well (Invitrogen) which contained preheated 1X TBE buffer Thermo Scientific (89mM
Tris, 89mM boric acid and 2mM EDTA).
15

3. X-cell sure lock module (Novex) was fastened in place and electrophoresis
performed at the following conditions; 260V, 90 Amps for 40 minutes at room temperature.

4. Detection and visualization of DNA in the gel was carried out with Chemidoc MP
20 (BioRad) using Cy3 LEDS. Visualization and analysis was carried out on the Image lab 2.0
platform.

Results and Conclusions

25 Cleavage results from Example 3 showed that a significantly high yield of cleaved DNA
could be obtained with Endonuclease V in the presence or absence of the helper strand
(Figure 19c).

Step 3: Ligation

The third step describes ligation of polynucleotides with DNA ligase in the presence of a helper strand. A diagrammatic illustration is shown in Figure 20a.

5

Materials and Methods

Materials

- 10 1. Oligonucleotides utilized in Example 3 were designed in-house and synthesised by Sigma Aldrich (see table in Figure 20b for sequences).
2. The oligonucleotides were diluted to a stock concentration of 100 uM using sterile distilled water (ELGA VEOLIA).

15

Methods

Ligation reaction on oligonucleotides was carried out using the procedure below

- 20 1. A pipette (Gilson) was used to transfer 16 µl sterile distilled water (ELGA VEOLIA) into a 1.5ml Eppendorf tube.
2. 10 µl of 2X Quick Ligation Reaction buffer NEB (132 mM Tris-HCl, 20mM MgCl₂, 2mM dithiothreitol, 2mM ATP, 15% Polyethylene glycol (PEG6000) and pH 7.6 at 25°C)
25 was then added into the same Eppendorf tube.
3. 1 µl each of oligonucleotides (Figure 20b); TAMRA or any fluorescently tagged phosphate strand (SEQ ID NO: 35), primer with T (SEQ ID NO: 36) and inosine strand (SEQ ID NO: 37) and helper strand (SEQ ID NO: 38) all having an amount of 5 pmols was added
30 into the same tube.

4. 1 µl of Quick T4 DNA Ligase NEB (400units/µl) was added into the same tube.
5. Reaction mixture was then gently mixed by resuspension with a pipette, centrifuged
5 at 13,000 rpm for 5 seconds and incubated at room temperature for 20 minutes.
6. Typically after the incubation time had elapsed, the reaction was terminated with the addition of TBE-Urea sample Buffer (Novex).
- 10 7. The reaction mixture was purified using the protocol outlined in purification steps 1-7 as described above.

Measurement of purified DNA concentration was determined using the protocol below:

- 15 1. NanoDrop one (Thermo Scientific) was equilibrated by adding 2 µl of sterile distilled water (ELGA VEOLIA) onto the pedestal.
2. After equilibration, the water was gently wiped off using a lint-free lens cleaning tissue (Whatman).
- 20 3. NanoDrop one was blanked by adding 2 µl of Buffer EB QIAGEN (10mM Tris.CL, pH 8.5). Then step 2 was repeated after blanking.
4. DNA concentration was measured by adding 2 µl of the sample onto the pedestal
25 and selecting the measure icon on the touch screen.
5. Purified DNA was run on a polyacrylamide gel and visualized in accordance with the procedure in steps 5-8 described above. No change in conditions or reagents was introduced.

30

Gel Electrophoresis and DNA Visualization:

1. 5 µl of DNA and TBE-Urea sample buffer (Novex) was added into a sterile 1.5 ml Eppendorf tube and heated to 95°C for 2 minutes using a heat thermoblock (Eppendorf).
5
2. The DNA mixtures were then loaded into the wells of a 15% TBE-Urea gel 1.0mm x 10 well (Invitrogen) which contained preheated 1X TBE buffer Thermo Scientific (89mM Tris, 89mM boric acid and 2mM EDTA).
- 10 3. X-cell sure lock module (Novex) was fastened in place and electrophoresis performed at the following conditions; 260V, 90 Amps for 40 minutes at room temperature.
4. Detection and visualization of DNA in the gel was carried out with ChemiDoc MP (BioRad) using Cy3 LEDS. Visualization and analysis was carried out on the Image lab 2.0
15 platform.

Step 4: Deprotection

- Deprotection step (Figure 21a) was studied on DNA model bearing 3'-O-azidomethyl group that is introduced to DNA by incorporation of 3'-O-azidomethyl-dNTPs by
20 Terminator IX DNA polymerase. Deprotection was carried out by tris(carboxyethyl)phosphine (TCEP) and monitored by extension reaction when mixture of all natural dNTPs is added to the solution of the purified deprotected DNA.

25 Materials and Methods

Materials

1. Oligonucleotides utilized in Example 3 were designed in-house and synthesised by
30 Sigma Aldrich (see Figure 21i for sequences).

2. The oligonucleotides were diluted to a stock concentration of 100 uM using sterile distilled water (ELGA VEOLIA).

5 3. Enzymes were purchased from New England BioLabs.

Methods

1. 2 µl of 10x Thermopol® buffer (20 mM Tris-HCl, 10 mM (NH₄)₂SO₄, 10 mM KCl,
10 2 mM MgSO₄, 0.1% Triton® X-100, pH 8.8, New England BioLabs) was mixed with 12.25 µl of sterile deionized water (ELGA VEOLIA) in 1.5 ml Eppendorf tube.

2. 1 µl of 10 µM primer (10 pmol, 1 equiv) (SEQ ID NO: 39, Figure 21i) and 1.5 µl of either 10 µM template-A/G/T/C (15 pmol, 1.5equiv) (SEQ ID NOS: 40 to 43, Figure
15 21i) were added to the reaction mixture.

3. 3'-O-modified-dTTP/dCTP/dATP/dGTP (2 µl of 100 µM) and MnCl₂ (1 µl of 40mM) were added.

20 4. 1.5 µl of Therminator IX DNA polymerase (15 U, New England BioLabs) was then added.

5. The reaction was incubated for 5 minutes at 37°C.

25 6. 4 µL of the sample was taken out and mixed with 0.5 ul of 5mM dNTP mix and allowed to react for 10 minutes for control reaction.

7. 40 µL of the 500 mM TCEP in 1M TRIS buffer pH 7.4 was added to the reaction mixture and allowed to react for 10 minutes at 37°C.

30

8. The reaction mixture was purified using QIAGEN Nucleotide removal kit eluting by 20 μ L of 1x Thermopol® buffer.

9. 1 μ L of 5mM dNTP mix and 1 μ L of DeepVent (exo-) DNA polymerase were
5 added to the purified reaction mixture and allowed to react 10 minutes.

10. The reaction was stopped by addition of TBE-Urea sample buffer (Novex).

11. The reaction was separated on polyacrylamide gel (15%) TBE buffer and
10 visualized by ChemiDoc MP imaging system (BioRad).

Results and Conclusion

50mM TCEP was not sufficient to cleave 3'-*O*-azidomethyl group with high efficiency on
15 0.2 μ M DNA model (Figure 21h). In contrast, 300mM TCEP successfully cleaved 3'-*O*-azidomethyl group with 95% efficiency on 0.2 μ M DNA model (Figure 21h).

Example 4. Version 2 Chemistry with Double Hairpin Model.

20 This Example describes the synthesis of polynucleotides using 4 steps on a two-hairpin model: incorporation of 3'-*O*- modified dNTPs from a nick site; cleavage, ligation and deprotection with the first step taking place opposite a naturally complementary nucleotide which is positioned in the support strand adjacent to a universal nucleotide, in this particular case inosine.

25

Step1: Incorporation

The first step describes controlled addition of 3'-*O*-protected single nucleotide to oligonucleotide by enzymatic incorporation by DNA polymerase (Figure 22a).

30

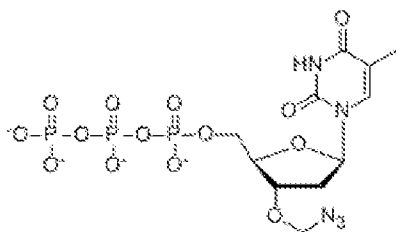
Materials and Methods

Materials

- 5 1. 3'-*O*-modified dNTPs were synthesised in-house according to the protocol described in PhD thesis Jian Wu: Molecular Engineering of Novel Nucleotide Analogues for DNA Sequencing by Synthesis. Columbia University, 2008. The protocol for synthesis is also described in the patent application publication: J. William Efcavitch, Julieta E. Sylvester, Modified Template-Independent Enzymes for Polydeoxynucleotide Synthesis, Molecular
10 Assemblies US2016/0108382A1.
2. Oligonucleotides were designed in house and obtained from Sigma-Aldrich (Figure 22c). The stock solutions were prepared in concentration of 100 μ M.
- 15 3. Terminator IX DNA polymerase was used that has been engineered by New England BioLabs with enhanced ability to incorporate 3'-*O*-modified dNTPs.

3'-*O*-azidomethyl-dTTP was tested for incorporation:

- 20 3'-*O*-azidomethyl-dTTP:



Method

- 25 1. 2 μ l of 10x Thermopol® buffer (20 mM Tris-HCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 10 mM KCl, 2 mM MgSO_4 , 0.1% Triton® X-100, pH 8.8, New England BioLabs) was mixed with 10.25 μ l of sterile deionized water (ELGA VEOLIA) in 1.5ml Eppendorf tube.

2. 0.5 μ l of 10 μ M hairpin oligonucleotide (5 pmol, 1 equiv) (SEQ ID NO: 44, Figure 22c) was added to the reaction mixture.
- 5 3. 3'-*O*-modified-dTTP (2 μ l of 100 μ M) and MnCl₂ (1 μ l of 40 mM) were added.
4. 1.5 μ l of Terminator IX DNA polymerase (15 U, New England BioLabs) was then added.
- 10 5. The reaction was incubated for 20 minutes at 65°C.
6. The reaction was stopped by addition of TBE-Urea sample buffer (Novex).
7. The reaction was separated on polyacrylamide gel (15%) TBE buffer and visualized
15 by ChemiDoc MP imaging system (BioRad).

Gel Electrophoresis and DNA Visualization:

1. 5 μ l of reaction mixture was added to 5 μ l of TBE-Urea sample buffer (Novex) in a
20 sterile 1.5ml Eppendorf tube and heated to 95°C for 5 minutes using a heat ThermoMixer (Eppendorf).
2. 5 μ l of the sample were then loaded into the wells of a 15% TBE-Urea gel 1.0mm x 10 well (Invitrogen) which contained preheated 1X TBE buffer Thermo Scientific (89mM
25 Tris, 89mM boric acid and 2mM EDTA).
3. X-cell sure lock module (Novex) was fastened in place and electrophoresis performed at the following conditions; 260V, 90 Amps for 40 minutes at room temperature.

4. The gel was visualized by ChemiDoc MP (BioRad) using Cy3 LEDS. Visualization and analysis was carried out on the Image lab 2.0 platform.

Results

5

DNA polymerases incorporate 3'-O-modified-dTTPs opposite its naturally complementary base in a hairpin construct.

Step2: Cleavage

10

The second step describes a one-step cleavage of a hairpin model in this particular case with Endonuclease V (Figure 23a).

Materials and Methods

15

Materials

1. Oligonucleotides utilized in Example 4 were designed in-house and synthesised by Sigma Aldrich (see Figure 23c for sequences).

20

2. The oligonucleotides were diluted to a stock concentration of 100uM using sterile distilled water (ELGA VEOLIA).

Methods

25

Cleavage reaction on hairpin oligonucleotides was carried out using the procedure below:

1. A pipette (Gilson) was used to transfer 43µl sterile distilled water (ELGA VEOLIA) into a 1.5ml Eppendorf tube.

30

2. 5 µl of 10X Reaction buffer® NEB (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9 @ 25°C) was then added into the same Eppendorf tube.
- 5 3. 1 µl of hairpin oligonucleotide (SEQ ID NO: 45, Figure 23c) having an amount of 5 pmols was added into the same tube.
4. 1 µl of Human Endonuclease V (Endo V) NEB (30 units/µl) was added into the same tube.
- 10 5. The reaction mixture was then gently mixed by resuspension with a pipette, centrifuged at 13,000 rpm for 5 seconds and incubated at 37°C for 1 hour.
6. Typically after incubation time had elapsed, the reaction was terminated by
15 enzymatic heat inactivation (*i.e.* 65°C for 20 minutes).

The sample mixture was purified using the protocol outlined below:

1. 500 µl of buffer PNI QIAGEN (5M guanidinium chloride) was added to the sample
20 and mixed by gentle resuspension with a pipette.
2. The mixture was transferred into a QIAquick spin column (QIAGEN) and centrifuged for 1 min at 6000 rpm.
- 25 3. After centrifugation, flow-through was discarded and 750 µl of buffer PE QIAGEN (10mM Tris-HCl pH 7.5 and 80% ethanol) was added into the spin column and centrifuged for 1 min at 6000 rpm.
4. The flow-through was discarded and the spin column was centrifuged for an
30 additional 1 min at 13000 rpm to remove residual PE buffer.

5. The spin column was then placed in a sterile 1.5ml Eppendorf tube.
6. For DNA elution, 50 µl of Buffer EB QIAGEN (10mM Tris.CL, pH 8.5) was added
5 to the centre of the column membrane and left to stand for 1min at room temperature.
7. The tube was then centrifuged at 13000 rpm for 1 minute. Eluted DNA concentration was measured and stored at -20°C for subsequent use.
- 10 Measurement of purified DNA concentration was determined using the protocol below:
 1. NanoDrop One (Thermo Scientific) was equilibrated by adding 2 µl of sterile distilled water (ELGA VEOLIA) onto the pedestal.
 - 15 2. After equilibration, the water was gently wiped off using a lint-free lens cleaning tissue (Whatman).
 3. NanoDrop One was blanked by adding 2 µl of Buffer EB QIAGEN (10mM Tris.CL, pH 8.5). Then step 2 was repeated after blanking.
 - 20 4. DNA concentration was measured by adding 2 µl of the sample onto the pedestal and selecting the measure icon on the touch screen.

Gel Electrophoresis and DNA Visualization:

25

1. 5 µl of DNA and TBE-Urea sample buffer (Novex) was added into a sterile 1.5ml Eppendorf tube and heated to 95°C for 2 minutes using a heat ThermoMixer(Eppendorf).

2. The DNA mixtures were then loaded into the wells of a 15% TBE-Urea gel 1.0mm x 10 well (Invitrogen) which contained preheated 1X TBE buffer Thermo Scientific (89mM Tris, 89mM boric acid and 2mM EDTA).

5 3. X-cell sure lock module (Novex) was fastened in place and electrophoresis performed at the following conditions; 260V, 90 Amps for 40 minutes at room temperature.

4. Detection and visualization of DNA in the gel was carried out with ChemiDoc MP (BioRad) using Cy3 LEDS. Visualization and analysis was carried out on the Image lab 2.0
10 platform.

Results and Conclusion

Cleavage results from Example 4 showed that a significantly high yield of digested hairpin
15 DNA was obtained with Endonuclease V at 37°C (Figure 23b).

Step 3: Ligation

The third step describes ligation of a hairpin model with DNA ligase. Diagrammatic
20 illustration is shown in Figure 24a.

Materials and Methods

Materials

25

1. Oligonucleotides utilized in Example 4 were designed in-house and synthesised by Sigma Aldrich (see Figure 24c for sequences).

2 The oligonucleotides were diluted to a stock concentration of 100uM using sterile
30 distilled water (ELGA VEOLIA).

Method

Ligation reaction on oligonucleotides was carried out using the procedure below:

5

1. A pipette (Gilson) was used to transfer 1 μ l (5pmols) of TAMRA or any fluorescently tagged phosphate hairpin oligo (SEQ ID NO: 46) into a 1.5ml Eppendorf tube.

10 2. 15 μ l (100pmols) of inosine-containing hairpin construct (SEQ ID NO: 47) was then added into the same tube and gently mixed by resuspension with a pipette for 3 seconds.

3. 40 μ l of Blunt/TA DNA Ligase NEB (180 units/ μ l) was added into the same tube.

15 4. Reaction mixture was then gently mixed by resuspension with a pipette, centrifuged at 13,000 rpm for 5 seconds and incubated at room temperature for 20 minutes.

5. Typically after incubation time had elapsed, the reaction was terminated with the addition of TBE-Urea sample buffer (Novex).

20

6. The reaction mixture was purified using the protocol outlined in purification steps 1-7 above.

Measurement of purified DNA concentration was determined using the protocol below:

25

1. NanoDrop One (Thermo Scientific) was equilibrated by adding 2 μ l of sterile distilled water (ELGA VEOLIA) onto the pedestal.

2. After equilibration, the water was gently wiped off using a lint-free lens cleaning
30 tissue (Whatman).

3. NanoDrop One was blanked by adding 2 μ l of Buffer EB QIAGEN (10mM Tris.CL, pH 8.5). Then step 2 was repeated after blanking.
- 5 4. DNA concentration was measured by adding 2 μ l of the sample onto the pedestal and selecting the measure icon on the touch screen.
5. Purified DNA was run on a polyacrylamide gel and visualized in accordance with the procedure in steps 5-8 as described above. No change in conditions or reagents was
10 introduced.

Gel Electrophoresis and DNA Visualization.

1. 5 μ l of DNA and TBE-Urea sample buffer (Novex) was added into a sterile 1.5 ml
15 Eppendorf tube and heated to 95°C for 2 minutes using a heat ThermoMixer (Eppendorf).
2. The DNA mixtures were then loaded into the wells of a 15% TBE-Urea gel 1.0mm x 10 well (Invitrogen) which contained preheated 1X TBE buffer Thermo Scientific (89mM Tris, 89mM boric acid and 2mM EDTA).
20
3. X-cell sure lock module (Novex) was fastened in place and electrophoresis performed at the following conditions; 260V, 90 Amps for 40 minutes at room temperature.
- 25 4. Detection and visualization of DNA in the gel was carried out with ChemiDoc MP (BioRad) using Cy3 LEDS. Visualization and analysis was carried out on the Image lab 2.0 platform.

Results

Ligation of hairpin oligonucleotides with blunt/TA DNA ligase at room temperature (24°C) in the presence of a helper strand resulted high yield of ligated product. Ligated hairpin oligonucleotide after 1 minute showed a high yield of ligated DNA product with a ratio of ~ 85%. The ligated hairpin oligonucleotide after 2 minutes showed a high yield of ligated DNA with a ratio of ~ 85%. The ligated hairpin oligonucleotide after 3 minutes showed a high yield of ligated DNA product with a ratio of ~ 85%. The ligated hairpin oligonucleotide after 4 minutes showed a high yield of ligated DNA product with a ratio of ~ >85% (Figure 24b).

Example 5. Version 2 Chemistry - Complete Cycle on Double Hairpin Model.

This Example describes the synthesis of polynucleotides using 4 steps on a double hairpin model: incorporation of 3'-O-modified dNTPs from the nick site; cleavage, ligation and deprotection with the first step taking place opposite a naturally complementary nucleotide which is positioned in the support strand adjacent to a universal nucleotide, in this particular case inosine. One end of the hairpin serves as an attachment anchor.

The method starts by controlled addition of a 3'-O-protected single nucleotide to an oligonucleotide by enzymatic incorporation by DNA polymerase followed by inosine cleavage, ligation and deprotection (Figure 25a).

Materials and Methods

25

Materials

1. 3'-O-modified dNTPs were synthesised in-house according to the protocol described in PhD thesis Jian Wu: Molecular Engineering of Novel Nucleotide Analogues for DNA Sequencing by Synthesis. Columbia University, 2008. The protocol for synthesis

is also described in the patent application publication: J. William Efcavitch, Julieta E. Sylvester, Modified Template-Independent Enzymes for Polydeoxynucleotide Synthesis, Molecular Assemblies US2016/0108382A1.

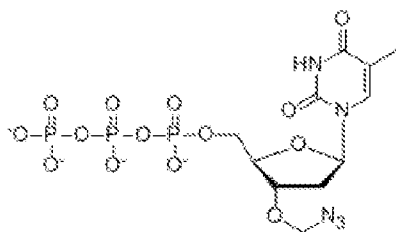
2. Oligonucleotides were designed in house and obtained from Sigma-Aldrich (Figure 25c). The stock solutions are prepared in concentration of 100 μ M.

3. Terminator IX DNA polymerase was used that has been engineered by New England BioLabs with enhanced ability to incorporate 3'-O-modified dNTPs.

10

3'-O-azidomethyl-dTTP was tested for incorporation:

3'-O-azidomethyl-dTTP:



15

Method

1. 2 μ l of 10x Thermopol® buffer (20 mM Tris-HCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 10 mM KCl, 2 mM MgSO_4 , 0.1% Triton® X-100, pH 8.8, New England BioLabs) was mixed with 12.5 μ l of sterile deionized water (ELGA VEOLIA) in 1.5ml Eppendorf tube.

20

2. 2 μ l of 10 μ M double hairpin model oligonucleotide (20 pmol, 1 equiv) (SEQ ID NO: 48, Figure 25c) were added to the reaction mixture.

25 3. 3'-O-modified-dTTP (2 μ l of 100 μ M) and MnCl_2 (1 μ l of 40 mM) were added.

4. 1.5 µl of Terminator IX DNA polymerase (15 U, New England BioLabs) was then added.
5. The reaction was incubated for 10 minutes at 37°C.
- 5 6. The aliquot (5 µl) was taken out of the reaction mixture and 0.5 µl of natural dNTP mix was added and allowed to react for 10 minutes. The reaction was analysed by gel electrophoresis.
- 10 7. The reaction mixture was purified using the protocol outlined in purification steps 1-7.
8. The DNA sample was eluted by 20 µl of NEB reaction buffer® (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9 @ 25°C) into
15 clean Eppendorf tube.
9. 1 µl of Human Endonuclease V (Endo V) NEB (30 units/µl) was added into the same tube.
- 20 10. Reaction mixture was then gently mixed by resuspension with a pipette, centrifuged at 13,000 rpm for 5 seconds and incubated at 37°C for 1 hour.
11. After incubation time had elapsed, reaction was terminated by enzymatic heat inactivation (*i.e.* 65°C for 20 minutes).
- 25 12. The aliquot (5 µl) was taken out of the reaction mixture and analysed on polyacrylamide gel (15%) using TBE buffer and visualized by ChemiDoc MP imaging system (BioRad).

13. Reaction mixture was purified using the protocol outlined in purification steps 1-7 above.

14. The DNA sample was eluted by 20 µl of NEB Reaction buffer® (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9 @ 25°C) into a clean Eppendorf tube.

15. 10 µl of 100 µM strand for ligation (1 nmol) (SEQ ID NO: 49, Figure 25c) were added to the reaction mixture.

10

16. 40 µl of Blunt/TA DNA Ligase NEB (180 units/µl) was added into the purified DNA sample.

17. Reaction mixture was then gently mixed by resuspension with a pipette, centrifuged at 13,000 rpm for 5 seconds and incubated at room temperature for 20 minutes.

18. 40 µL of the 500 mM TCEP in 1M TRIS buffer pH 7.4 was added to the reaction mixture and allowed to react for 10 minutes at 37°C.

19. The reaction mixture was purified using QIAGEN nucleotide removal kit eluting by 20 µL of 1x Thermopol® buffer.

Gel Electrophoresis and DNA Visualization:

1. 5 µl of reaction mixture was added to 5 µl of TBE-Urea sample buffer (Novex) in a sterile 1.5ml Eppendorf tube and heated to 95°C for 5 minutes using a heat ThermoMixer (Eppendorf).

2. 5 µl of the sample were then loaded into the wells of a 15% TBE-Urea gel 1.0mm x 10 well (Invitrogen) which contained preheated 1X TBE buffer Thermo Scientific (89mM Tris, 89mM boric acid and 2mM EDTA).

5 3. X-cell sure lock module (Novex) was fastened in place and electrophoresis performed at the following conditions; 260V, 90 Amps for 40 minutes at room temperature.

10 4. The gel was visualized by ChemiDoc MP (BioRad) using Cy3 LEDS. Visualization and analysis was carried out on the Image lab 2.0 platform.

Measurement of purified DNA concentration was determined using the protocol below:

15 1. NanoDrop One (Thermo Scientific) was equilibrated by adding 2 µl of sterile distilled water (ELGA VEOLIA) onto the pedestal.

2. After equilibration, the water was gently wiped off using a lint-free lens cleaning tissue (Whatman).

20 3. NanoDrop One was blanked by adding 2 µl of Buffer EB QIAGEN (10mM Tris.CL, pH 8.5). Then step 2 was repeated after blanking.

4. DNA concentration was measured by adding 2 µl of the sample onto the pedestal and selecting the measure icon on the touch screen.
25

5. Purified DNA was run on a polyacrylamide gel and visualized in accordance with the procedure in section 2 steps 5-8. No change in conditions or reagents was introduced.

The sample mixture was purified after each step using the protocol outlined below:

30

1. 500 µl of buffer PNI QIAGEN (5M guanidinium chloride) was added to the sample and mixed by gentle resuspension with a pipette.
2. The mixture was transferred into a QIAquick spin column (QIAGEN) and
5 centrifuged for 1 min at 6000 rpm.
3. After centrifugation, flow-through was discarded and 750 µl of buffer PE QIAGEN (10mM Tris-HCl pH 7.5 and 80% ethanol) was added into the spin column and centrifuged for 1 min at 6000 rpm.
10
4. The flow-through was discarded and the spin column was centrifuged for an additional 1min at 13000 rpm to remove residual PE buffer.
5. The spin column was then placed in a sterile 1.5 ml Eppendorf tube.
15
6. For DNA elution, 20 µl of appropriate buffer for the reaction was added to the centre of the column membrane and left to stand for 1min at room temperature.
7. The tube was then centrifuged at 13000 rpm for 1 minute. Eluted DNA
20 concentration was measured and stored at -20°C for subsequent use.

Results

DNA polymerase incorporates 3'-O-modified-dTTPs opposite its naturally complementary
25 base in a double hairpin construct (Figure 25b).

Example 6. Version 2 Chemistry - Complete Cycle on Single Hairpin Model Using Helper Strand.

This Example describes the synthesis of polynucleotides using 4 steps on single-hairpin
5 model: incorporation of 3'-*O*-modified dNTPs from nick site; cleavage, ligation and
deprotection with the first step taking place opposite a naturally complementary base. The
DNA synthesis uses a helper strand in the process.

The method starts by controlled addition of a 3'-*O*-protected single nucleotide to an
10 oligonucleotide by enzymatic incorporation by DNA polymerase followed by inosine
cleavage, ligation and deprotection (Figure 26a).

Materials and Methods

15 **Materials**

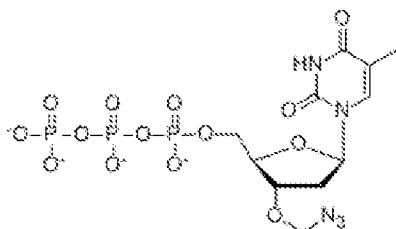
1. 3'-*O*-modified dNTPs were synthesised in-house according to the protocol
described in PhD thesis Jian Wu: Molecular Engineering of Novel Nucleotide Analogues
for DNA Sequencing by Synthesis. Columbia University, 2008. The protocol for synthesis
20 is also described in the patent application publication: J. William Efcavitch, Julieta E.
Sylvester, Modified Template-Independent Enzymes for Polydeoxynucleotide Synthesis,
Molecular Assemblies US2016/0108382A1.

2. Oligonucleotides were designed in house and obtained from Sigma Aldrich (Figure
25 26b). The stock solutions are prepared in concentration of 100 μ M.

3. Terminator IX DNA polymerase was used that has been engineered by New
England BioLabs with enhanced ability to incorporate 3'-*O*-modified dNTPs.

30 3'-*O*-azidomethyl-dTTP was tested for incorporation:

3'-*O*-azidomethyl-dTTP:



5 Method

1. 2 μ l of 10x Thermopol® buffer (20 mM Tris-HCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 10 mM KCl, 2 mM MgSO_4 , 0.1% Triton® X-100, pH 8.8, New England BioLabs) was mixed with 12.5 μ l of sterile deionized water (ELGA VEOLIA) in 1.5ml Eppendorf tube.
2. 2 μ l of 10 μ M Single hairpin model oligonucleotide (20 pmol, 1 equiv) (SEQ ID NO: 50, Figure 26b) and Helper strand (30 pmol, 1.5 equiv) (SEQ ID NO: 51, Figure 26b) were added to the reaction mixture.
3. 3'-*O*-modified-dTTP (2 μ l of 100 μ M) and MnCl_2 (1 μ l of 40 mM) were added
4. 1.5 μ l of Terminator IX DNA polymerase (15 U, New England BioLabs) was then added.
5. The reaction was incubated for 10 minutes at 37°C.
6. The aliquot (5 μ l) was taken out of the reaction mixture and 0.5 μ l of natural dNTP mix was added and allowed to react for 10 minutes. The reaction was analysed by gel electrophoresis.
7. The reaction mixture was purified using the protocol outlined in purification steps 1-7 above.

8. The DNA sample was eluted by 20µl of NEB reaction buffer® (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9 @ 25°C) into a clean Eppendorf tube.
- 5
9. 1µl of Human Endonuclease V (Endo V) NEB (30units/µl) was added into the same tube.
10. Reaction mixture was then gently mixed by resuspension with a pipette, centrifuged at 13,000 rpm for 5 seconds and incubated at 37°C for 1 hour.
- 10
11. After incubation time had elapsed, the reaction was terminated by enzymatic heat inactivation (*i.e.* 65°C for 20 minutes).
12. The aliquot (5 µl) was taken out of the reaction mixture and analysed on polyacrylamide gel (15%) using TBE buffer and visualized by ChemiDoc MP imaging system (BioRad).
- 15
13. The reaction mixture was purified using the protocol outlined in purification steps 1-7 above.
- 20
14. The DNA sample was eluted by 20 µl of NEB reaction buffer® (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9 @ 25°C) into clean Eppendorf tube.
- 25
15. 10 µl of 100 µM strand for ligation (1 nmol) (SEQ ID NO: 52, Figure 26b) and 10 µl of 100 µM helper strand for ligation (1 nmol) (SEQ ID NO: 53, Figure 26b) were added to the reaction mixture.
16. 40µl of Blunt/TA DNA Ligase NEB (180 units/µl) was added into the same tube.
- 30

17. Reaction mixture was then gently mixed by resuspension with a pipette, centrifuged at 13,000rpm for 5 seconds and incubated at room temperature for 20 minutes.

5 18. 40 μ L of the 500 mM TCEP in 1M TRIS buffer pH 7.4 was added to the reaction mixture and allowed to react for 10 minutes at 37°C.

19. The reaction mixture was purified using QIAGEN Nucleotide removal kit eluting by 20 μ L of 1x NEB Thermopol® buffer.

10

20. Typically after incubation time had elapsed, reaction was terminated with the addition of TBE-Urea sample Buffer (Novex).

Gel Electrophoresis and DNA Visualization:

15

1. 5 μ l of reaction mixture was added to 5 μ l of TBE-Urea sample buffer (Novex) in a sterile 1.5 ml Eppendorf tube and heated to 95°C for 5 minutes using a heat ThermoMixer (Eppendorf).

20 2. 5 μ l of the sample were then loaded into the wells of a 15% TBE-Urea gel 1.0mm x 10 well (Invitrogen) which contained preheated 1X TBE buffer Thermo Scientific (89mM Tris, 89mM boric acid and 2mM EDTA).

3. X-cell sure lock module (Novex) was fastened in place and electrophoresis
25 performed at the following conditions; 260V, 90 amps for 40 minutes at room temperature.

4. The gel was visualized by ChemiDoc MP (BioRad) using Cy3 LEDS. Visualization and analysis was carried out on the Image lab 2.0 platform.

30 Measurement of purified DNA concentration was determined using the protocol below:

1. NanoDrop One (Thermo Scientific) was equilibrated by adding 2 μ l of sterile distilled water (ELGA VEOLIA) onto the pedestal.
- 5 2. After equilibration, the water was gently wiped off using a lint-free lens cleaning tissue (Whatman).
3. NanoDrop One was blanked by adding 2 μ l of Buffer EB QIAGEN (10mM Tris.CL, pH 8.5). Then step 2 was repeated after blanking.
- 10 4. DNA concentration was measured by adding 2 μ l of the sample unto the pedestal and selecting the measure icon on the touch screen.
5. Purified DNA was run on a polyacrylamide gel and visualized in accordance with
15 the procedure noted above in steps 5-8. No change in conditions or reagents was introduced.

The sample mixture was purified after each step using the protocol outlined below:

- 20 1. 500 μ l of buffer PNI QIAGEN (5M guanidinium chloride) was added to the sample and mixed by gentle resuspension with a pipette.
2. The mixture was transferred into a QIAquick spin column (QIAGEN) and centrifuged for 1min at 6000 rpm.
- 25 3. After centrifugation, flow-through was discarded and 750 μ l of buffer PE QIAGEN (10mM Tris-HCl pH 7.5 and 80% ethanol) was added into the spin column and centrifuged for 1min at 6000 rpm.

4. The flow-through was discarded and the spin column was centrifuged for an additional 1min at 13000 rpm to remove residual PE buffer.

5. The spin column was then placed in a sterile 1.5ml Eppendorf tube.

5

6. For DNA elution, 20 µl of appropriate buffer for the reaction was added to the centre of the column membrane and left to stand for 1 minute at room temperature.

7. The tube was then centrifuged at 13000 rpm for 1 minute. Eluted DNA
10 concentration was measured and stored at -20°C for subsequent use.

Example 7. Version 3 Chemistry - Complete Cycle on Double Hairpin Model.

This Example describes the synthesis of polynucleotides using 4 steps on a double- hairpin
15 construct model: incorporation of 3'-O-modified dNTPs from the nick site; cleavage, ligation and deprotection with the first step taking place opposite a universal nucleotide, in this particular case an inosine base.

The method starts by controlled addition of a 3'-O-protected single nucleotide to an
20 oligonucleotide by enzymatic incorporation by DNA polymerase followed by inosine cleavage, ligation and deprotection (Figure 27a).

Materials and Methods

25 Materials

1. 3'-O-modified dNTPs were synthesised in-house according to the protocol described in PhD thesis Jian Wu: Molecular Engineering of Novel Nucleotide Analogues for DNA Sequencing by Synthesis. Columbia University, 2008. The protocol for synthesis
30 is also described in the patent application publication: J. William Efcavitch, Julieta E.

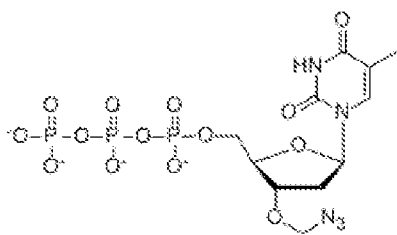
Sylvester, Modified Template-Independent Enzymes for Polydeoxynucleotide Synthesis, Molecular Assemblies US2016/0108382A1.

2. Oligonucleotides were designed in house and obtained from Sigma-Aldrich (Figure
5 27b). The stock solutions are prepared in concentration of 100 μ M.

3. Therminator IX DNA polymerase that has been engineered by New England BioLabs has enhanced ability to incorporate 3'-*O*-modified dNTPs.

10 3'-*O*-azidomethyl-dTTP was tested for incorporation:

3'-*O*-azidomethyl-dTTP:



15 Method

1. 2 μ l of 10x Thermopol® buffer (20 mM Tris-HCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 10 mM KCl, 2 mM MgSO_4 , 0.1% Triton® X-100, pH 8.8, New England BioLabs) was mixed with 12.5 μ l of sterile deionized water (ELGA VEOLIA) in 1.5ml Eppendorf tube.

20

2. 2 μ l of 10 μ M double hairpin model oligonucleotide (20 pmol, 1 equiv) (SEQ ID NO: 54, Figure 27b) were added to the reaction mixture.

3. 3'-*O*-modified-dTTP (2 μ l of 100 μ M) and MnCl_2 (1 μ l of 40 mM) were added.

25

4. 1.5 μ l of Therminator IX DNA polymerase (15 U, New England BioLabs) was then added.

5. The reaction was incubated for 10 minutes at 37°C.
6. The aliquot (5 µl) was taken out of the reaction mixture and 0.5 µl of natural dNTP
5 mix was added and allowed to react for 10 minutes. The reaction was analysed by gel electrophoresis.
7. The reaction mixture was purified using the protocol outlined in purification steps
1-7.
- 10 8. The DNA sample was eluted by 20 µl of NEB Reaction buffer® (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9 @ 25°C) into clean Eppendorf tube.
- 15 9. 1µl of Human Endonuclease V (Endo V) NEB (30units/µl) was added into the same tube.
10. Reaction mixture was then gently mixed by resuspension with a pipette, centrifuged
at 13,000 rpm for 5 seconds and incubated at 37°C for 1 hour.
- 20 11. After the incubation time had elapsed, the reaction was terminated by enzymatic heat inactivation (*i.e.* 65°C for 20 minutes).
12. The aliquot (5 µl) was taken out of the reaction mixture and analysed on
25 polyacrylamide gel (15%) using TBE buffer and visualized by ChemiDoc MP imaging system (BioRad).
13. Reaction mixture was purified using the protocol outlined in purification steps 1-7
above.

30

14. The DNA sample was eluted by 20 µl of NEB Reaction buffer® (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9 @ 25°C) into a clean Eppendorf tube.
- 5 15. 10 µl of 100 µM strand for ligation (1 nmol) (SEQ ID NO: 55, Figure 27b), were added to the reaction mixture.
16. 40µl of Blunt/TA DNA Ligase NEB (180 units/µl) was added into the same tube.
- 10 17. Reaction mixture was then gently mixed by resuspension with a pipette, centrifuged at 13,000rpm for 5 seconds and incubated at room temperature for 20 minutes.
18. 40 µL of the 500 mM TCEP in 1M TRIS buffer pH 7.4 was added to the reaction mixture and allowed to react for 10 minutes at 37°C.
- 15 19. The reaction mixture was purified using QIAGEN Nucleotide removal kit eluting by 20 µL of 1x NEB Thermopol® buffer.
20. Typically after incubation time had elapsed, reaction was terminated with the
20 addition of TBE-Urea sample Buffer (Novex).

Gel Electrophoresis and DNA Visualization:

1. 5 µl of reaction mixture was added to 5 µl of TBE-Urea sample buffer (Novex) in a
25 sterile 1.5ml Eppendorf tube and heated to 95°C for 5 minutes using a heat ThermoMixer (Eppendorf).
2. 5 µl of the sample were then loaded into the wells of a 15% TBE-Urea gel 1.0mm x 10 well (Invitrogen) which contained preheated 1X TBE buffer Thermo Scientific (89mM
30 Tris, 89mM boric acid and 2mM EDTA).

3. X-cell sure lock module (Novex) was fastened in place and electrophoresis performed at the following conditions; 260V, 90 amps for 40 minutes at room temperature.

5 4. The gel was visualized by ChemiDoc MP (BioRad) using Cy3 LEDS. Visualization and analysis was carried out on the Image lab 2.0 platform.

Measurement of purified DNA concentration was determined using the protocol below:

10 1. NanoDrop One (Thermo Scientific) was equilibrated by adding 2 µl of sterile distilled water (ELGA VEOLIA) onto the pedestal.

2. After equilibration, the water was gently wiped off using a lint-free lens cleaning tissue (Whatman).

15

3. NanoDrop One was blanked by adding 2 µl of Buffer EB QIAGEN (10mM Tris.CL, pH 8.5). Step 2 was then repeated after blanking.

4. DNA concentration was measured by adding 2 µl of the sample unto the pedestal
20 and selecting the measure icon on the touch screen.

5. Purified DNA was run on a polyacrylamide gel and visualized in accordance with the procedure in section 2 steps 5-8. No change in conditions or reagents was introduced.

25 The sample mixture was purified after each step using the protocol outlined below:

1. 500 µl of buffer PNI QIAGEN (5M guanidinium chloride) was added to the sample and mixed by gentle resuspension with a pipette.

2. The mixture was transferred into a QIAquick spin column (QIAGEN) and centrifuged for 1min at 6000 rpm.

3. After centrifugation, flow-through was discarded and 750 µl of buffer PE QIAGEN
5 (10mM Tris-HCl pH 7.5 and 80% ethanol) was added into the spin column and centrifuged for 1min at 6000 rpm.

4. The flow-through was discarded and the spin column was centrifuged for an additional 1min at 13000 rpm to remove residual PE buffer.

10

5. The spin column was then placed in a sterile 1.5ml Eppendorf tube.

6. For DNA elution, 20 µl of appropriate buffer for the reaction was added to the centre of the column membrane and left to stand for 1min at room temperature.

15

7. The tube was then centrifuged at 13000 rpm for 1minutes. Eluted DNA concentration was measured and stored at -20°C for subsequent use.

20 **Example 8. Version 2 Chemistry - Complete Two-Cycle Experiment on Double-Hairpin Model.**

This example describes a complete two-cycle experiment for the synthesis of polynucleotides using 4 steps on a double-hairpin model: incorporation of 3'-O-modified dNTPs from the nick site; deprotection, cleavage, and ligation with the first step taking
25 place opposite a complementary base.

The method starts by controlled addition of a 3'-O-protected single nucleotide to an oligonucleotide by enzymatic incorporation by DNA polymerase followed by deprotection, inosine cleavage and ligation, as depicted in the reaction schematic for the first cycle
30 shown in Figure 28a. Figure 28b shows a reaction schematic for the second cycle.

Materials and Methods

Materials

5

1. 3'-*O*-modified dNTPs were synthesised in-house according to the protocol described in PhD thesis Jian Wu: Molecular Engineering of Novel Nucleotide Analogues for DNA Sequencing by Synthesis. Columbia University, 2008. The protocol for synthesis is also described in the patent application publication: J. William Efcavitch, Julieta E. Sylvester, Modified Template-Independent Enzymes for Polydeoxynucleotide Synthesis, Molecular Assemblies US2016/0108382A1.

15

2. Oligonucleotides were designed in house and obtained from Sigma-Aldrich (Figure 28d). The stock solutions are prepared in concentration of 100 μ M.

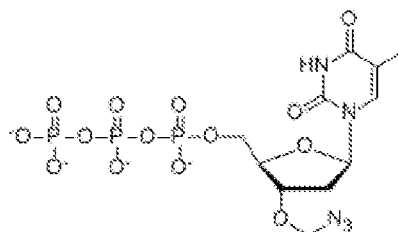
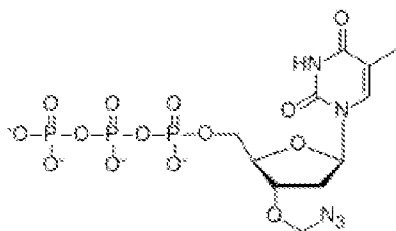
3. Terminator IX DNA polymerase that has been engineered by New England BioLabs has enhanced ability to incorporate 3'-*O*-modified dNTPs.

3'-*O*-azidomethyl-dTTP and 3'-*O*-azidomethyl-dCTP were used for incorporation:

20

3'-*O*-azidomethyl-dTTP:

3'-*O*-azidomethyl-dCTP:



25

Method

1st cycle:

- 5 1. 2 µl of 10x Thermopol® buffer (20 mM Tris-HCl, 10 mM (NH₄)₂SO₄, 10 mM KCl, 2 mM MgSO₄, 0.1% Triton® X-100, pH 8.8, New England BioLabs) was mixed with 12.5 µl of sterile deionized water (ELGA VEOLIA) in 1.5ml Eppendorf tube.
2. 2 µl of 10 µM double hairpin model oligonucleotide (20 pmol, 1 equiv) (SEQ ID
10 NO: 56, Figure 28d) were added to the reaction mixture.
3. 3'-O-modified-dTTP (2 µl of 100 µM) and MnCl₂ (1 µl of 40 mM) were added.
4. 1.5 µl of Terminator IX DNA polymerase (15 U, New England BioLabs) was then
15 added.
5. The reaction was incubated for 10 minutes at 37°C.
6. The aliquot (5 µl) was taken out of the reaction mixture and 0.5 µl of natural dNTP
20 mix was added and allowed to react for 10 min. The reaction was analysed by gel electrophoresis.
7. 40 µL of the 500 mM TCEP in 1M TRIS buffer pH=7.4 was added to the reaction mixture and allowed to react for 10 minutes at 37°C.
25
8. The reaction mixture was purified using the protocol outlined in purification steps 1-7.

9. The DNA sample was eluted by 20 µl of NEB Reaction buffer® (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9 @ 25°C) into a clean Eppendorf tube.
- 5 10. 1 µl of Human Endonuclease V (Endo V) NEB (30units/µl) was added into the same tube.
11. Reaction mixture was then gently mixed by resuspension with a pipette, centrifuged at 13,000 rpm for 5 seconds and incubated at 37°C for 1 hour.
- 10 12. After incubation time had elapsed, the reaction was terminated by enzymatic heat inactivation (*i.e.* 65°C for 20mins).
13. The aliquot (5 µl) was taken out of the reaction mixture and analysed on
15 polyacrylamide gel (15%) using TBE buffer and visualized by ChemiDoc MP imaging system (BioRad).
14. Reaction mixture was purified by QIAGEN Nucleotide Removal kit using the protocol outlined in purification steps 1-7.
- 20 15. The DNA sample was eluted by 20 µl of NEB Reaction buffer® (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9 @ 25°C) into a clean Eppendorf tube.
- 25 16. 10 µl of 100 µM strand for ligation (1 nmol) (SEQ ID NO: 57, Figure 28d), were added to the reaction mixture.
17. 40µl of Blunt/TA DNA Ligase NEB (180 units/µl) was added into the same tube.
- 30 18. Reaction mixture was then gently mixed by resuspension with a pipette, centrifuged at 13,000rpm for 5 seconds and incubated at room temperature for 20 mins.

19. Reaction mixture was purified by Streptavidin Magnetic Beads kit using the protocol outlined in purification steps 1-5.

5 20. Unligated oligonucleotide was digested by Lambda Exonuclease.

21. Reaction mixture was purified by QIAGEN Nucleotide Removal kit using the protocol outlined in purification steps 1-7.

10 22. The DNA sample was eluted by 20 µl of NEB Reaction buffer® (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9 @ 25°C) into a clean Eppendorf tube.

2nd cycle:

15

23. 3'-O-modified-dCTP (2 µl of 100 µM) and MnCl₂ (1 µl of 40 mM) were added.

24. 1.5 µl of Terminator IX DNA polymerase (15 U, New England BioLabs) was then added.

20

25. The reaction was incubated for 10 minutes at 37°C.

26. The aliquot (5 µl) was taken out of the reaction mixture and 0.5 µl of natural dNTP mix was added and reacted for 10 min. The reaction was analysed by gel electrophoresis.

25

27. 40 µL of the 500 mM TCEP in 1M TRIS buffer pH=7.4 was added to the reaction mixture and reacted for 10 minutes at 37°C.

28. The reaction mixture was purified using the protocol outlined in purification steps
30 1-7.

29. The DNA sample was eluted by 20 µl of NEB Reaction buffer® (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9 @ 25°C) into a clean Eppendorf tube.
- 5 30. 1 µl of Human Endonuclease V (Endo V) NEB (30units/µl) was added into the same tube.
31. The reaction mixture was then gently mixed by resuspension with a pipette, centrifuged at 13,000 rpm for 5 seconds and incubated at 37°C for 1 hour.
- 10 32. After incubation time had elapsed, the reaction was terminated by enzymatic heat inactivation (*i.e.* 65°C for 20mins).
- 15 33. The aliquot (5 µl) was taken out of the reaction mixture and analysed on polyacrylamide gel (15%) using TBE buffer and visualized by ChemiDoc MP imaging system (BioRad).
34. The reaction mixture was purified using the protocol outlined in purification steps 1-7.
- 20 35. The DNA sample was eluted by 20 µl of NEB Reaction buffer® (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9 @ 25°C) into clean Eppendorf tube.
- 25 36. 10 µl of 100 µM strand for ligation (1 nmol) (SEQ ID NO: 58, Figure 28d), were added to the reaction mixture.
37. 40µl of Blunt/TA DNA Ligase NEB (180 units/µl) was added into the same tube.
- 30 38. Reaction mixture was then gently mixed by resuspension with a pipette, centrifuged at 13,000rpm for 5seconds and incubated at room temperature for 10mins.

39. After incubation time had elapsed, the reaction was terminated with the addition of TBE-Urea sample Buffer (Novex).

5 Gel Electrophoresis and DNA Visualization:

1. 5 µl of reaction mixture was added to 5 µl of TBE-Urea sample buffer (Novex) in a sterile 1.5ml Eppendorf tube and heated to 95°C for 5 mins using a heat ThermoMixer (Eppendorf).

10

2. 5 µl of the sample were then loaded into the wells of a 15% TBE-Urea gel 1.0mm x 10 well (Invitrogen) which contained preheated 1X TBE buffer Thermo Scientific (89mM Tris, 89mM boric acid and 2mM EDTA).

15 3. X-cell sure lock module (Novex) was fastened in place and subjected to electrophoresis by applying the following conditions; 260V, 90 amps for 40 mins at room temperature.

20 4. The gel was visualized by ChemiDoc MP (BioRad) using Cy3 LEDS. Visualization and analysis was carried out on the Image lab 2.0 platform.

Measurement of purified DNA concentration was determined using the protocol below:

25 1. NanoDrop One (Thermo Scientific) was equilibrated by adding 2 µl of sterile distilled water (ELGA VEOLIA) onto the pedestal.

2. After equilibration, the water was gently wiped off using a lint-free lens cleaning tissue (Whatman).

3. NanoDrop One was blanked by adding 2 µl of Buffer EB QIAGEN (10mM Tris.CL, pH 8.5). Then step 2 was repeated after blanking.

4. DNA concentration was measured by adding 2 µl of the sample unto the pedestal
5 and selecting the measure icon on the touch screen.

The sample mixture was purified by QIAGEN Nucleotide Removal kit using the protocol outlined below:

10 1. 500 µl of buffer PNI QIAGEN (5M guanidinium chloride) was added to the sample and mixed by gentle resuspension with a pipette.

2. The mixture was transferred into a QIAquick spin column (QIAGEN) and centrifuged for 1min at 6000 rpm.

15

3. After centrifugation, flow-through was discarded and 750 µl of buffer PE QIAGEN (10mM Tris-HCl pH 7.5 and 80% ethanol) was added into the spin column and centrifuged for 1min at 6000 rpm.

20 4. The flow-through was discarded and the spin column was centrifuged for an additional 1min at 13000 rpm to remove residual PE buffer.

5. The spin column was then placed in a sterile 1.5ml Eppendorf tube.

25 6. For DNA elution, 20 µl of appropriate buffer for the reaction was added to the centre of the column membrane and left to stand for 1min at room temperature.

7. The tube was then centrifuged at 13000 rpm for 1min.

After the ligation step, the sample mixture was purified using Streptavidin Magnetic Beads via the protocol outlined below:

1. 100 μ l of Streptavidin Magnetic Beads (New England BioLabs) were washed 3 times by 200 μ l of binding buffer (20mM TRIS, 500 mM NaCl, pH = 7.4).
2. Reaction mixture after ligation step is mixed with 10 volumes of binding buffer (20mM TRIS, 500 mM NaCl, pH = 7.4) and incubated with Streptavidin Magnetic Beads for 15 minutes at 20 °C.
3. Streptavidin Magnetic Beads were washed 3 times by 200 μ l of binding buffer (20mM TRIS, 500 mM NaCl, pH = 7.4).
4. Streptavidin Magnetic Beads were washed 3 times by deionized water.
5. The oligonucleotides were eluted by 40 μ l of deionized water by heating to 95°C for 3 minutes.

The results shown in Figure 28c demonstrate the performance two complete synthesis cycles using an exemplary method of the invention.

Example 9. Version 2 Chemistry - Complete Three-Cycle Experiment on Single-Hairpin Model.

This example describes a complete three-cycle experiment for the synthesis of polynucleotides using 5 steps on a double-hairpin model: incorporation of 3'-O-modified dNTPs from the nick site, deprotection, cleavage, ligation and denaturation step with the first step taking place opposite a complementary base.

Exemplary schematic overviews of the method are shown in Figures 33, 34 and 35.

The method starts by the controlled addition of a 3'-*O*-protected single nucleotide to an oligonucleotide by enzymatic incorporation by DNA polymerase followed by deprotection, cleavage, ligation, and denaturation of the helper strand. Figure 33 shows the 1st full cycle involving enzymatic incorporation, deprotection, cleavage, ligation and denaturation steps. In this example the oligonucleotide is extended by T nucleotide. Figure 34 shows the 2nd full cycle following the 1st cycle involving enzymatic incorporation, deprotection, cleavage, ligation steps, and denaturation steps. In this example the oligonucleotide is extended by T nucleotide. Figure 35 shows the 3rd full cycle following the 2nd cycle involving enzymatic incorporation, deprotection, cleavage, ligation, and denaturation steps. In this example the oligonucleotide is extended by T nucleotide.

Materials and Methods

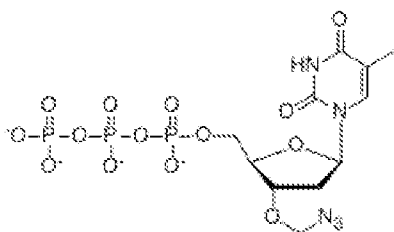
Materials

1. 3'-*O*-modified dNTPs were synthesised in-house according to the protocol described in PhD thesis Jian Wu: Molecular Engineering of Novel Nucleotide Analogues for DNA Sequencing by Synthesis, Columbia University, 2008. The protocol for synthesis is also described in the patent application publication: J. William Efcavitch, Julieta E. Sylvester, Modified Template-Independent Enzymes for Polydeoxynucleotide Synthesis, Molecular Assemblies US2016/0108382A1.

2. Oligonucleotides were designed in house and obtained from Integrated DNA Technologies, Sigma-Aldrich (Figure 36). The stock solutions are prepared in concentration of 100 μ M.

3. Terminator X DNA polymerase was used that has been engineered by New England BioLabs with enhanced ability to incorporate 3-*O*-modified dNTPs. Any DNA polymerase or other enzyme that could incorporate modified dNTPs could alternatively be used.

3'-*O*-azidomethyl-dTTP was used for incorporation:



5

Method

1st cycle:

- 10 1. 20 μ l of 10x Thermopol® buffer (20 mM Tris-HCl, 10 mM (NH₄)₂SO₄, 10 mM KCl, 2 mM MgSO₄, 0.1% Triton® X-100, pH 8.8, New England BioLabs) and MnCl₂ solution (10 μ l of 40 mM) were mixed with 139 μ l of sterile deionized water (ELGA VEOLIA) in 1.5ml Eppendorf tube.
- 15 2. 20 μ l of 100 μ M single hairpin model oligonucleotide (2 nmol, 1 equiv) (SEQ ID NO: 59, Figure 36) was added to the reaction mixture.
3. The aliquot (4 μ l) was taken out of the reaction mixture and 0.5 μ l of natural dNTP mix (4mM) and 0.5 μ l of Bst DNA polymerase and 0.5 μ l of Sulfolobus DNA polymerase
20 IV were added and allowed to react for 10 min. The reaction was analysed by gel electrophoresis.
4. 3'-*O*-modified-dTTP (10 μ l of 2 mM) was added.
- 25 5. 5 μ l of Terminator X DNA polymerase (50 U, New England BioLabs) was then added. However, any DNA polymerase or other enzyme that could incorporate modified dNTPs could be used.

6. The reaction was incubated for 30 minutes at 37°C.
7. The reaction mixture was purified using QIAGEN Nucleotide Removal kit outlined
5 in purification steps 66-72.
8. The DNA sample was eluted by 200 µl of TE buffer into a clean Eppendorf tube.
9. The aliquot (4 µl) was taken out of the reaction mixture and 0.5 µl of natural dNTP
10 mix (4mM) and 0.5 µl of Bst DNA polymerase and 0.5 µl of Sulfolobus DNA polymerase
IV were added and allowed to react for 10 min. The reaction was analysed by gel
electrophoresis.
10. 400 µL of the 500 mM TCEP was added to the reaction mixture and allowed to react
15 for 10 minutes at 37°C.
11. The reaction mixture was purified using QIAGEN Nucleotide Removal kit outlined
in purification steps 66-72.
12. The DNA sample was eluted by 150 µl of NEB Reaction buffer® (50 mM potassium
20 acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9 @ 25°C) into
clean Eppendorf tube.
13. The aliquot (4 µl) was taken out of the reaction mixture and 0.5 µl of natural dNTP
mix (4mM) and 0.5 µl of Bst DNA polymerase and 0.5 µl of Sulfolobus DNA polymerase
25 IV were added and allowed to react for 10 min. The reaction was analysed by gel
electrophoresis.
14. 5 µl of Human Endonuclease V (Endo V) NEB (30units/µl) was added to the eluate
and incubated at 37°C for 30 minutes. Any suitable alternative endonuclease could be used.
30

15. After incubation time had elapsed, the reaction was terminated by enzymatic heat inactivation at 65°C for 20mins.
16. An aliquot (5 µl) was taken out of the reaction mixture and analysed on a
5 polyacrylamide gel.
17. The reaction mixture was purified by QIAGEN Nucleotide Removal kit using the protocol outlined in purification steps 66-72.
- 10 18. The DNA sample was eluted by 100 µl of T3 DNA ligase buffer (2x concentrate) into a clean Eppendorf tube.
19. 20 µl of 100 µM inosine strand for ligation (2 nmol) and 20 µl of 100 µM helper strand for ligation (2 nmol) (SEQ ID NO: 60, 51, Figure 36), and 40 µl of water were added
15 to the reaction mixture.
20. 20 µl of T3 DNA Ligase NEB (3000 units/µl) was added into the same tube (this could include any DNA ligating enzyme) and incubated at room temperature for 30 mins.
- 20 The reaction mixture was purified using the protocol for Streptavidin Magnetic Beads kit including the denaturation step outlined in purification steps 73-78.
21. The reaction mixture was purified using the protocol for QIAGEN Nucleotide Removal kit outlined in purification steps 66-72.
- 25 22. The DNA sample was eluted by 100 µl of TE buffer into a clean Eppendorf tube.

2nd cycle:

23. 15 µl of 10x Thermopol® buffer (20 mM Tris-HCl, 10 mM (NH₄)₂SO₄, 10 mM KCl, 2 mM MgSO₄, 0.1% Triton® X-100, pH 8.8, New England BioLabs), MnCl₂ solution (7.5
5 µl of 40 mM) and 19 µl of deionized water was added.

24. An aliquot (4 µl) was taken out of the reaction mixture and 0.5 µl of natural dNTP mix (4mM) and 0.5 µl of Bst DNA polymerase and 0.5 µl of Sulfolobus DNA polymerase IV were added and allowed to react for 10 min. The reaction was analysed by gel
10 electrophoresis.

25. 3'-O-modified-dTTP (7.5 µl of 2 mM) was added.

26. 5 µl of Terminator X DNA polymerase (50 U, New England BioLabs) was then
15 added. Any DNA polymerase that could incorporate modified dNTPs could be used.

27. The reaction was incubated for 30 minutes at 37°C.

28. The reaction mixture was purified using QIAGEN Nucleotide Removal kit outlined
20 in purification steps 66-72.

29. The DNA sample was eluted by 100 µl of TE buffer into a clean Eppendorf tube.

30. An aliquot (4 µl) was taken out of the reaction mixture and 0.5 µl of natural dNTP
25 mix (4mM) and 0.5 µl of Bst DNA polymerase and 0.5 µl of Sulfolobus DNA polymerase IV were added and allowed to react for 10 min. The reaction was analysed by gel electrophoresis.

31. 200 µL of the 500 mM TCEP was added to the reaction mixture and allowed to react
30 for 10 minutes at 37°C.

32. The reaction mixture was purified using QIAGEN Nucleotide Removal kit outlined in purification steps 66-72.

33. The DNA sample was eluted by 100 µl of NEB Reaction buffer® (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9 @ 25°C) into
5 a clean Eppendorf tube.

34. The aliquot (4 µl) was taken out of the reaction mixture and 0.5 µl of natural dNTP mix (4mM) and 0.5 µl of Bst DNA polymerase and 0.5 µl of Sulfolobus DNA polymerase IV were added and allowed to react for 10 min. The reaction was analysed by gel
10 electrophoresis.

35. 5 µl of Human Endonuclease V (Endo V) NEB (30units/µl) was added to the eluate. and incubated at 37°C for 30 minutes. Any suitable alternative endonuclease could be used.

15 36. After incubation time had elapsed, the reaction was terminated by enzymatic heat inactivation at 65°C for 20mins.

37. The aliquot (5 µl) was taken out of the reaction mixture and analysed on a polyacrylamide gel.
20

38. The reaction mixture was purified by QIAGEN Nucleotide Removal kit using the protocol outlined in purification steps 66-72.

39. The DNA sample was eluted by 60 µl of T3 DNA ligase buffer (2x concentrate) into
25 a clean Eppendorf tube.

40. 20 µl of 100 µM inosine strand for ligation (2 nmol) and 20 µl of 100 µM helper strand for ligation (2 nmol) (SEQ ID NO: 60, 51, Figure 36), and 10 µl of deionized water were added to the reaction mixture.
30

41. 10 µl of T3 DNA Ligase NEB (3000 units/µl) was added into the same tube and incubated at room temperature for 30 mins. Any suitable DNA ligase could be used.

42. The reaction mixture was purified using the protocol for Streptavidin Magnetic
5 Beads kit including denaturation step outlined in purification steps 73-78.

43. The reaction mixture was purified using the protocol for QIAGEN Nucleotide Removal kit outlined in purification steps 66-72.

10 44. The DNA sample was eluted by 46 µl of TE buffer into a clean Eppendorf tube.

3rd cycle:

45. 6 µl of 10x Thermopol® buffer (20 mM Tris-HCl, 10 mM (NH₄)₂SO₄, 10 mM KCl,
15 2 mM MgSO₄, 0.1% Triton® X-100, pH 8.8, New England BioLabs), MnCl₂ solution (3 µl of 40 mM) was added.

46. An aliquot (4 µl) was taken out of the reaction mixture and 0.5 µl of natural dNTP mix (4mM) and 0.5 µl of Bst DNA polymerase and 0.5 µl of Sulfolobus DNA polymerase
20 IV were added and allowed to react for 10 min. The reaction was analysed by gel electrophoresis.

47. 3'-O-modified-dTTP (6 µl of 200 µM) was added.

25 48. 3 µl of Terminator X DNA polymerase (30 U, New England BioLabs) was then added. Any DNA polymerase or other suitable enzyme that could incorporate modified dNTPs could be used.

49. The reaction was incubated for 30 minutes at 37°C.

30

50. The reaction mixture was purified using QIAGEN Nucleotide Removal kit outlined in purification steps 66-72.

51. The DNA sample was eluted by 50 µl of TE buffer into a clean Eppendorf tube.

5

52. The aliquot (4 µl) was taken out of the reaction mixture and 0.5 µl of natural dNTP mix (4mM) and 0.5 µl of Bst DNA polymerase and 0.5 µl of Sulfolobus DNA polymerase IV were added and allowed to react for 10 min. The reaction was analysed by gel electrophoresis.

10

53. 100 µL of the 500 mM TCEP was added to the reaction mixture and allowed to react for 10 minutes at 37°C.

54. The reaction mixture was purified using QIAGEN Nucleotide Removal kit outlined in purification steps 66-72.

15

55. The DNA sample was eluted by 49 µl of NEB Reaction buffer® (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9 @ 25°C) into a clean Eppendorf tube.

20

56. An aliquot (4 µl) was taken out of the reaction mixture and 0.5 µl of natural dNTP mix (4mM) and 0.5 µl of Bst DNA polymerase and 0.5 µl of Sulfolobus DNA polymerase IV were added and allowed to react for 10 min. The reaction was analysed by gel electrophoresis.

25

57. 5 µl of Human Endonuclease V (Endo V) NEB (30units/µl) was added to the eluate and incubated at 37°C for 30 minutes. Any suitable endonuclease could alternatively be used.

58. After incubation time had elapsed, the reaction was terminated by enzymatic heat inactivation at 65°C for 20mins.

30

59. The aliquot (5 µl) was taken out of the reaction mixture and analysed on a polyacrylamide gel.

5 60. The reaction mixture was purified by QIAGEN Nucleotide Removal kit using the protocol outlined in purification steps 66-72.

61. The DNA sample was eluted by 30 µl of T3 DNA ligase buffer (2x concentrate) into a clean Eppendorf tube.

10 62. 10 µl of 100 µM inosine strand for ligation (2 nmol), 10 µl of 100 µM helper strand for ligation (2 nmol) (SEQ ID NO: 60, 51, Figure 36) and 5 µl of water were added to the reaction mixture.

63. 5 µl of T3 DNA Ligase NEB (3000 units/µl) was added into the same tube. (This could include any DNA ligating enzyme) and incubated at room temperature for 30 mins.

15

64. The reaction mixture was analysed by gel electrophoresis.

Purification of the reaction mixture by QIAGEN Nucleotide Removal kit after incorporation, deblock and cleavage steps using the protocol outlined below:

20

65. 10 volumes of buffer PNI QIAGEN (5M guanidinium chloride) was added to the sample and mixed by gentle resuspension with a pipette.

66. The mixture was transferred into a QIAquick spin column (QIAGEN) and
25 centrifuged for 1min at 6000 rpm.

67. After centrifugation, flow-through was discarded and 750 µl of buffer PE QIAGEN (10mM Tris-HCl pH 7.5 and 80% ethanol) was added into the spin column and centrifuged for 1min at 6000 rpm.

30

68. The flow-through was discarded and the spin column was centrifuged for an additional 1min at 13000 rpm to remove residual PE buffer.

69. The spin column was then placed in a sterile 1.5ml Eppendorf tube.

5

70. For DNA elution, 20-200 µl of appropriate buffer for the reaction was added to the centre of the column membrane and left to stand for 1 min at room temperature.

71. The tube was then centrifuged at 13000 rpm for 1min.

10

Purification of the reaction after the ligation step using Streptavidin Magnetic Beads involving denaturation step was performed via the protocol outlined below:

72. 100 µl of Streptavidin Magnetic Beads (New England BioLabs) were washed 3 times
15 by 200 µl of binding buffer (20mM TRIS, 500 mM NaCl, pH = 7.4).

73. Reaction mixture after ligation step is mixed with 10 volumes of binding buffer (20mM TRIS, 500 mM NaCl, pH = 7.4) and allowed to incubate with Streptavidin Magnetic Beads for 15 minutes at 20 °C.

20

74. Streptavidin Magnetic Beads were washed 3 times by 200 µl of binding buffer (20mM TRIS, 500 mM NaCl, pH = 7.4).

75. To remove the helper strand, Streptavidin Magnetic Beads were heated to 80°C in
25 200 µl of binding buffer (20mM TRIS, 500 mM NaCl, pH = 7.4), placed to magnet and supernatant was quickly discarded.

76. Streptavidin Magnetic Beads were washed 3 times with deionized water.

30 77. The oligonucleotides were eluted by 50-100 µl of deionized water by heating to 95°C for 3 minutes.

Results and conclusion

Figure 37 depicts a gel showing reaction products corresponding to a full three-cycle experiment comprising: incorporation, deblock, cleavage and ligation steps. The results shown demonstrate the performance of three complete synthesis cycles using an exemplary method of the invention.

Example 10. Derivatization of a polyacrylamide surface and subsequent immobilisation of molecules.

This example describes the presentation of bromoacetyl groups on a polyacrylamide surface using N-(5- bromoacetamidylpentyl) acrylamide (BRAPA) and the subsequent surface immobilisation of thiolated molecules by their covalent coupling to bromoacetyl groups.

Materials and Methods

Glass microscope slides and coverslips were cleaned by ultrasonication in acetone, ethanol and water sequentially for 10 mins each and dried with Argon. Clean glass coverslips were silanised with Trichloro(1H,1H,2H,2H-perfluorooctyl)silane in vapor phase in a polystyrene petri dish, sonicated twice in ethanol and dried with Ar ('fluorinated coverslips' hereafter). On glass microscope slides, 4% acrylamide/N,N'-Methylenebisacrylamide (19:1) solution was mixed with 100 µl of 10% (w/v) ammonium persulphate (APS), 10 µl of tetramethylethylenediamine (TEMED) spiked with N-(5-bromoacetamidylpentyl) acrylamide (BRAPA) at 0, 0.1, 0.2, and 0.3% (w/v) and quickly dispensed into a 4 mm diameter rubber gasket and subsequently sandwiched with a fluorinated coverslip with the fluorinated side facing towards the acrylamide solution and polymerised for 10 mins. After 10 mins, the surfaces were immersed in deionised water

and left immersed for a total of 4 hrs, during which time the fluorinated coverslips were carefully removed. The polymerised polyacrylamide surfaces were dried with Argon.

The polyacrylamide surfaces were subsequently exposed to thiolated polyethylene glycol (1kDa) fluorescein (FITC-PEG-SH), and carboxylated polyethylene glycol (1 kDa) fluorescein (FITC-PEG-COOH) as a negative control in sodium phosphate buffer (10mM, pH 8) for 1 hr and subsequently washed sequentially with sodium phosphate buffer (10mM, pH 7) and the same buffer containing 0.05% Tween20/0.5M NaCl to eliminate non-specifically adsorbed thiolated and carboxylated fluorophores. The surfaces were subsequently imaged by ChemiDoc (Bio-Rad) in the fluorescein channel.

Results and conclusion

Figure 38 shows fluorescence signals and Figure 39 shown measured fluorescence from polyacrylamide gel surfaces spiked with different amount of BRAPA exposed to FITC-PEG-SH and FITC-PEG-COOH. Immobilisation of fluorescein was only successful with polyacrylamide surfaces that were spiked with BRAPA and solely with thiolated fluorescein, with close to zero non-specific adsorption of the carboxylated fluorescein.

Significantly high positive fluorescence signals were obtained from polyacrylamide surfaces containing BRAPA (BRAPA 0.1, 0.2 and 0.3%) and only from thiolated molecules (FITC-PEG-SH) compared to those polyacrylamide surfaces without BRAPA (BRAPA 0%) and those polyacrylamide surfaces containing BRAPA and carboxylated molecules (FITC-PEG-COOH). The results indicate that specific covalent coupling has occurred between the bromoacetyl moiety from the surface and the thiol moiety from the fluorescein tagged molecules.

The results demonstrate that molecules, such as a molecule comprising a support strand and a synthesis strand for use in the methods of the present invention, can readily be

immobilised on a surface substrate compatible with the polynucleotide synthesis reactions described herein.

5 **Example 11. Surface immobilisation of hairpin DNA oligomers and subsequent incorporation of fluorescently labelled deoxynucleoside triphosphates.**

This example describes:

- (1) a method of presenting bromoacetyl groups on a thin polyacrylamide surface;
- 10 (2) the subsequent immobilisation of hairpin DNA via covalent coupling of thiophosphate functionalised hairpin DNA with or without a linker; and
- (3) the incorporation of 2'-deoxynucleotide triphosphate (dNTP) into hairpin DNA.

The method is compatible with virtually any type of material surface (*e.g.* metals, polymers
15 etc).

(1): Fabrication of a bromoacetyl functionalised thin polyacrylamide surface

Materials and Methods

20

Glass microscope slides were first cleaned by ultrasonication in neat Decon 90 (30 mins), water (30 mins), 1M NaOH (15 mins), water (30 mins), 0.1M HCl (15 mins), water (30 mins) and finally dried with Argon.

25 2% (w/v) acrylamide monomer solution was first made by dissolving 1 g of acrylamide monomer in 50 ml of water. The acrylamide monomer solution was vortexed and degassed in argon for 15 mins. N-(5-bromoacetamidylpentyl) acrylamide (BRAPA, 82.5 mg) was dissolved in 825 µl of DMF and added to the acrylamide monomer solution and vortexed further. Finally, 1 ml of 5% (w/v) potassium persulphate (KPS) and 115 µl of
30 neat tetramethylethylenediamine (TEMED) were added to the acrylamide solution,

vortexed and the clean glass microscope slides were exposed to this acrylamide polymerisation mixture for 90 mins. After 90 mins, the surfaces were washed with deionised water and dried with argon. These surfaces will be referred to as 'BRAPA modified surfaces' in this example hereafter. As a negative control, polyacrylamide surfaces without BRAPA was also made in a similar manner as described above by excluding the addition of BRAPA solution into the acrylamide monomer solution. These surfaces will be referred to as 'BRAPA control surface' in this example hereafter.

(2): Covalent coupling of thiophosphate functionalised hairpin DNA onto polyacrylamide surfaces

Materials and Methods

Rubber gaskets with a 4 mm diameter circular opening were placed and secured onto BRAPA modified and BRAPA control surfaces. The surfaces were first primed with sodium phosphate buffer (10 mM, pH 7) for 10 mins. The buffer was subsequently removed and the surfaces were exposed to 5'-fluorescently labelled (Alexa 647) hairpin DNA oligomers with and without a linker modified with six and single thiophosphates respectively at a 1 μ M concentration and incubated for 1 hr in the dark. BRAPA modified surfaces were also incubated with DNA oligomers with and without linker but without thiophosphates as a control (referred to 'oligomer control surfaces' in this example hereafter). After incubation, the surfaces were rinsed in sodium phosphate (100 mM, pH 7) followed by Tris-EDTA buffer (10mM Tris, 10mM EDTA, pH 8) and finally with water. To remove any non-specifically adsorbed DNA oligomers, the surfaces were subsequently washed with water containing 1M sodium chloride and 0.05% (v/v) Tween20, washed with water and dried with argon. The surfaces were scanned on ChemiDoc imager in the Alexa 647 channel.

Figure 40a shows the sequences of hairpin DNA without a linker immobilised on different samples. Figure 40b shows the sequences of hairpin DNA with a linker immobilised on different samples.

5 Results

Results are shown in Figures 41 and 42. Figure 41 shows fluorescence signals originating from hairpin DNA oligomers with and without a linker immobilised onto bromoacetyl functionalised polyacrylamide surfaces, but not from BRAPA or oligomer controls.

10

Figure 42 shows measured fluorescence intensity following DNA immobilisation on polyacrylamide surface. The Figure shows the surface fluorescence signals obtained from various polyacrylamide surfaces and shows that significantly higher signals were obtained from hairpin DNA oligomers immobilised onto BRAPA modified surfaces compared to
15 BRAPA and oligomer control surfaces (as described in (2)), due to successful covalent immobilisation of DNA onto bromoacetyl functionalised polyacrylamide surfaces.

Conclusion

20 Fluorescence signals from DNA were only prominently present from BRAPA modified surfaces that were spiked with BRAPA, indicative of successful covalent coupling of DNA onto the surface via the thiophosphate functionality. Homogenous and higher signals were obtained from DNA with the linker compared to DNA without the linker.

25 **(3): Incorporation of triphosphates into hairpin DNA oligomer with a linker**

Materials and Methods

Rubber gaskets with a 9 mm diameter circular opening were placed on the BRAPA
30 modified surfaces immobilised with the DNA oligomer with the linker and primed with

incorporation buffer (50 mM TRIS pH 8, 1 mM EDTA, 6 mM MgSO₄, 0.05% tween20 and 2 mM MnCl₂) for 10 mins. The surfaces were subsequently exposed to incorporation buffer containing DNA polymerase (0.5U/μl Terminator X DNA polymerase) and triphosphates (20 μM Alexa 488 labelled dUTP) and incubated for 1 hr (referred to as
5 'polymerase surface' in this example hereafter). Additional set of surfaces were also exposed to incorporation buffer without Terminator X DNA polymerase for 1 hr as a negative control (referred to as 'negative surface' in this example hereafter). After 1 hr, both types of sample were washed in water, subsequently exposed to water containing 1M sodium chloride and 0.05% (v/v) Tween20, and washed again with water. Fluorescence
10 signals from the surfaces were measured using ChemiDoc in the Alexa 647 and Alexa 488 channels to monitor both the presence of hairpin DNA (Alexa 647) and incorporation of dUTP (Alexa 488).

Results

15

Figure 43 shows fluorescence signals detected from Alexa 647 and Alexa 488 channels before and after incorporation of Alexa 488-labelled dUTP. Unchanged positive signals from Alexa 647 before and after incorporation indicates that the surface immobilised hairpin DNA is stable during the incorporation reaction, while positive signals from Alexa
20 488 were only observed from the polymerase surfaces after incorporation reaction showing the successful incorporation of dUTPs only with the presence of polymerase.

Figure 44 shows measured fluorescence signals in the Alexa 647 (hairpin DNA) and Alexa 488 (dUTP) channels obtained from 'polymerase surfaces' and 'negative surfaces' before
25 and after incorporation of Alexa 488-labelled dUTP as described in (3). A significant increase in the Alexa 488 fluorescence signals was obtained after the incorporation reaction from the polymerase surface as a result of the successful incorporation, while the signals from negative surfaces remained the same after the incorporation reaction due to the absence of polymerase. Fluorescence signals in the Alexa 647 channel remained
30 virtually unchanged after the incorporation reaction, indicating the presence of hairpin

DNA on the surface. The slight reduction in the fluorescence signal maybe attributed to the effect of photo-bleaching due to the second round of light exposure.

Conclusion

5

The results demonstrate that a molecule comprising a support strand and a synthesis strand for use in the methods of the present invention, can readily be immobilised on a surface substrate compatible with the polynucleotide synthesis reactions described herein. The results further demonstrate that such a molecule can accept the incorporation of a new dNTP so as to extend the synthesis strand, whilst at the same time the molecule remains stable and attached to the substrate.

10

Example 12. Cleavage and ligation of hairpin DNA oligomers immobilised to derivatized surfaces via a linker and thiophosphate covalent linkage.

15

This example describes the covalent coupling to derivatized surfaces of thiophosphate functionalised hairpin DNA with a linker, followed by cleavage and ligation reactions. The substrate preparation and coupling of hairpin DNA was carried out as described in Example 11.

20

(1): Cleavage of immobilised hairpin DNA oligomers with a linker

Materials and Methods

25

Hairpin DNA was immobilised on surface BRAPA modified surfaces as described in Example 11. Four sets of triplicate surfaces including all the experimental controls for cleavage and ligation reactions were prepared. The experimental conditions are described in Figure 45a. Figure 45b shows the sequences of hairpin DNA immobilised on different samples.

30

After the DNA immobilisation step, rubber gaskets with a 9 mm diameter circular opening were placed on all surfaces that were immobilised with DNA labelled with Alexa 647 at the 5' end and primed with 1X NEBuffer 4 (50 mM Potassium Acetate, 20 mM Tris-acetate, 10 mM Magnesium Acetate, 1 mM DTT, pH 7.9) for 10 mins. Note that for sample D, the immobilised hairpin DNA does not contain inosine and inosine is replaced by guanine. All the samples were subsequently exposed to either NEBuffer 4 containing 1.5 U/ μ l Endonuclease V (sample A, B and D) or NEBuffer 4 without Endonuclease V (sample C) for 1 hr. All the samples were subsequently washed with 1X T3 DNA Ligase buffer (66 mM Tris-HCl, 10mM MgCl₂, 1 mM ATP, 7.5% PEG6000, 1 mM DTT, pH 7.6), 1X T3 DNA Ligase buffer containing 1M sodium chloride and 0.05% (v/v) Tween20, washed again with 1X T3 DNA Ligase buffer and scanned on ChemiDoc Imager in the Alexa 647 channel.

Results

Figure 46 shows fluorescence signals from hairpin DNA oligomers before and after cleavage reactions.

Figure 47 shows measured fluorescence signals before and after cleavage reactions obtained from DNA immobilised surfaces as described above. Successful cleavage reactions were only observed from samples A and B, while fluorescence signal intensities remained almost the same for samples C and D due to absence of either Endonuclease V (sample C) or inosine in the sequence (sample D).

Significant reductions in the fluorescence signals were observed from samples A and B as a result of successful cleavage reactions at the inosine site within the DNA strand with the presence of Endonuclease V. For samples C and D, absence of Endonuclease V and lack of inosine in the DNA respectively resulted in the fluorescence signals to remain almost the same level as the initial signals obtained after DNA immobilisation.

(2): Ligation reactions

Materials and Methods

5 After the cleavage reaction as described in (1), samples A and B (as described in Figure 45a) were exposed to 1X T3 DNA Ligase buffer containing MnCl_2 (2 mM), inosine strands labelled with Alexa 647 at the 5' end (16 μM) and complimentary 'helper' strands (16 μM) (the sequences are shown in Figure 48 below) with T3 DNA ligase (250 U/ μl) for sample A, and without T3 DNA Ligase as a negative control for sample B. Samples were
10 incubated in the respective solutions for 1 hr. After 1 hr, the surfaces were washed in water, subsequently exposed to water containing 1M sodium chloride and 0.05% (v/v) Tween20, and washed again with water. Fluorescence signals from the surfaces were measured using ChemiDoc in the Alexa 647 channels. Figure 48 shows the sequences for the inosine-containing strand and the complimentary 'helper' strand for ligation reactions.

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Results

Figure 49 shows results relating to the monitoring of ligation reactions. Fluorescence signals detected from Alexa 647 channel before and after ligation reactions. An increase in
20 fluorescence signals in the Alexa 647 channels after ligation were only obtained from sample A with T3 DNA ligase, while fluorescence signals remained at the same level after ligation reaction for sample B due to the absence of T3 DNA ligase.

Figure 50 shows that a significant increase in the Alexa 647 fluorescence signal was
25 obtained after ligation reaction from sample A as a result of the successful ligation, where the signal level recovers to the initial signal level after DNA immobilisation and prior to cleavage reaction as shown in Figure 47. The fluorescence signals from the sample B remained the same after the ligation reaction due to the absence of T3 DNA ligase.

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Conclusion

The results in this Example demonstrate that a molecule comprising a support strand and a synthesis strand for use in the methods of the present invention, can readily be immobilised on a surface substrate compatible with the polynucleotide synthesis reactions described herein and can be subjected to cleavage and ligation reactions whilst at the same time remaining stable and attached to the substrate.

Example 13. Incorporation of 3'-O-azidomethyl-dNTPs to the 3' terminal end of blunt-ended DNA.

This example describes the incorporation of 3'-O-azidomethyl-dNTPs to the 3' end of blunt-ended double-stranded DNA.

The steps below demonstrate the controlled addition of a 3'-O-protected single nucleotide to a blunt-ended double-stranded oligonucleotide by enzymatic incorporation by DNA polymerase. The steps are in accordance with incorporation step 3 as shown in each of Figures 1 and 2 (103, 108, 203, 208).

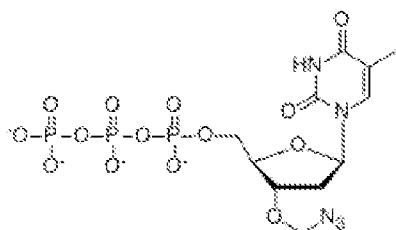
Materials and Methods

Materials

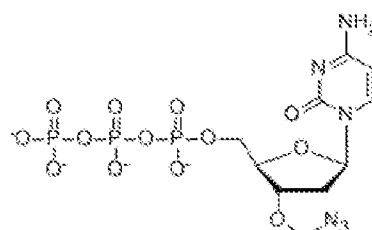
1. In-house synthesized 3'-O-azidomethyl-dNTPs.
2. Terminator X DNA polymerase that has been engineered by New England Biolabs to possess enhanced ability to incorporate 3-O-modified dNTPs.
3. Blunt-ended double-stranded DNA oligonucleotide.

Four types of reversible terminators were tested:

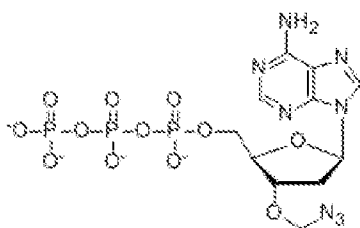
3'-*O*-azidomethyl-dTTP:



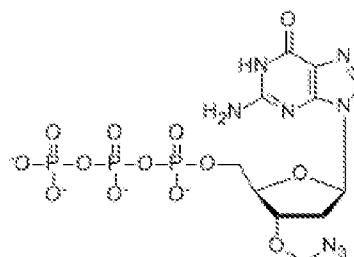
3'-*O*-azidomethyl-dCTP:



5 3'-*O*-azidomethyl-dATP:



3'-*O*-azidomethyl-dGTP:



Method

- 10 1. 5 μ l of 10x Thermopol® buffer (20 mM Tris-HCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 10 mM KCl, 2 mM MgSO_4 , 0.1% Triton® X-100, pH 8.8, New England Biolabs) was mixed with 33.5 μ l of sterile deionized water (ELGA VEOLIA) in a 1.5ml Eppendorf tube.
2. 2 μ l of 20 μ M primer (40 pmol, 1 equiv) (SEQ ID: NO: 68, Figure 51a) and 3 μ l of
15 20 μ M template (60 pmol, 1.5equiv) (SEQ ID: NO: 69, Figure 51a) were added to the reaction mixture.
3. 3'-*O*-modified-dTTP (2 μ l of 100 μ M) and MnCl_2 (2.5 μ l of 40 mM) were added.
- 20 4. 2 μ l of Therminator X DNA polymerase (20 U, New England BioLabs) was then added.

5. The reaction was incubated for 30 minutes at 37°C.
6. The reaction was stopped by addition of TBE-Urea sample buffer (Novex).
7. The reaction was separated on polyacrylamide gel (15%) TBE buffer and visualized by ChemiDoc MP imaging system (BioRad).

Results

Figure 51b depicts a gel showing results of incorporation of 3'-*O*-modified-dNTPs by Terminator X DNA polymerase in the presence of Mn²⁺ ions at 37°C. The data show that Terminator X DNA polymerase was successfully able to incorporate 3'-*O*-modified-dNTPs to the 3' terminal end of the blunt ended DNA oligonucleotide to create a single base overhang.

In the above Examples, all oligonucleotides presented in SEQ ID NOS 1-69 have a hydroxyl group at the 3' terminus. All oligonucleotides presented in SEQ ID NOS 1-69 lack a phosphate group at the 5' terminus except for SEQ ID NO 7, SEQ ID NO 18 and SEQ ID NO 35.

It is to be understood that different applications of the disclosed methods and products may be tailored to the specific needs in the art. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments of the invention only, and is not intended to be limiting.

As used in this specification and the appended claims, the singular forms "a", "an", and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to "a ligation polynucleotide" includes two or more such polynucleotides, reference to "a scaffold polynucleotide" includes two or more such scaffold polynucleotides, and the like.

All publications, patents and patent applications cited herein are hereby incorporated by reference in their entirety.

CLAIMS

1. An *in vitro* method of synthesising a double-stranded polynucleotide having a predefined sequence, the method comprising performing cycles of synthesis wherein each cycle comprises cleaving a double-stranded scaffold polynucleotide and extending the cleaved double-stranded scaffold polynucleotide by incorporating a nucleotide pair, wherein a terminal end of a first strand of the cleaved double-stranded scaffold polynucleotide is extended by the addition of a nucleotide of the predefined sequence and a terminal end of the second strand of the cleaved double-stranded scaffold polynucleotide which is hybridized to the first strand is extended by the addition of a partner nucleotide thereby forming a nucleotide pair with the incorporated nucleotide of the first strand, wherein in each cycle the nucleotides are incorporated into the cleaved scaffold polynucleotide, and wherein each cycle comprises ligating a ligation polynucleotide to the cleaved scaffold polynucleotide, the ligation polynucleotide comprising a partner nucleotide for the nucleotide of the predefined sequence, wherein upon ligation the nucleotide of the predefined sequence pairs with the partner nucleotide.
2. The method according to claim 1, wherein each cycle comprises extending the first strand by adding the nucleotide of the predefined sequence together with an attached reversible blocking group followed by extending the second strand, wherein the reversible blocking group is removed before or after the second strand is extended.
3. The method according to claim 1 or claim 2, wherein each cycle comprises:
 - (1) providing a scaffold polynucleotide;
 - (2) cleaving the scaffold polynucleotide at a cleavage site;
 - (3) adding to the cleaved scaffold polynucleotide by the action of a nucleotide transferase or polymerase enzyme a nucleotide of the predefined sequence, the

nucleotide comprising a reversible terminator group which prevents further extension by the enzyme;

- (4) removing the reversible terminator group from the nucleotide of the predefined sequence; and
- (5) ligating a ligation polynucleotide to the cleaved scaffold polynucleotide, the ligation polynucleotide comprising a partner nucleotide for the nucleotide of the predefined sequence, wherein upon ligation the nucleotide of the predefined sequence pairs with the partner nucleotide.

4. The method according to claim 3, wherein step (1) comprises providing a scaffold polynucleotide comprising a synthesis strand and a support strand hybridized thereto, wherein the synthesis strand comprises a primer strand portion, and the support strand comprises a universal nucleotide; wherein step (2) comprises cleaving the scaffold polynucleotide at a cleavage site, the site defined by a sequence comprising the universal nucleotide in the support strand, wherein cleavage comprises cleaving the support strand and removing the universal nucleotide from the scaffold polynucleotide; and wherein in step (5) the ligation polynucleotide comprises a support strand comprising the partner nucleotide and a universal nucleotide which defines a cleavage site for use in the next cycle, and wherein the ligation polynucleotide is ligated to the support strand of the cleaved scaffold polynucleotide thereby forming the nucleotide pair.

5. The method according to claim 3 or claim 4, the method comprising:

- (1) providing a scaffold polynucleotide comprising a synthesis strand and a support strand hybridized thereto, wherein 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) cleaving the scaffold polynucleotide at a cleavage site, the site defined by a sequence comprising the universal nucleotide in the support strand, wherein cleavage comprises cleaving the support strand and removing the universal nucleotide from the scaffold polynucleotide to provide a cleaved double-stranded scaffold polynucleotide comprising a support strand and a synthesis strand comprising the primer strand portion;
- (3) extending the terminal end of the primer strand portion of the synthesis strand of the cleaved double-stranded scaffold polynucleotide with a first nucleotide of the predefined sequence by the action of a nucleotide transferase or polymerase enzyme, the first nucleotide comprising a reversible terminator group which prevents further extension by the enzyme;
- (4) removing the reversible terminator group from the first nucleotide;
- (5) ligating a double-stranded ligation polynucleotide to the cleaved scaffold polynucleotide, the ligation polynucleotide comprising a support strand and a helper strand hybridised thereto and further comprising a complementary ligation end, the ligation end comprising:
 - (i) in the support strand a universal nucleotide and a partner nucleotide for the first nucleotide, wherein the partner nucleotide for the first nucleotide overhangs the helper strand; and
 - (ii) in the helper strand a terminal nucleotide lacking a phosphate group; wherein upon ligation of the support strands the first nucleotide pairs with the partner nucleotide;
- (6) cleaving the scaffold polynucleotide at a cleavage site, the site defined by a sequence comprising the universal nucleotide in the support strand, wherein cleavage comprises cleaving the support strand and removing the universal nucleotide from the scaffold polynucleotide to provide a cleaved double-

stranded scaffold polynucleotide comprising a support strand and a synthesis strand comprising a primer strand portion;

- (7) extending the terminal end of the primer strand portion of the synthesis strand of the cleaved double-stranded scaffold polynucleotide with the next nucleotide of the predefined nucleotide sequence by the action of a nucleotide transferase or polymerase enzyme, the next nucleotide comprising a reversible terminator group which prevents further extension by the enzyme;
 - (8) removing the terminator group from the next nucleotide; and
 - (9) ligating a double-stranded ligation polynucleotide to the cleaved scaffold polynucleotide, the ligation polynucleotide comprising a support strand and a helper strand hybridised thereto and further comprising a complementary ligation end, the ligation end comprising:
 - (i) in the support strand a universal nucleotide and a partner nucleotide for the next nucleotide, wherein the partner nucleotide for the next nucleotide overhangs the helper strand; and
 - (ii) in the helper strand a terminal nucleotide lacking a phosphate group; wherein upon ligation of the support strands the next nucleotide pairs with the partner nucleotide;
 - (10) repeating steps 6 to 9 multiple times to provide the double-stranded polynucleotide having a predefined nucleotide sequence.
6. The method according to claim 5, wherein:
- (I) a) prior to and at the cleavage step of the first cycle (step 2) the universal nucleotide occupies position n in the support strand of the scaffold polynucleotide, wherein 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 first nucleotide of

the predefined sequence upon its addition to the terminal end of the primer strand portion in that cycle, wherein the nucleotide at position n in the support strand is opposite the terminal nucleotide of the helper strand and is paired therewith;

- b) in the cleavage step of the first cycle (step 2) the support strand of the scaffold polynucleotide is cleaved between positions n and $n-1$, wherein 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 portion;
- c) in the ligation step of the first cycle (step 5) the complementary ligation end of the ligation polynucleotide is structured such that the partner nucleotide for the first nucleotide of the predefined sequence is the terminal nucleotide of the support strand and occupies position n , wherein the universal nucleotide occupies position $n+1$ in the support strand and is paired with the terminal nucleotide of the helper strand, wherein position n is the nucleotide position which will be opposite the first nucleotide of the predefined sequence upon ligation of the ligation polynucleotide to the cleaved scaffold polynucleotide in step 5;
- d) in the cleavage step of the second cycle (step 6) and in cleavage steps of all subsequent cycles:
 - i. the universal nucleotide occupies position n in the support strand of the scaffold polynucleotide, wherein 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 addition to the terminal end of the primer strand portion in that cycle; and
 - ii. the support strand of the scaffold polynucleotide is cleaved between positions n and $n-1$, wherein $n-1$ 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 portion; and

- e) in the ligation step of the second cycle (step 9) and in ligation steps of all subsequent cycles the complementary ligation end of the ligation polynucleotide is structured such that the partner nucleotide for the next nucleotide of the predefined sequence in that cycle is the terminal nucleotide of the support strand and occupies position n , and the universal nucleotide occupies position $n+1$ in the support strand and is paired with the terminal nucleotide of the helper strand; wherein position n is the nucleotide position which upon ligation of the ligation polynucleotide to the cleaved scaffold polynucleotide will be opposite the next nucleotide of the predefined sequence incorporated in that cycle (step 7); or
- (II) a) prior to and at the cleavage step of the first cycle (step 2) the universal nucleotide occupies position $n+1$ in the support strand of the scaffold polynucleotide, wherein 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 first nucleotide of the predefined sequence upon its addition to the terminal end of the primer strand portion in that cycle, wherein the nucleotide at position n in the support strand is opposite the terminal nucleotide of the helper strand and is paired therewith, and wherein $n+1$ 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 portion;
- b) in the cleavage step of the first cycle (step 2) the support strand of the scaffold polynucleotide is cleaved between positions n and $n-1$, wherein 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 portion;
- c) in the ligation step of the first cycle (step 5) the complementary ligation end of the ligation polynucleotide is structured such that the partner nucleotide for the first nucleotide of the predefined sequence is the terminal nucleotide of the

support strand and occupies position n , wherein the universal nucleotide occupies position $n+2$ in the support strand and is paired with the penultimate nucleotide of the helper strand, wherein position n is the nucleotide position which will be opposite the first nucleotide of the predefined sequence upon ligation of the ligation polynucleotide to the cleaved scaffold polynucleotide in step 5 and position $n+2$ is the second position in the support strand relative to position n in the direction proximal to the helper strand/distal to the primer strand portion;

- d) in the cleavage step of the second cycle (step 6) and in cleavage steps of all subsequent cycles:
 - i. the universal nucleotide occupies position $n+1$ in the support strand of the scaffold polynucleotide, wherein 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 addition to the terminal end of the primer strand portion in that cycle, and wherein $n+1$ 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 portion; and
 - ii. the support strand of the scaffold polynucleotide is cleaved between positions n and $n-1$, wherein $n-1$ 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 portion; and
- e) in the ligation step of the second cycle (step 9) and in ligation steps of all subsequent cycles the complementary ligation end of the ligation polynucleotide is structured such that the partner nucleotide for the next nucleotide of the predefined sequence in that cycle is the terminal nucleotide of the support strand and occupies position n , and the universal nucleotide occupies position $n+2$ in the support strand and is paired with the penultimate nucleotide of the helper

strand; wherein position n is the nucleotide position which upon ligation of the ligation polynucleotide to the cleaved scaffold polynucleotide will be opposite the next nucleotide of the predefined sequence incorporated in that cycle (step 7) and position $n+2$ is the second position in the support strand relative to position n in the direction proximal to the helper strand/distal to the primer strand portion; or

- (III) a) prior to and at the cleavage step of the first cycle (step 2) the universal nucleotide occupies position n in the support strand of the scaffold polynucleotide, wherein 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 first nucleotide of the predefined sequence upon its addition to the terminal end of the primer strand portion in that cycle, wherein the nucleotide at position n in the support strand is opposite the terminal nucleotide of the helper strand and is paired therewith;
- b) in the cleavage step of the first cycle (step 2) the support strand of the scaffold polynucleotide is cleaved between positions $n-1$ and $n-2$, wherein positions $n-1$ and $n-2$ are respectively the next and subsequent nucleotide positions in the support strand relative to position n in the direction distal to the helper strand/proximal to the primer strand portion;
- c) in the ligation step of the first cycle (step 5) the complementary ligation end of the ligation polynucleotide is structured such that the partner nucleotide for the first nucleotide of the predefined sequence is the penultimate nucleotide of the support strand and occupies position n , wherein the universal nucleotide occupies position $n+1$ in the support strand and is paired with the terminal nucleotide of the helper strand, wherein position n is the nucleotide position which will be opposite the first nucleotide of the predefined sequence upon ligation of the ligation polynucleotide to the cleaved scaffold polynucleotide in

step 5, and wherein 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 portion;

- d) in the cleavage step of the second cycle (step 6) and in cleavage steps of all subsequent cycles:
- i. the universal nucleotide occupies position n in the support strand of the scaffold polynucleotide, wherein 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 addition to the terminal end of the primer strand portion in that cycle; and
 - ii. the support strand of the scaffold polynucleotide is cleaved between positions $n-1$ and $n-2$, wherein positions $n-1$ and $n-2$ are respectively the next and subsequent nucleotide positions in the support strand relative to position n in the direction distal to the helper strand/proximal to the primer strand portion; and
- e) in the ligation step of the second cycle (step 9) and in ligation steps of all subsequent cycles the complementary ligation end of the ligation polynucleotide is structured such that the partner nucleotide for the next nucleotide of the predefined sequence in that cycle is the penultimate nucleotide of the support strand and occupies position n , and the universal nucleotide occupies position $n+1$ in the support strand and is paired with the terminal nucleotide of the helper strand; wherein position n is the nucleotide position which upon ligation of the ligation polynucleotide to the cleaved scaffold polynucleotide will be opposite the next nucleotide of the predefined sequence incorporated in that cycle (step 7), and wherein 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 portion; or

- (IV) a) prior to and at the cleavage step of the first cycle (step 2) the universal nucleotide occupies position $n+2$ in the support strand of the scaffold polynucleotide, wherein 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 first nucleotide of the predefined sequence upon its addition to the terminal end of the primer strand portion in that cycle, wherein the nucleotide at position n in the support strand is opposite the terminal nucleotide of the helper strand and is paired therewith, and wherein $n+2$ is the second nucleotide position in the support strand relative to position n in the direction proximal to the helper strand/distal to the primer strand portion;
- b) in the cleavage step of the first cycle (step 2) the support strand of the scaffold polynucleotide is cleaved between positions n and $n-1$, wherein 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 portion;
- c) in the ligation step of the first cycle (step 5) the complementary ligation end of the ligation polynucleotide is structured such that the partner nucleotide for the first nucleotide of the predefined sequence is the terminal nucleotide of the support strand and occupies position n , wherein the universal nucleotide occupies position $n+3$ in the support strand and is paired with the nucleotide which is two positions removed from the terminal nucleotide of the helper strand in the direction distal to the primer strand portion, wherein position n is the nucleotide position which will be opposite the first nucleotide of the predefined sequence upon ligation of the ligation polynucleotide to the cleaved scaffold polynucleotide in step 5 and position $n+3$ is the third position in the support strand relative to position n in the direction proximal to the helper strand/distal to the primer strand portion;
- d) in the cleavage step of the second cycle (step 6) and in cleavage steps of all subsequent cycles:

- i. the universal nucleotide occupies position $n+2$ in the support strand of the scaffold polynucleotide, wherein 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 addition to the terminal end of the primer strand portion in that cycle, and wherein $n+2$ is the second nucleotide position in the support strand relative to position n in the direction proximal to the helper strand/distal to the primer strand portion; and
 - ii. the support strand of the scaffold polynucleotide is cleaved between positions n and $n-1$, wherein 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 portion; and
- e) in the ligation step of the second cycle (step 9) and in ligation steps of all subsequent cycles the complementary ligation end of the ligation polynucleotide is structured such that the partner nucleotide for the next nucleotide of the predefined sequence in that cycle is the terminal nucleotide of the support strand and occupies position n , and the universal nucleotide occupies position $n+3$ in the support strand and is paired with the nucleotide which is two positions removed from the terminal nucleotide of the helper strand in the direction distal to the primer strand portion; wherein position n is the nucleotide position which upon ligation of the ligation polynucleotide to the cleaved scaffold polynucleotide will be opposite the next nucleotide of the predefined sequence incorporated in that cycle (step 7) and position $n+3$ is the third position in the support strand relative to position n in the direction proximal to the helper strand/distal to the primer strand portion.

7. The method according to claim 6 (iv), wherein:

- (i) a) in the cleavage step of the first cycle (step 2) the universal nucleotide instead occupies position $n+3$ in the support strand of the scaffold polynucleotide, wherein $n+3$ is the third nucleotide position in the support strand relative to position n in the direction proximal to the helper strand/distal to the primer strand portion; and the support strand of the scaffold polynucleotide is cleaved between positions n and $n-1$;
- b) in the ligation step of the first cycle (step 5) the complementary ligation end of the ligation polynucleotide is structured such that the universal nucleotide instead occupies position $n+4$ in the support strand and is paired with the nucleotide which is 3 positions removed from the terminal nucleotide of the helper strand at the complementary ligation end; wherein position $n+4$ is position 4 in the support strand relative to position n in the direction proximal to the helper strand/distal to the primer strand portion;
- c) in the cleavage step of the second cycle (step 6) and in cleavage steps of all subsequent cycles the universal nucleotide occupies position $n+3$ in the support strand of the scaffold polynucleotide, and the support strand of the scaffold polynucleotide is cleaved between positions n and $n-1$; and
- d) in the ligation step of the second cycle (step 9) and in ligation steps of all subsequent cycles the complementary ligation end of the ligation polynucleotide is structured such that the universal nucleotide occupies position $n+4$ in the support strand and is paired with the nucleotide which is 2 positions removed from the terminal nucleotide of the helper strand at the complementary ligation end; or
- (ii) a) in the cleavage step of the first cycle (step 2) the universal nucleotide instead occupies position $n+3+x$ in the support strand of the scaffold polynucleotide, wherein $n+3$ is the third nucleotide position in the support strand relative to position n in the direction proximal to the helper strand/distal to the primer

strand portion; and the support strand of the scaffold polynucleotide is cleaved between positions n and $n-1$;

- b) in the ligation step of the first cycle (step 5) the complementary ligation end of the ligation polynucleotide is structured such that the universal nucleotide instead occupies position $n+4+x$ in the support strand and is paired with the nucleotide which is $3+x$ positions removed from the terminal nucleotide of the helper strand at the complementary ligation end; wherein position $n+4$ is position 4 in the support strand relative to position n in the direction proximal to the helper strand/distal to the primer strand portion;
- c) in the cleavage step of the second cycle (step 6) and in cleavage steps of all subsequent cycles the universal nucleotide occupies position $n+3+x$ in the support strand of the scaffold polynucleotide and the support strand of the scaffold polynucleotide is cleaved between positions n and $n-1$;
- d) in the ligation step of the second cycle (step 9) and in ligation steps of all subsequent cycles the complementary ligation end of the ligation polynucleotide is structured such that the universal nucleotide occupies position $n+4+x$ in the support strand and is paired with the nucleotide which is $2+x$ positions removed from the terminal nucleotide of the helper strand at the complementary ligation end; and
- e) wherein x is a whole number between 1 to 10 or more, and wherein x is the same whole number in steps (2), (5), (6) and (9).

8. The method according to claim 5, wherein:

- a) prior to and at the cleavage step of the first cycle (step 2) the universal nucleotide occupies position $n+1$ in the support strand of the scaffold polynucleotide, wherein 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 first nucleotide of the predefined sequence upon its addition to the terminal end of the primer strand portion in that cycle, wherein the nucleotide at position n in the support strand is opposite the terminal nucleotide of the helper strand and is paired therewith, and wherein $n+1$ 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 portion;

- b) in the cleavage step of the first cycle (step 2) the support strand of the scaffold polynucleotide is cleaved between positions $n-1$ and $n-2$, wherein positions $n-1$ and $n-2$ are respectively the next and subsequent nucleotide positions in the support strand relative to position n in the direction distal to the helper strand/proximal to the primer strand portion;
- c) in the ligation step of the first cycle (step 5) the complementary ligation end of the ligation polynucleotide is structured such that the partner nucleotide for the first nucleotide of the predefined sequence is the penultimate nucleotide of the support strand and occupies position n , wherein the universal nucleotide occupies position $n+2$ in the support strand and is paired with the penultimate nucleotide of the helper strand, wherein position n is the nucleotide position which will be opposite the first nucleotide of the predefined sequence upon ligation of the ligation polynucleotide to the cleaved scaffold polynucleotide, and wherein position $n+2$ is the second nucleotide position in the support strand relative to position n in the direction proximal to the helper strand/distal to the primer strand portion;
- d) in the cleavage step of the second cycle (step 6) and in cleavage steps of all subsequent cycles:
 - i. the universal nucleotide occupies position $n+1$ in the support strand of the scaffold polynucleotide, wherein 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 addition to the terminal end of the primer strand portion in that cycle, and wherein $n+1$ 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 portion; and
- ii. the support strand of the scaffold polynucleotide is cleaved between positions $n-1$ and $n-2$, wherein positions $n-1$ and $n-2$ are respectively the next and subsequent nucleotide positions in the support strand relative to position n in the direction distal to the helper strand/proximal to the primer strand portion;
- e) in the ligation step of the second cycle (step 9) and in ligation steps of all subsequent cycles the complementary ligation end of the ligation polynucleotide is structured such that the partner nucleotide for the next nucleotide of the predefined sequence in that cycle is the penultimate nucleotide of the support strand and occupies position n , and the universal nucleotide occupies position $n+2$ in the support strand and is paired with the penultimate nucleotide of the helper strand; wherein position n is the nucleotide position which upon ligation of the ligation polynucleotide to the cleaved scaffold polynucleotide will be opposite the next nucleotide of the predefined sequence incorporated in that cycle (step 7), and wherein position $n+2$ is the second nucleotide position in the support strand relative to position n in the direction proximal to the helper strand/distal to the primer strand portion.

9. The method according to claim 8, wherein:

- (i) a) in the cleavage step of the first cycle (step 2) the universal nucleotide instead occupies position $n+2$ in the support strand of the scaffold polynucleotide,

wherein $n+2$ is the second nucleotide position in the support strand relative to position n in the direction proximal to the helper strand/distal to the primer strand portion; and the support strand of the scaffold polynucleotide is cleaved between positions n and $n-1$;

- b) in the ligation step of the first cycle (step 5) the complementary ligation end of the ligation polynucleotide is structured such that the universal nucleotide instead occupies position $n+3$ in the support strand and is paired with the nucleotide which is 2 positions removed from the terminal nucleotide of the helper strand at the complementary ligation end; wherein position $n+3$ is position 3 in the support strand relative to position n in the direction proximal to the helper strand/distal to the primer strand portion;
 - c) in the cleavage step of the second cycle (step 6) and in cleavage steps of all subsequent cycles the universal nucleotide occupies position $n+2$ in the support strand of the scaffold polynucleotide, and the support strand of the scaffold polynucleotide is cleaved between positions n and $n-1$; and
 - d) in the ligation step of the second cycle (step 9) and in ligation steps of all subsequent cycles the complementary ligation end of the ligation polynucleotide is structured such that the universal nucleotide occupies position $n+3$ in the support strand and is paired with the nucleotide which is 2 positions removed from the terminal nucleotide of the helper strand at the complementary ligation end; or
- (ii) a) in the cleavage step of the first cycle (step 2) the universal nucleotide instead occupies position $n+2+x$ in the support strand of the scaffold polynucleotide, wherein $n+2$ is the second nucleotide position in the support strand relative to position n in the direction proximal to the helper strand/distal to the primer strand portion; and the support strand of the scaffold polynucleotide is cleaved between positions n and $n-1$;

- b) in the ligation step of the first cycle (step 5) the complementary ligation end of the ligation polynucleotide is structured such that the universal nucleotide instead occupies position $n+3+x$ in the support strand and is paired with the nucleotide which is $2+x$ positions removed from the terminal nucleotide of the helper strand at the complementary ligation end; wherein position $n+3$ is position 3 in the support strand relative to position n in the direction proximal to the helper strand/distal to the primer strand portion;
- c) in the cleavage step of the second cycle (step 6) and in cleavage steps of all subsequent cycles the universal nucleotide occupies position $n+2+x$ in the support strand of the scaffold polynucleotide and the support strand of the scaffold polynucleotide is cleaved between positions n and $n-1$;
- d) in the ligation step of the second cycle (step 9) and in ligation steps of all subsequent cycles the complementary ligation end of the ligation polynucleotide is structured such that the universal nucleotide occupies position $n+3+x$ in the support strand and is paired with the nucleotide which is $2+x$ positions removed from the terminal nucleotide of the helper strand at the complementary ligation end; and
- e) wherein x is a whole number between 1 to 10 or more, and wherein x is the same whole number in steps (2), (5), (6) and (9).

10. The method according to any one of claims 1 to 9, wherein a partner 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. The method according to any one of claims 5 to 10, wherein:

- (i) in any one or more cycles of synthesis, or in all cycles of synthesis, prior to step (2) and/or (6) the scaffold polynucleotide is provided comprising a synthesis strand and a support strand hybridized thereto, and wherein the synthesis strand is provided without a helper strand; and/or
 - (ii) in any one or more cycles of synthesis, or in all cycles of synthesis, prior to step (2) and/or (6) the synthesis strand is removed from the scaffold polynucleotide.
12. The method according to any one of claims 3 to 6(i), claim 10 and claim 11, wherein:
- (a) each cleavage step comprises a two step cleavage process wherein 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 wherein the first step is performed with a nucleotide-excising enzyme, and/or wherein the second step is performed with an enzyme having abasic site lyase activity, optionally wherein the enzyme having abasic site lyase activity is.
 - (i) AP Endonuclease I;
 - (ii) Endonuclease III (Nth); or
 - (iii) Endonuclease VIII; or
 - (b) wherein each cleavage step comprises a one step cleavage process comprising removing the universal nucleotide with a cleavage enzyme wherein the enzyme is
 - (i) Endonuclease III;
 - (ii) Endonuclease VIII;
 - (iii) formamidopyrimidine DNA glycosylase (Fpg); or
 - (iv) 8-oxoguanine DNA glycosylase (hOGG1).

13. The method according to any one of claims 3 to 5, claim 6 (ii) and claim 6 (iii), wherein the cleavage step comprises cleaving the support strand with an enzyme; optionally wherein:

- (a) 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; and/or
- (b) the enzyme is Endonuclease V.

14. The method according to any one of claims 5 to 13, wherein in steps (1), (5) and/or (9):

- 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 wherein 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 complementary ligation end; more preferably wherein 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 complementary ligation end.

15. The method according to any one of claims 4 to 14, wherein 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; further optionally wherein the one or more covalent bonds is formed between a functional group on the common surface and a functional group on the scaffold molecule, wherein the functional group on the scaffold molecule is an amine group, a thiol group, a thiophosphate group or a thioamide group; more preferably wherein the functional group on the common surface is a bromoacetyl group, optionally wherein the bromoacetyl group

is provided on a polyacrylamide surface derived using N- (5-bromoacetamidylpentyl) acrylamide (BRAPA).

16. The method according to any one of claims 3 to 15, wherein the step of removing the reversible terminator group from a nucleotide of the predefined sequence is performed before or after the ligation step.

17. The method according to any one of claims 1 to 16, wherein:

- (a) synthesis cycles are performed in droplets within a microfluidic system; optionally wherein the microfluidic system is an electrowetting system; more preferably wherein the microfluidic system is an electrowetting-on-dielectric system (EWOD); and/or
- (b) following synthesis the strands of the double-stranded polynucleotides are separated to provide a single-stranded polynucleotide having a predefined sequence; and/or
- (c) following synthesis the double-stranded polynucleotide or a region thereof is amplified, preferably by PCR.

18. A method of assembling a polynucleotide having a predefined sequence, the method comprising performing the method of any one of the preceding claims to synthesise a first polynucleotide having a predefined sequence and one or more additional polynucleotides having a predefined sequence and joining together the first and one or more additional polynucleotides; optionally wherein the first polynucleotide and the one or more additional polynucleotides are cleaved to create compatible termini and joined together, preferably by ligation; further optionally wherein the first polynucleotide and the one or more additional polynucleotides are cleaved by a restriction enzyme at a cleavage site.

Figure 1

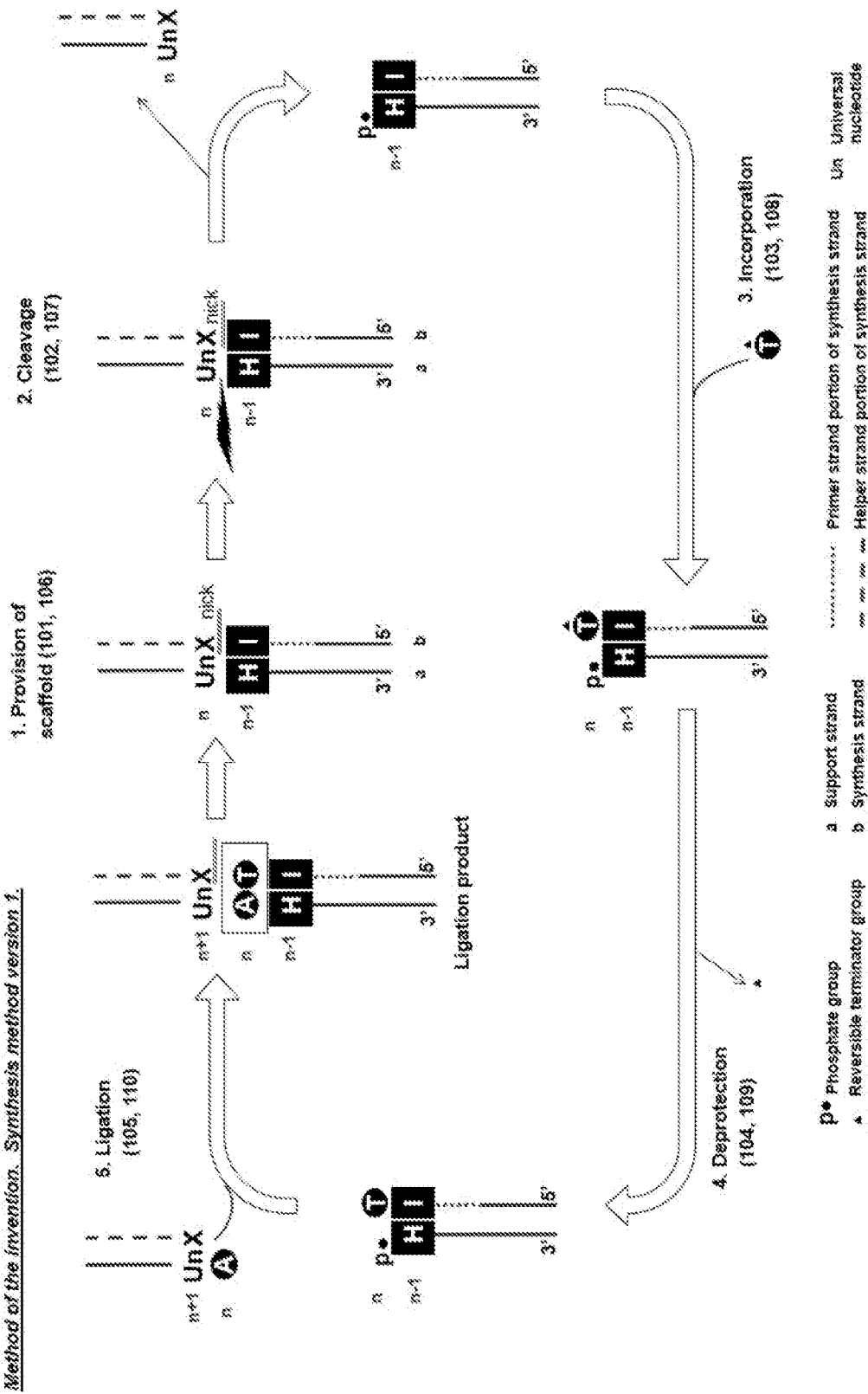


Figure 2

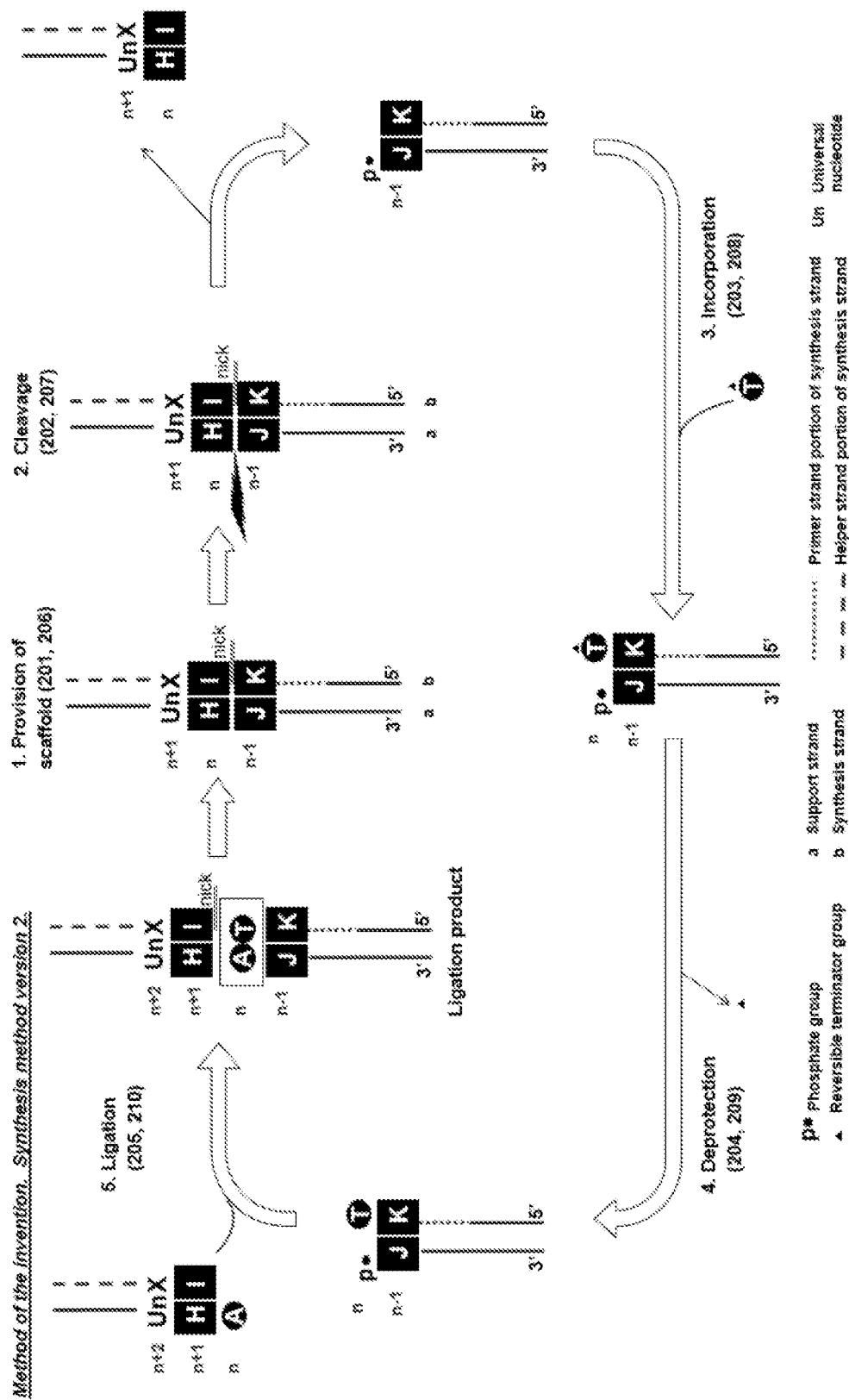


Figure 3

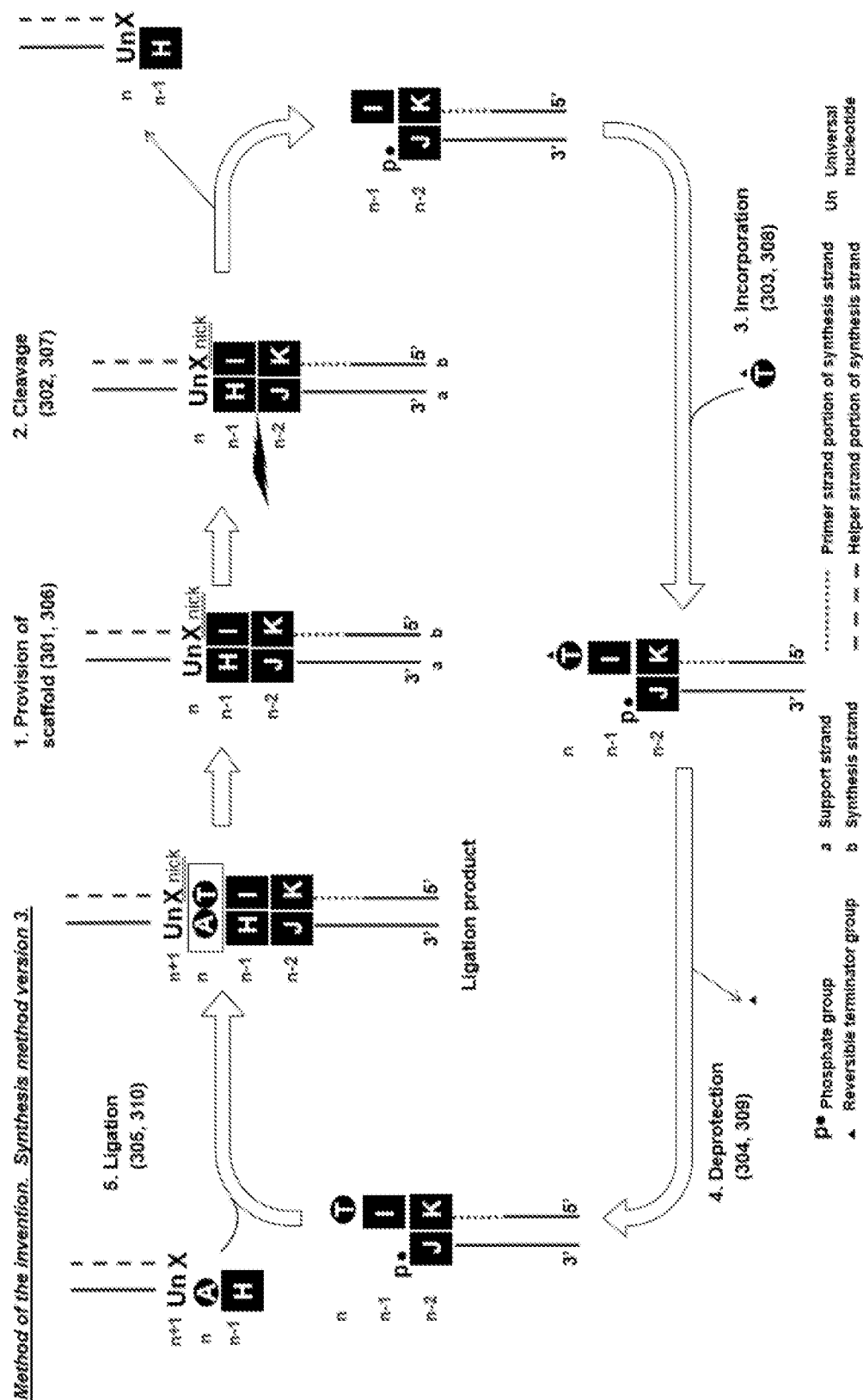


Figure 4

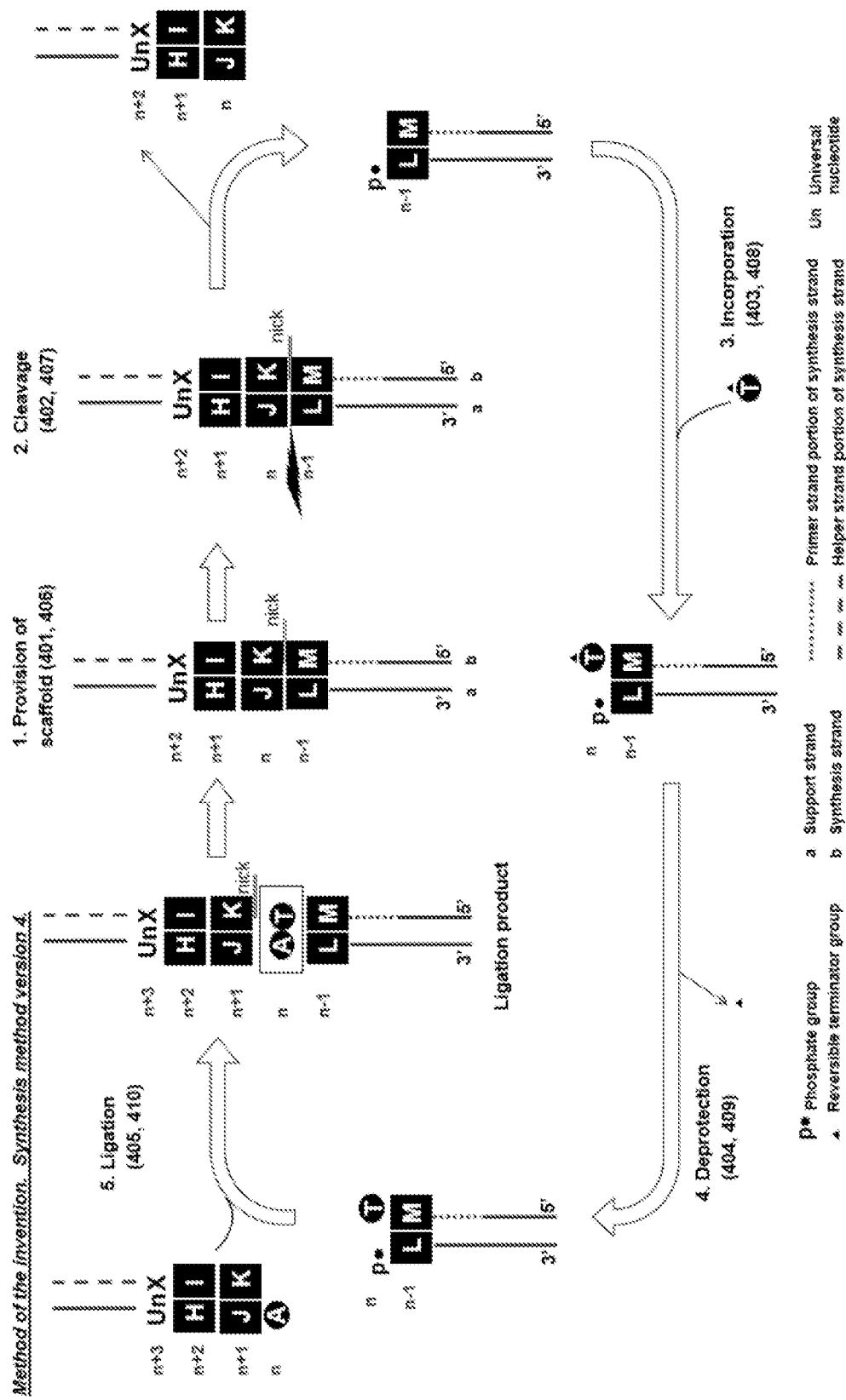


Figure 5

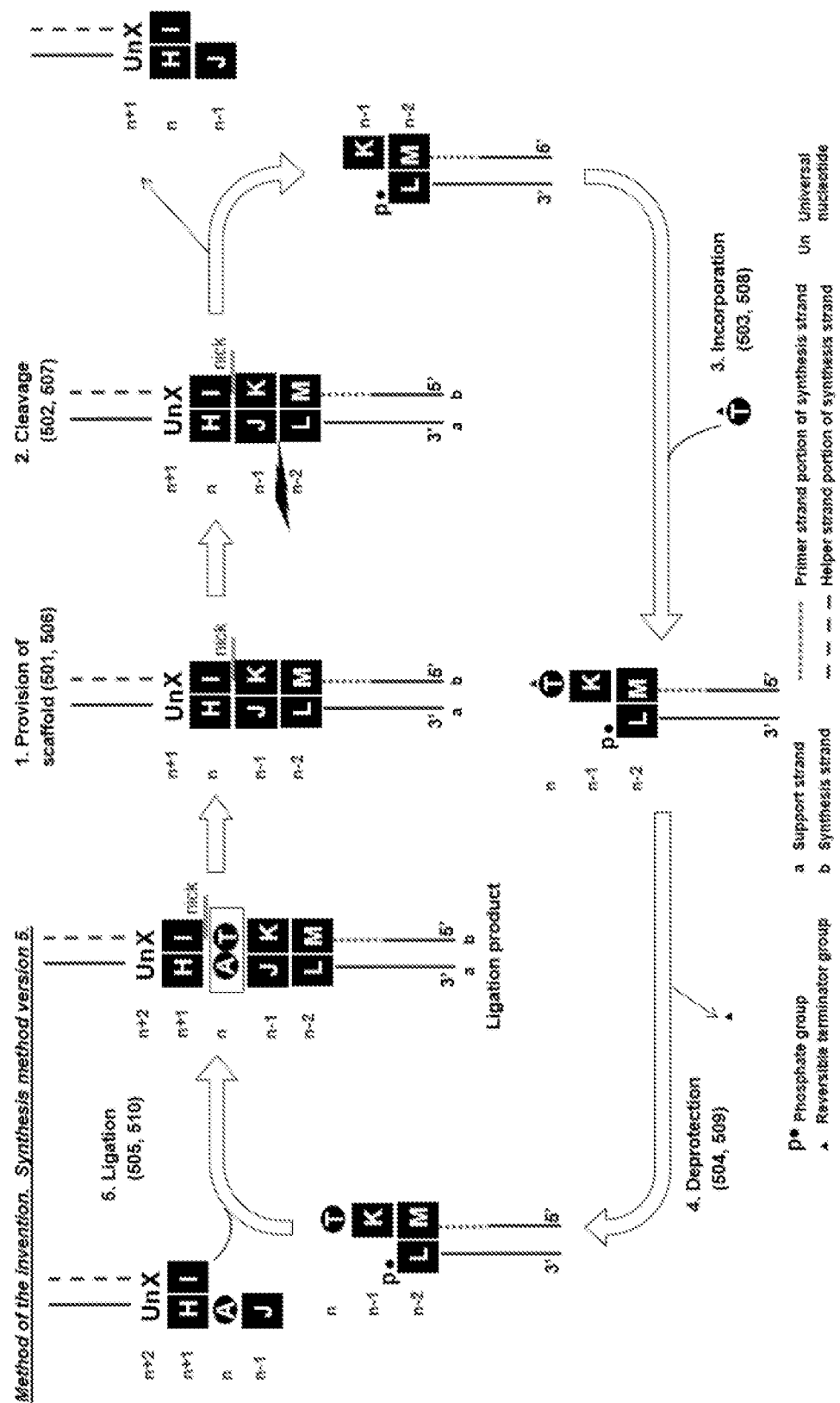


Figure 6

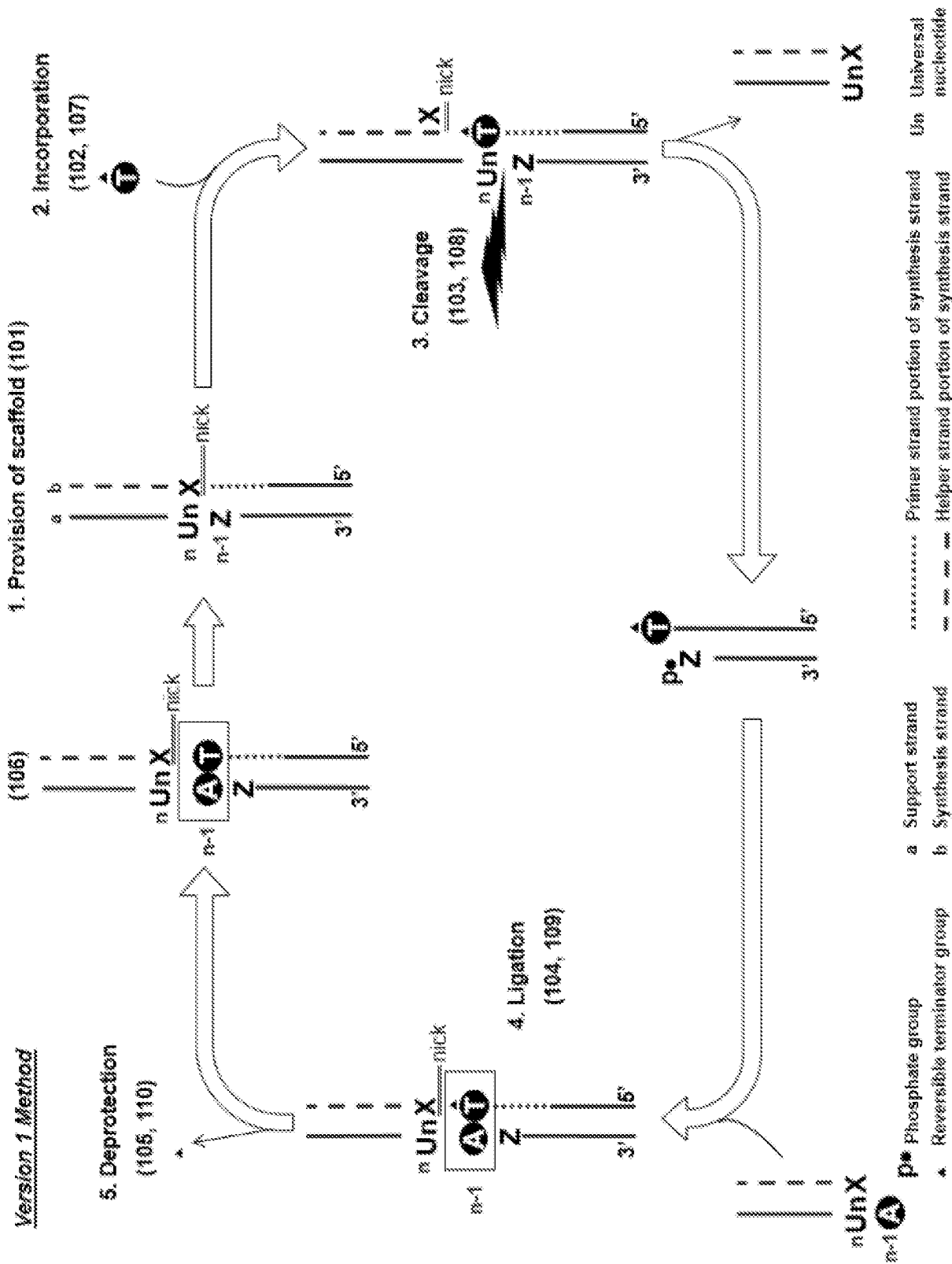


Figure 7

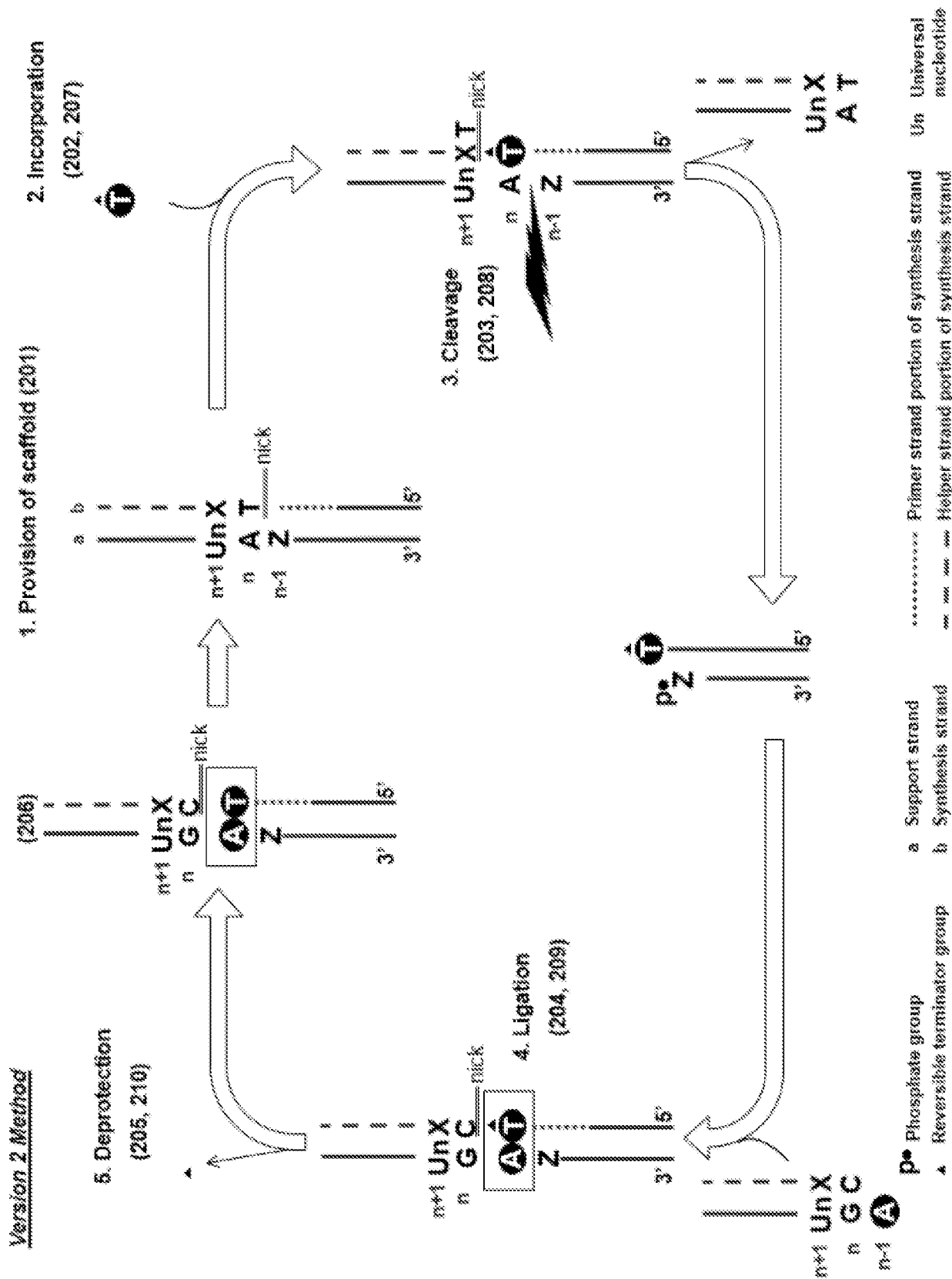


Figure 8

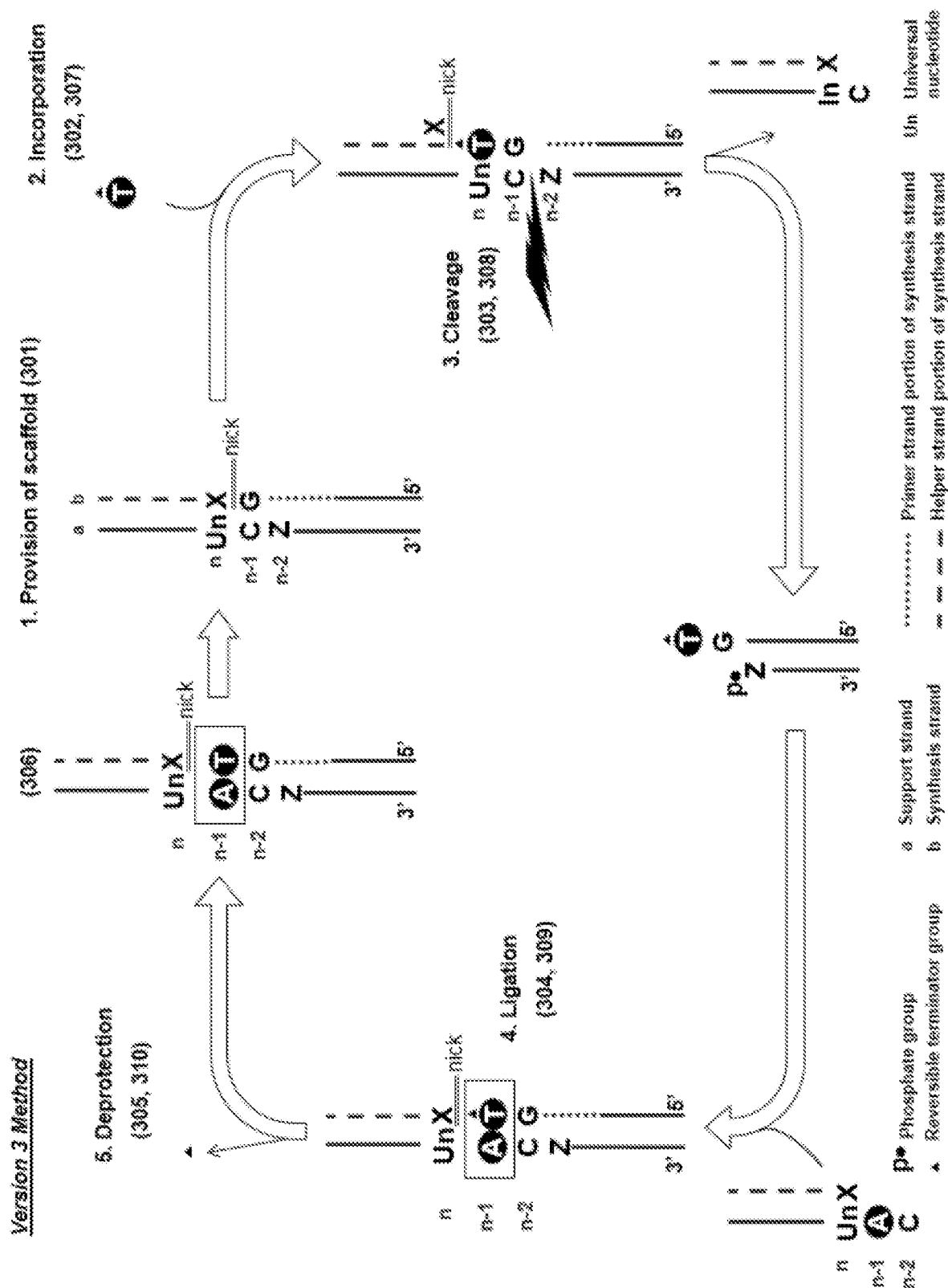


Figure 9

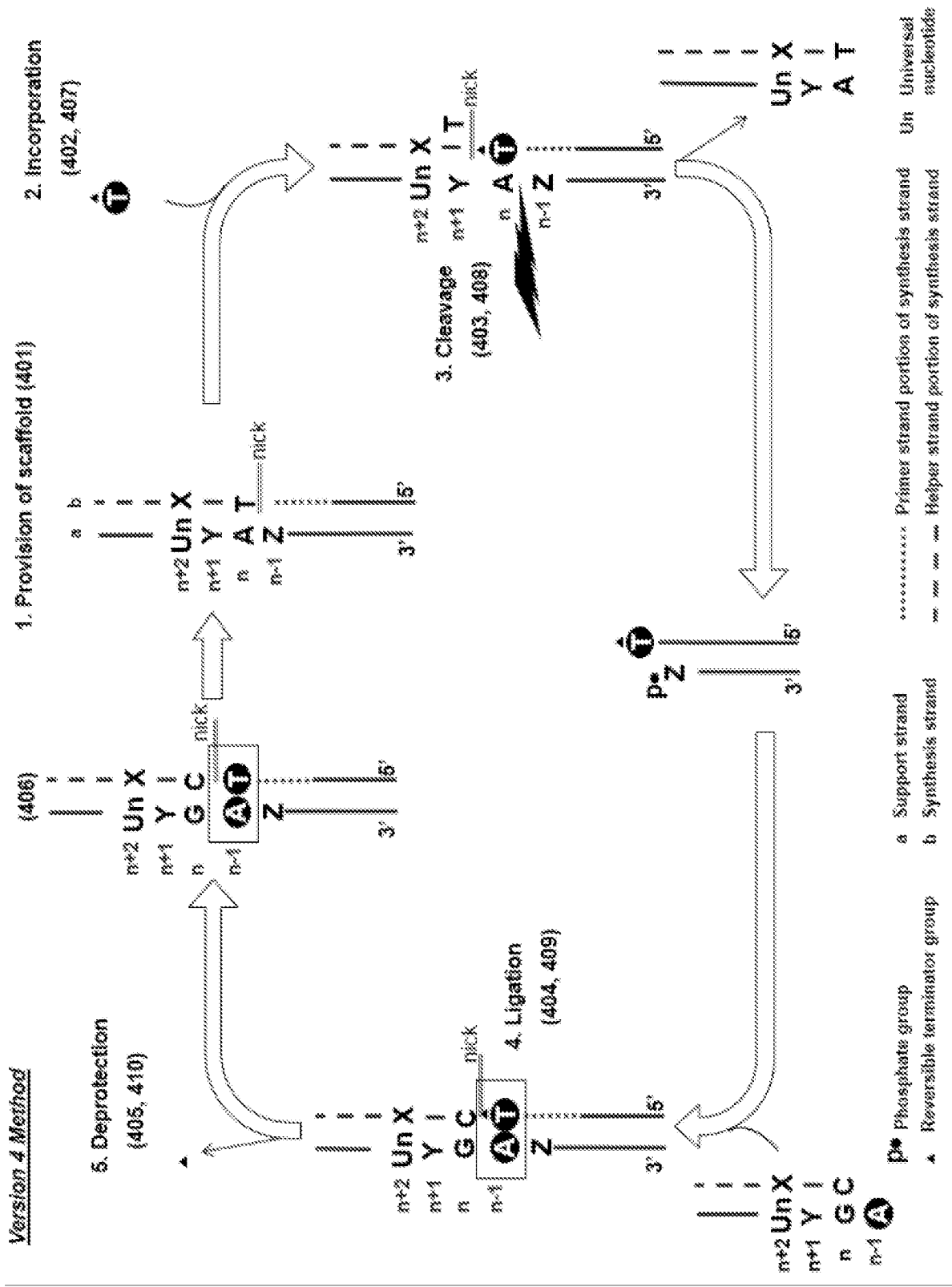


Figure 10

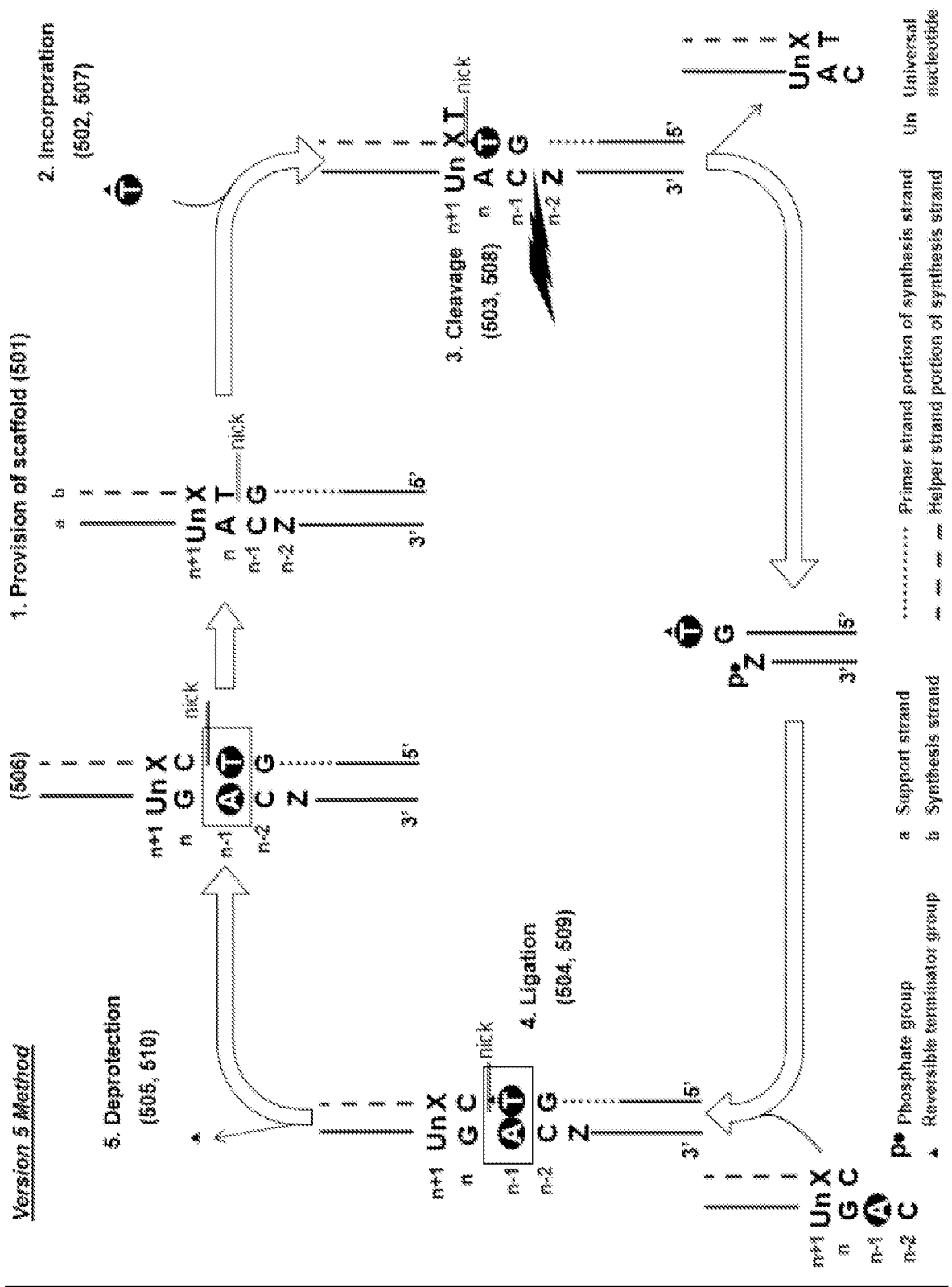
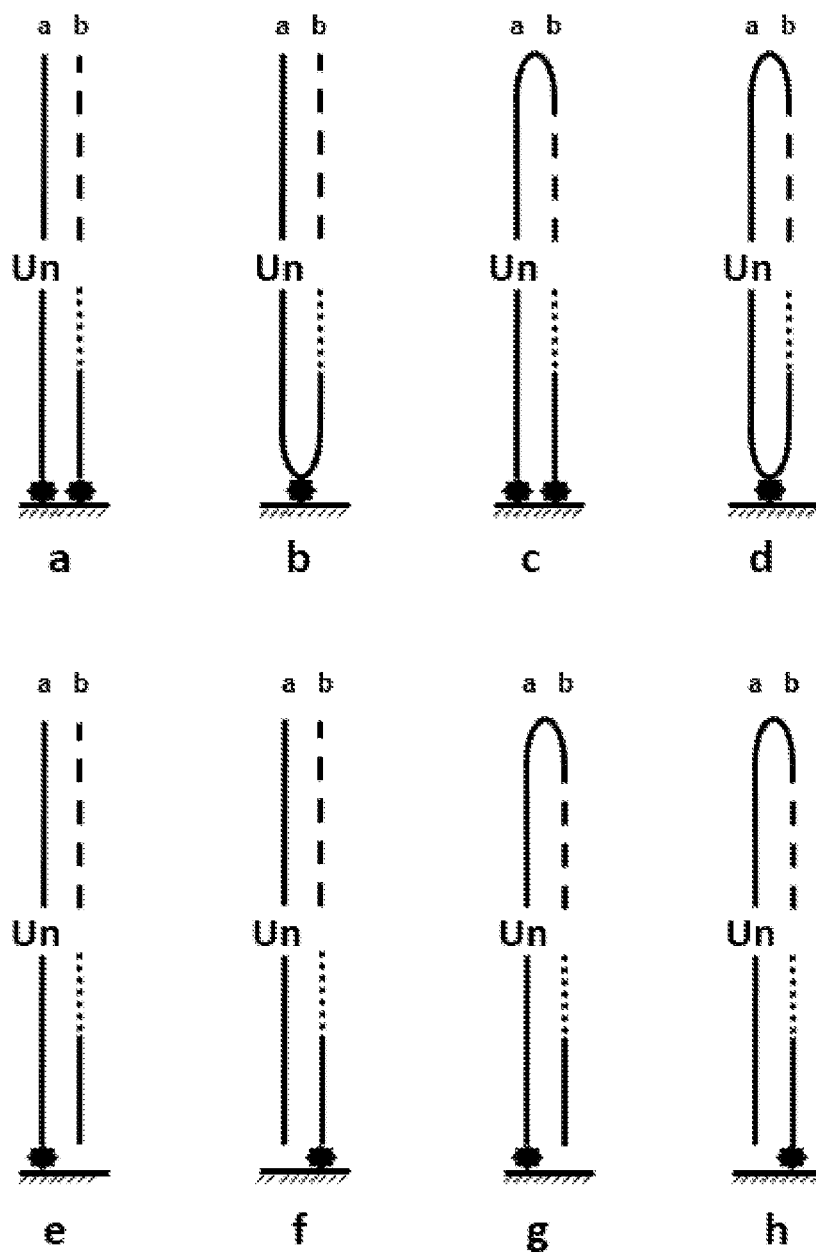


Figure 11



a Support strand

b Synthesis strand

Un Universal
nucleotide

..... Primer strand portion of synthesis strand

- - - - - Helper strand portion of synthesis strand

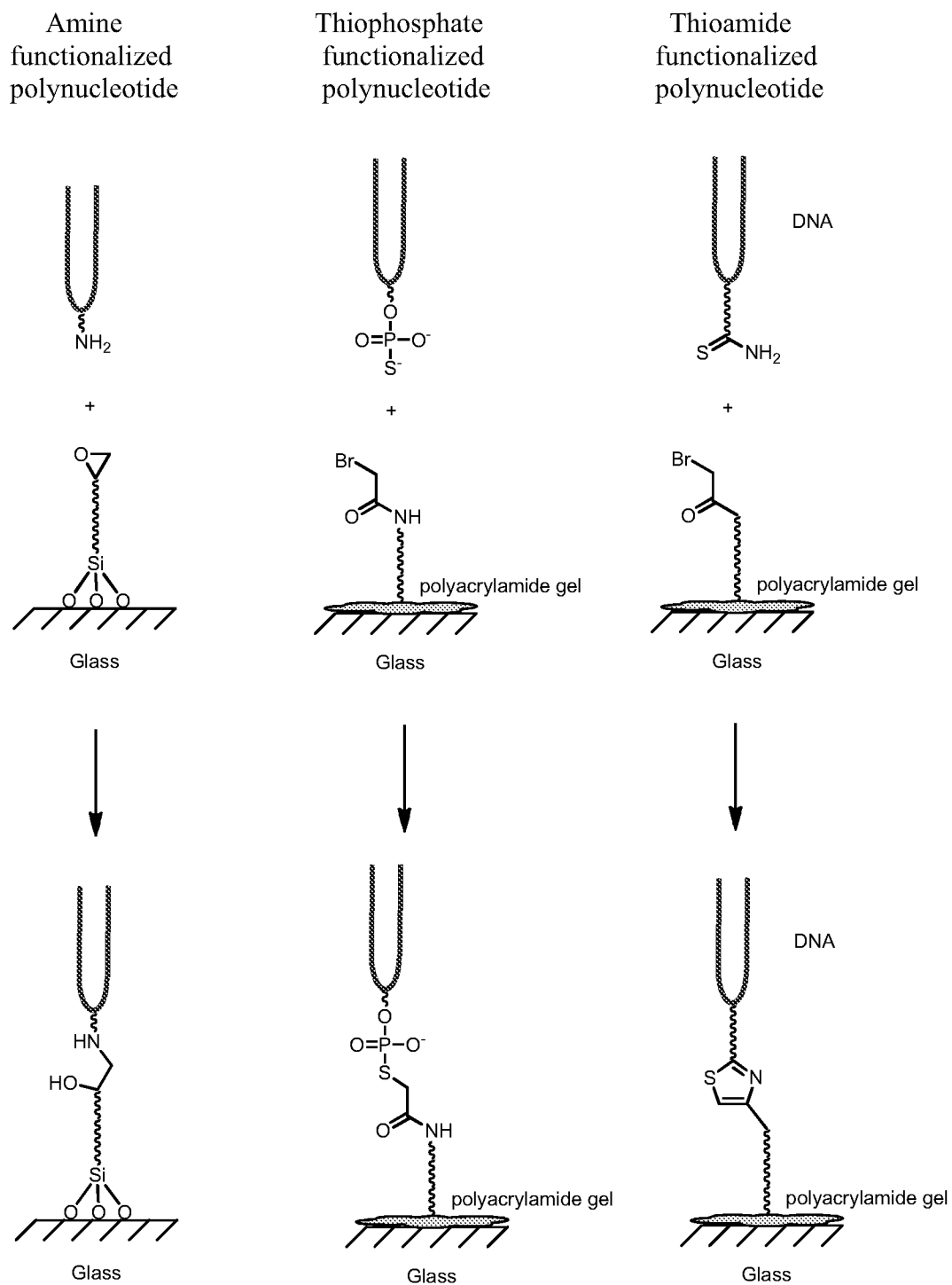
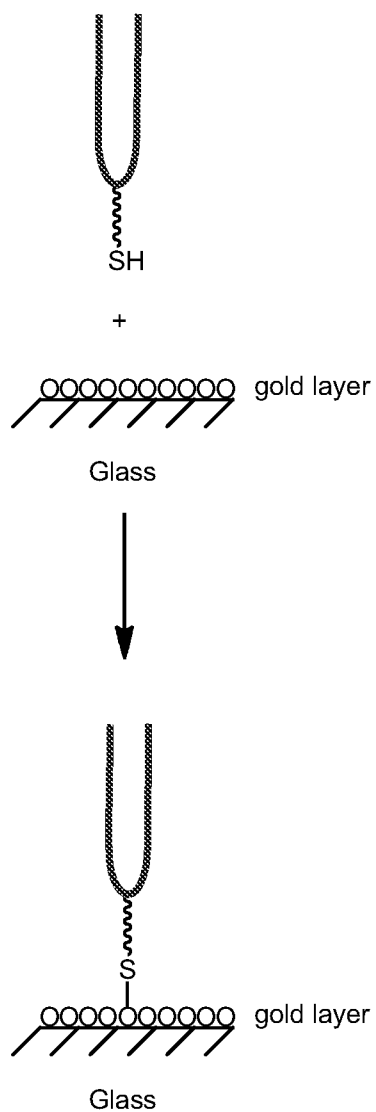
Figure 11i

Figure 11j

Example method for
attaching polynucleotide to
gold surface:



Example method for
attaching polynucleotide by
biotin-streptavidin binding:

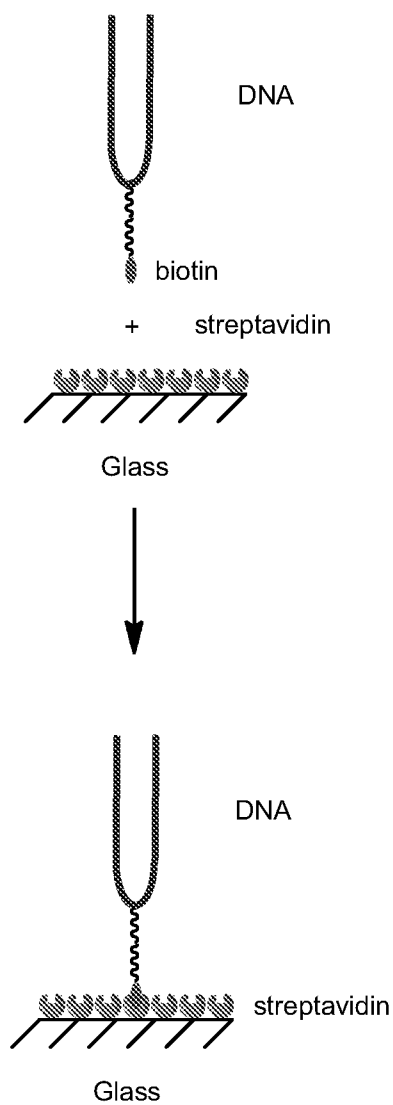


Figure 12a

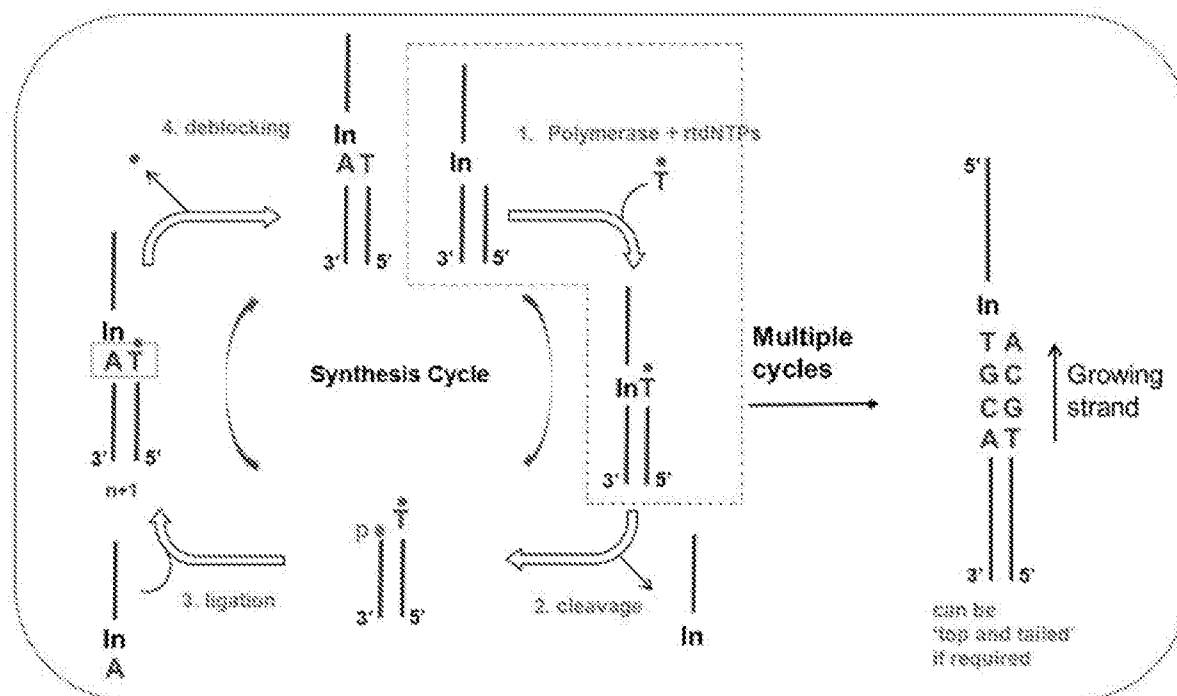


Figure 12b

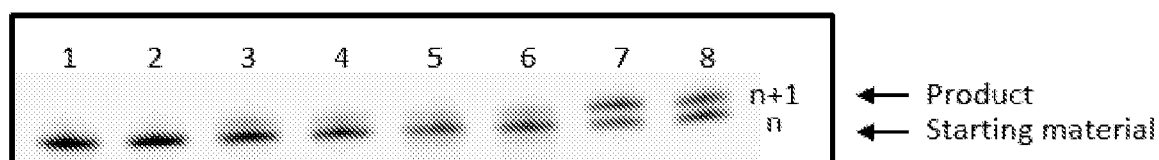


Figure 12c

Lane no.	Incorporation at 50°C	DNA polymerase	conversion
1	3'-O-allyl-dTTPs	Bst	<1%
2	3'-O-azidomethyl-dTTPs	Bst	<1%
3	3'-O-allyl-dTTPs	Deep Vent (exo-)	5%
4	3'-O-azidomethyl-dTTPs	Deep Vent (exo-)	5%
5	3'-O-allyl-dTTPs by	Therminator I	8%
6	3'-O-azidomethyl-dTTPs	Therminator I	8%
7	3'-O-allyl-dTTPs	Therminator IX	55%
8	3'-O-azidomethyl-dTTPs	Therminator IX	48%

Figure 12d

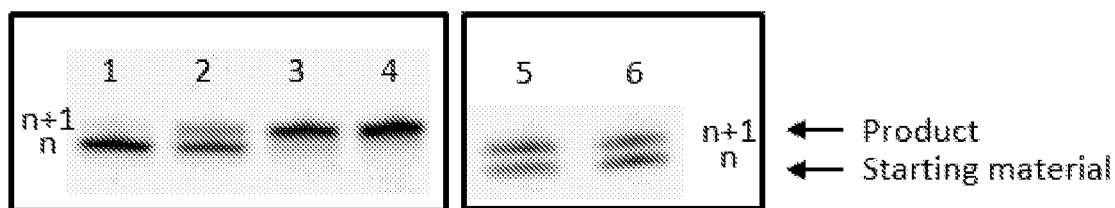


Figure 12e

Lane No.	Incorporation	Enzyme	Temp.	Conversion
1	3'-O-allyl-dTTPs	Therminator IX	37 °C	<5%
2	3'-O-azidomethyl-dTTPs	Therminator IX	37 °C	15%
3	3'-O-allyl-dTTPs	Therminator IX	50 °C	55%
4	3'-O-azidomethyl-dTTPs	Therminator IX	50 °C	48%
5	3'-O-allyl-dTTPs	Therminator IX	65 °C	>90%
6	3'-O-azidomethyl-dTTPs	Therminator IX	65 °C	>90%

Figure 12f

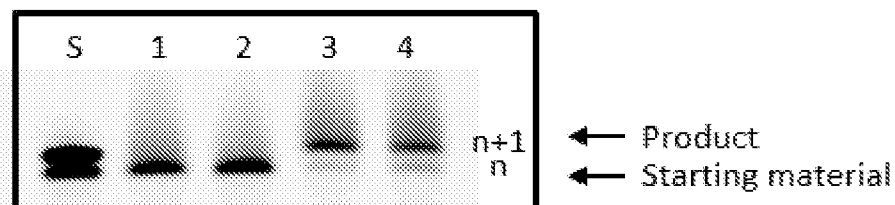


Figure 12g

Lane No.	incorporation	Enzyme	Temp	2mM Mn ²⁺	conversion
1	3'-O-allyl-dTTPs	Therminator IX	65 °C	No	<1%
2	3'-O-azidomethyl-dTTPs	Therminator IX	65 °C	No	<1%
3	3'-O-allyl-dTTPs	Therminator IX	65 °C	Yes	>90%
4	3'-O-azidomethyl-dTTPs	Therminator IX	65 °C	Yes	>90%

Figure 12h

SEQ ID NO:	Oligonucleotide name	Sequence	Modification
1	Primer	5'-GCGACAGGTGACTGCAGC-3'	TAMRA at 5'-end (attached to terminal guanine)
2	Template	5'-CACATCACGTCGTAGTCXGCTGCAG TCACCTGTCGC-3'	X = 2'- deoxyinosine

Figure 13a

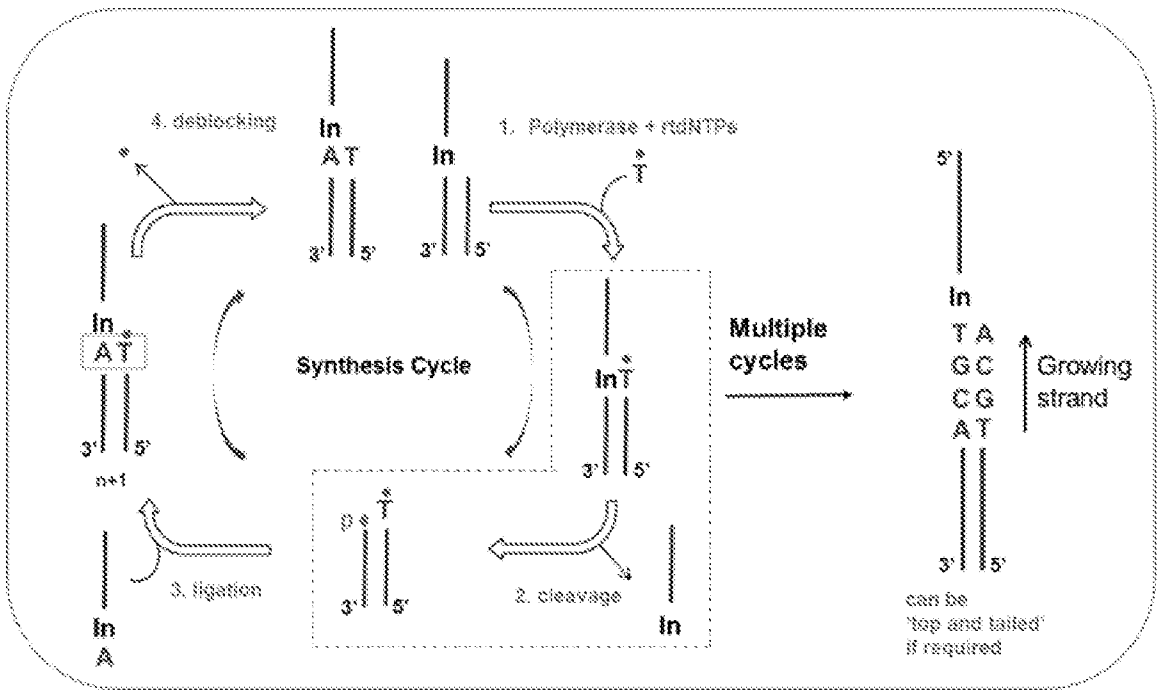


Figure 13b

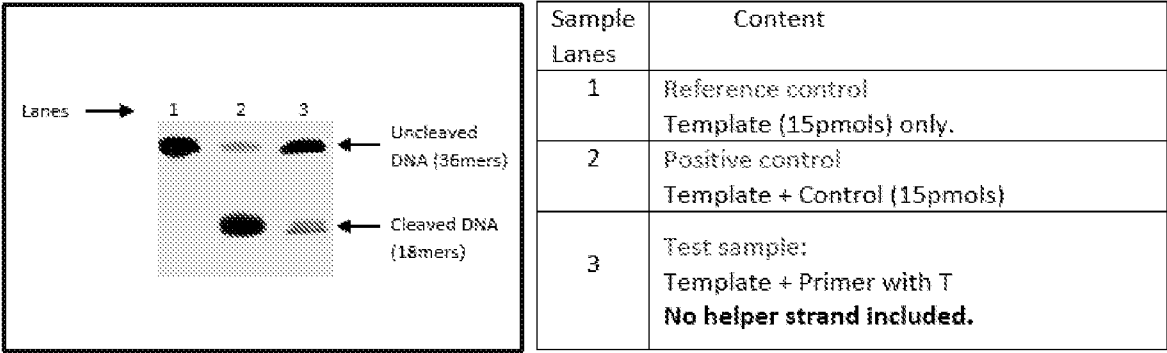


Figure 13c

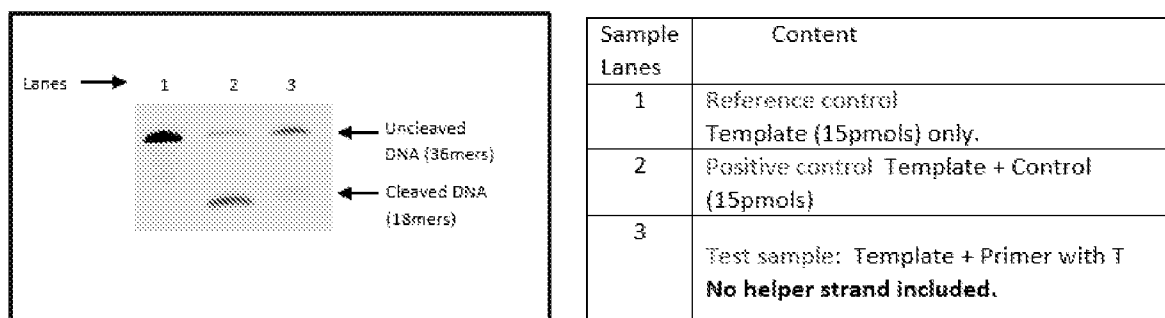


Figure 13d

Gel	Cleavage reaction combination	% Conversion of cleaved DNA without a helper strand
A	hAAG/ Chemical base	< 10
B	hAAG/Endo VIII	< 7

Figure 13e

SEQ ID NO:	Oligonucleotide name	Sequence	Modification
3	Template	5'-CACATCACGTCGTAGTCXGCT GCAGTCACCTGTCGC-3'	TAMRA at 5'-end X = 2' deoxyinosine
4	Primer with T	5'-GCGACAGGTGACTGCAGCT-3'	None
5	Control	5'-GCGACAGGTGACTGCAGCTGA CTACGACGTGATGTG-3'	None

Figure 15a

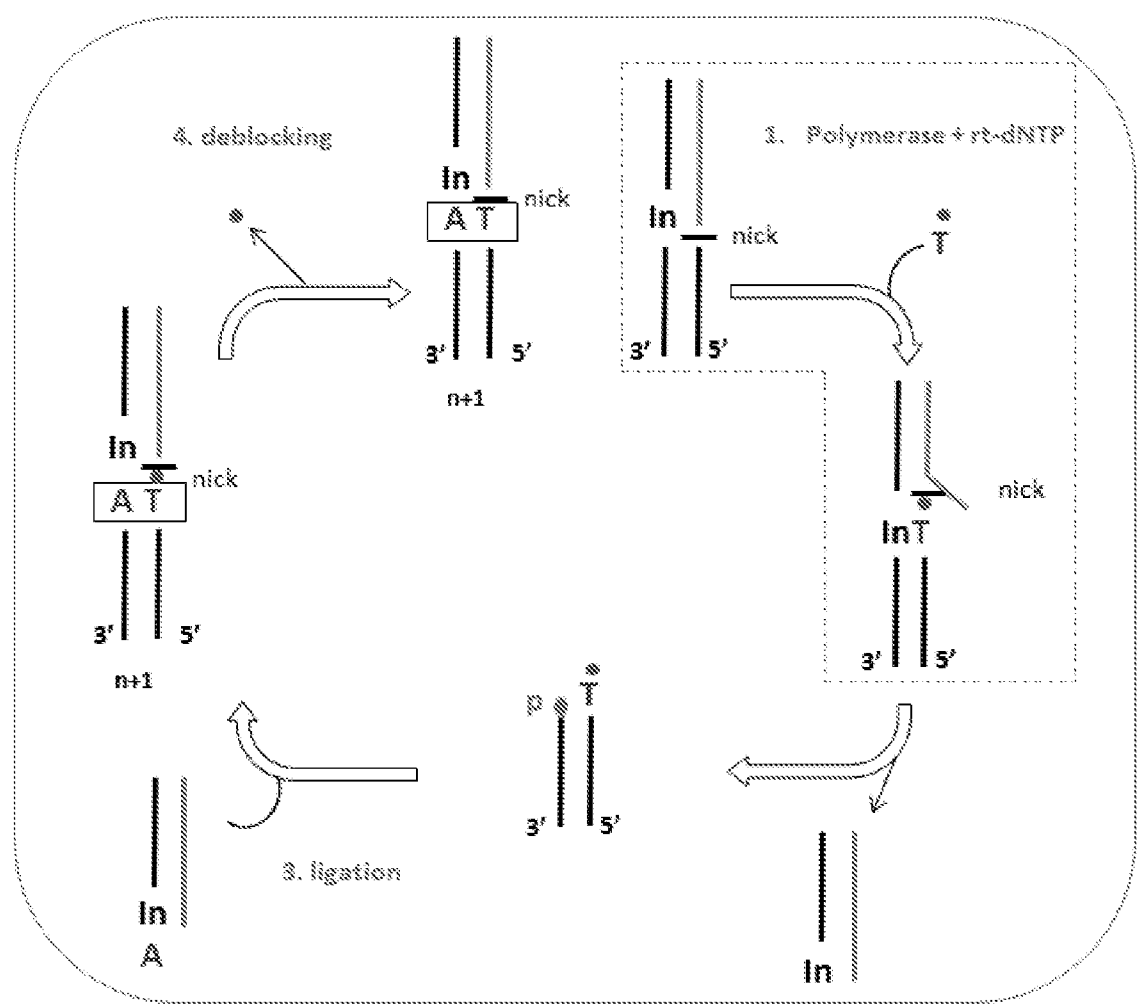


Figure 15b

SEQ ID NO:	Oligonucleotide name	Sequence	Modification
10	Primer	5'-GCGACAGGTGACTGCAGC-3'	TAMRA at 5'-end (attached to terminal guanine)
11	Template	5'-CACATCACGTCGTAGTCXGCTGCAG TCACCTGTCGC-3'	X = 2'-deoxyinosine
12	Helper strand	5'-CGACTACGACGTGATGTG-3'	None

Figure 16a

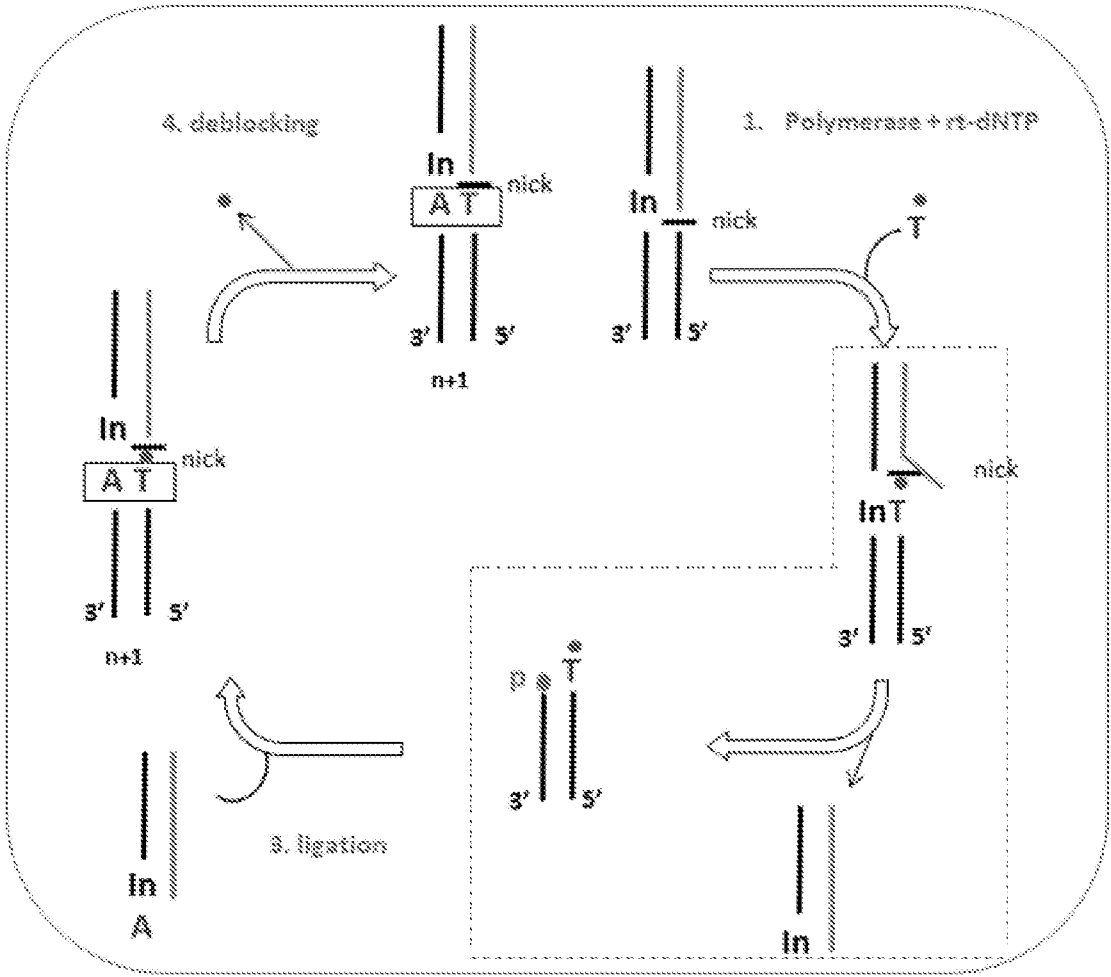
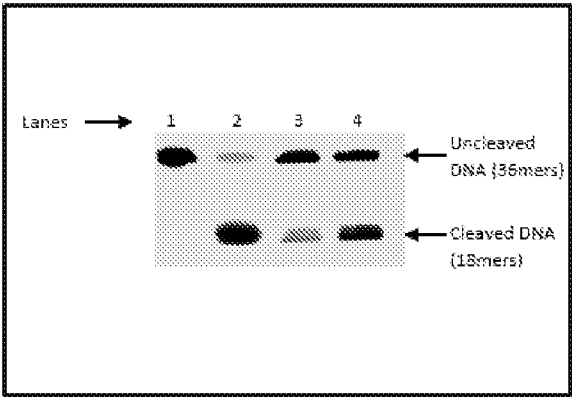
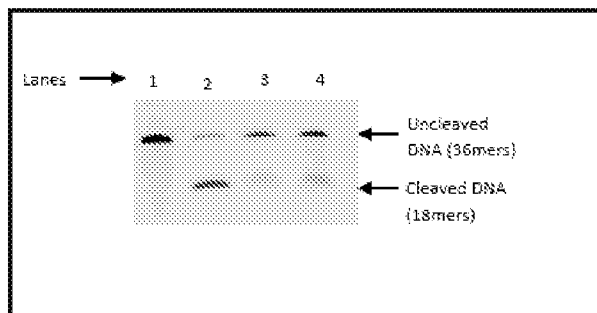


Figure 16b



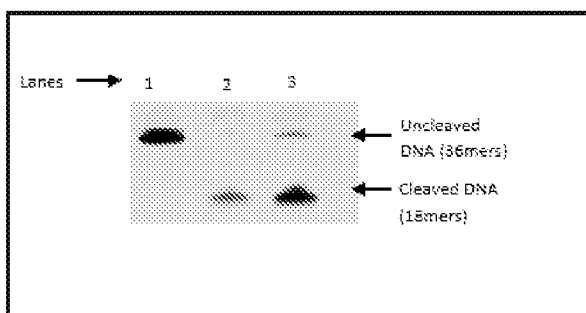
Sample Lanes	Content
1	Reference control Template (15pmols) only.
2	Positive control Template + Control (15pmols)
3	Test sample I: Template + Primer with T No helper strand included (15pmols)
4	Test Sample II : Template + Primer with T + Helper strand (15pmols)

Figure 16c



Sample Lanes	Content
1	Reference control Template (15pmols) only.
2	Positive control Template + Control (15pmols)
3	Test sample I: Template + Primer with T No helper strand included. (15pmols)
4	Test Sample II: Template + Primer with T + Helper strand (15pmols).

Figure 16d



Sample Lanes	Content
1	Reference control Template (15pmols) only.
2	Positive control Template + Control (15pmols)
3	Test Sample I: Template + Primer with T + Helper strand (15pmols).

Figure 16e

Gel	Cleavage reaction combination	% Conversion of cleaved DNA with a helper strand
A	hAAG/ Chemical base	50
B	hAAG/Endo VIII	< 10
C	hAAG/Alternative chemical base	90*

Figure 16f

SEQ ID NO:	Oligonucleotide name	Sequence	Modification
13	Template	5'-CACATCACGTCGTAGTCXGCT GCAGTCACCTGTCGC-3'	TAMRA at 5'-end (attached to terminal cytosine), X = deoxyinosine
14	Primer with T	5'-GCGACAGGTGACTGCAGCT-3'	None
15	Control	5'-GCGACAGGTGACTGCAGCTGA CTACGACGTGATGTG-3'	None
16	Helper Strand	5'-TGACTACGACGTGATGTG-3'	None

Figure 17a

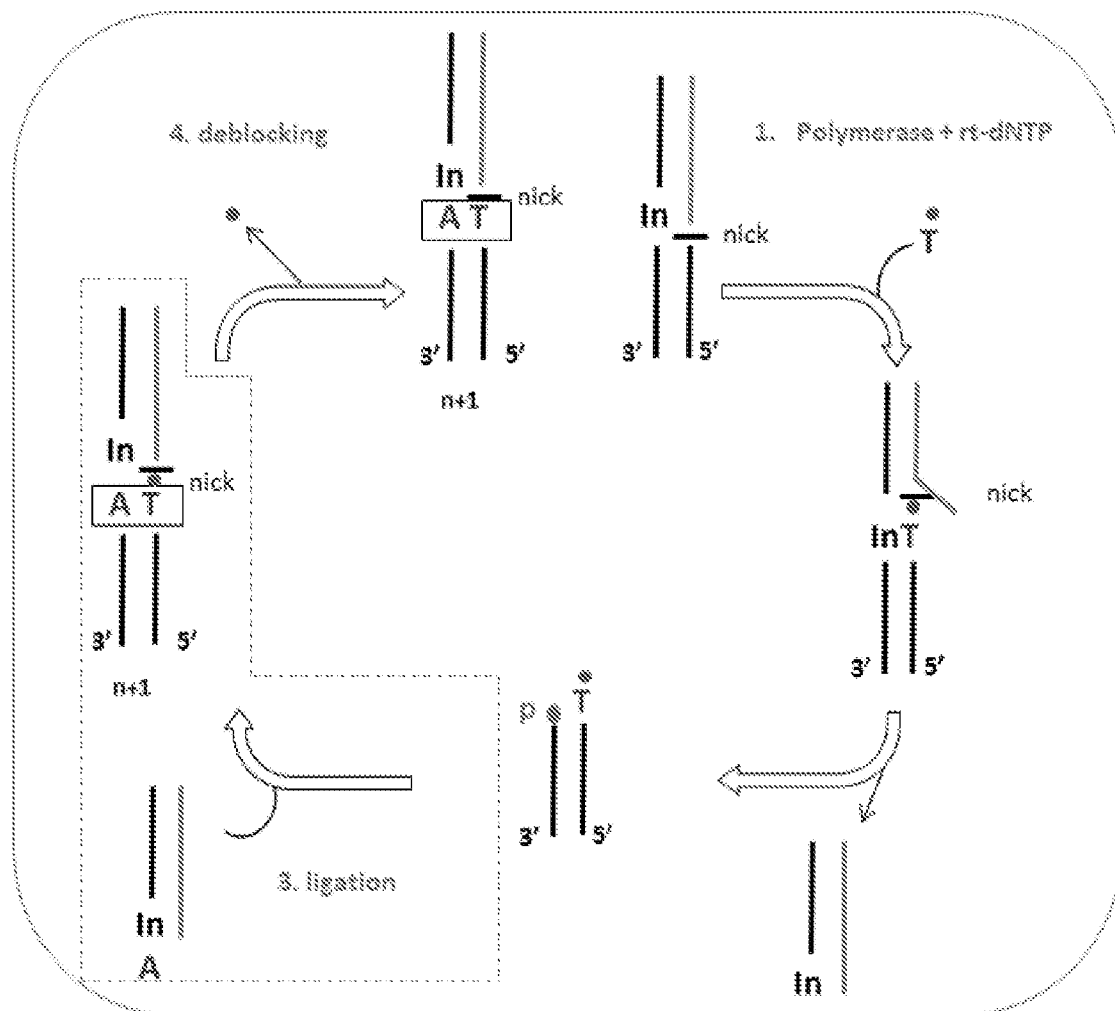


Figure 17b

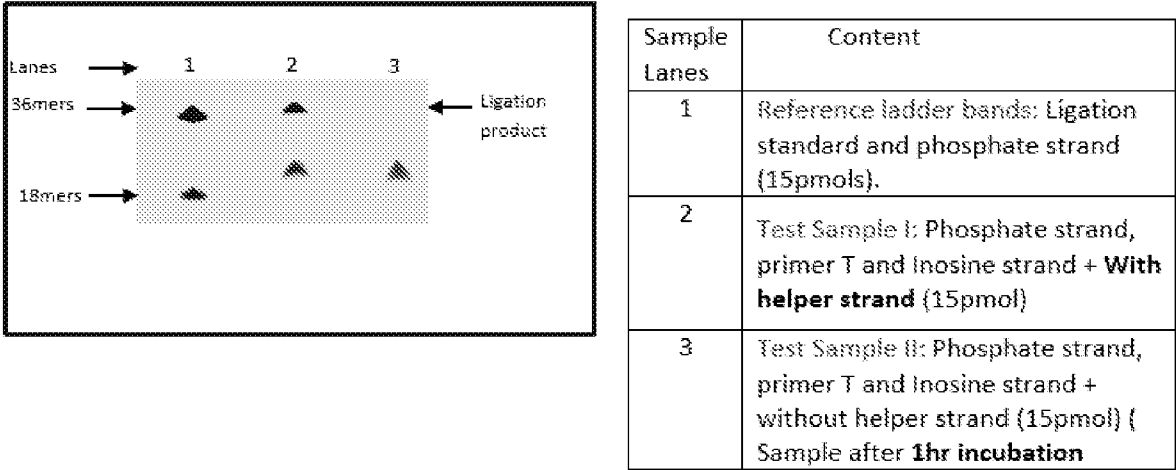


Figure 17d

SEQ ID NO:	Oligonucleotide name	Sequence	Modification
17	Ligation standard	5'-CACATCACGTCGTAGT X A GCTGCAGTCACCTGTCGC-3'	X = 2'-deoxyinosine
18	Phosphate strand	5'-GCTGCAGTCACCTGTCGC-3'	TAMRA at 3'-end, (attached to terminal cytosine), phosphate at 5' end
19	Primer with T	5'-GCGACAGGTGACTGCAGCT-3'	None
20	Inosine strand	5'-CACATCACGTCGTAGT X A-3'	X = 2'-deoxyinosine
21	Helper strand	5'-CACTACGACGTGATGTG-3'	None

Figure 18a

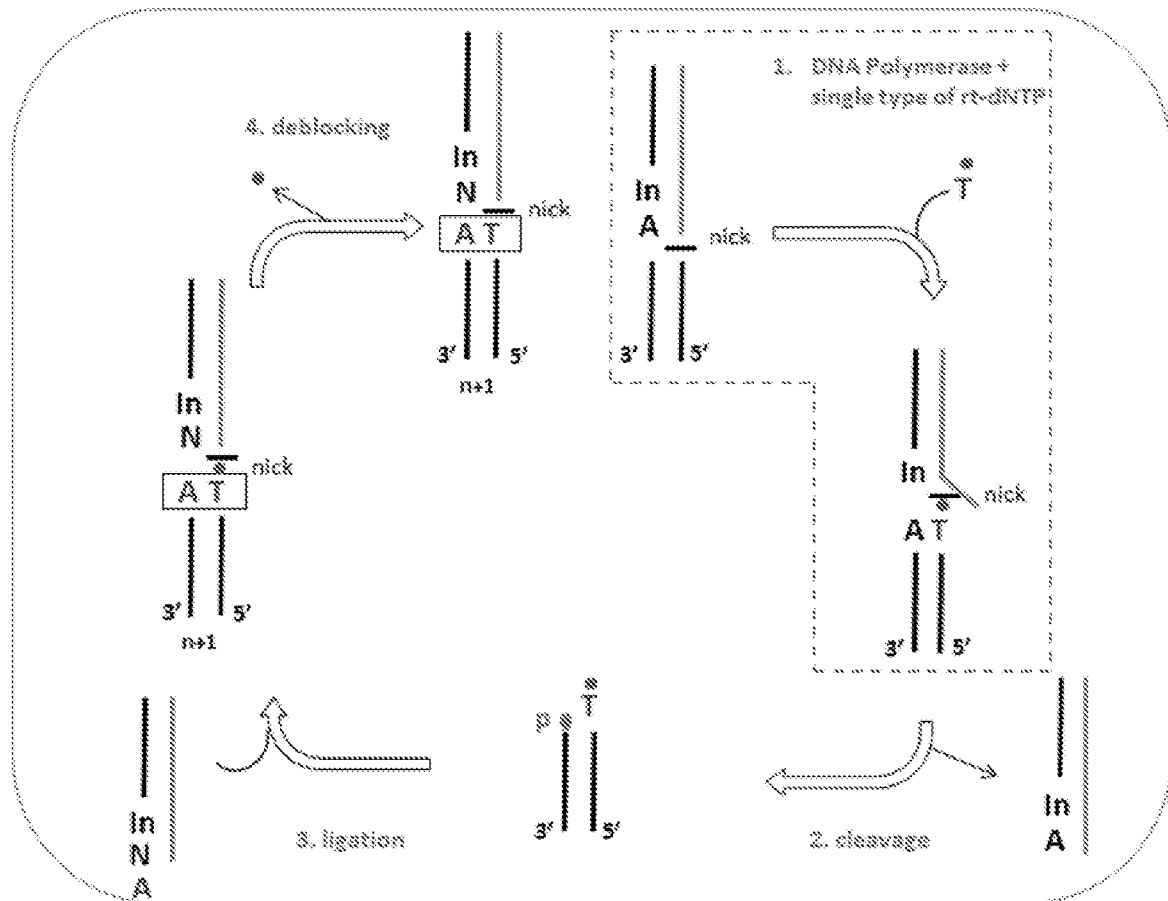


Figure 18b

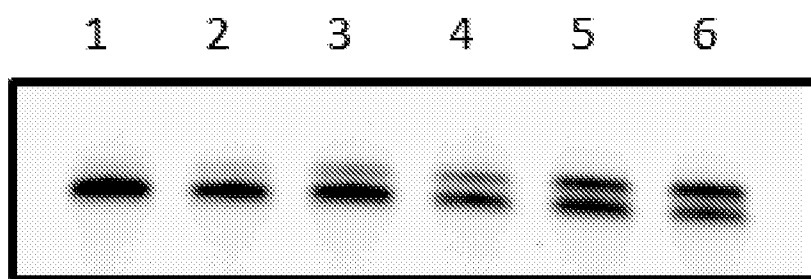


Figure 18c

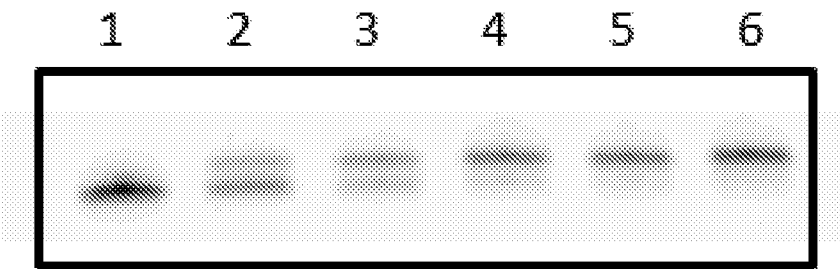


Figure 18d

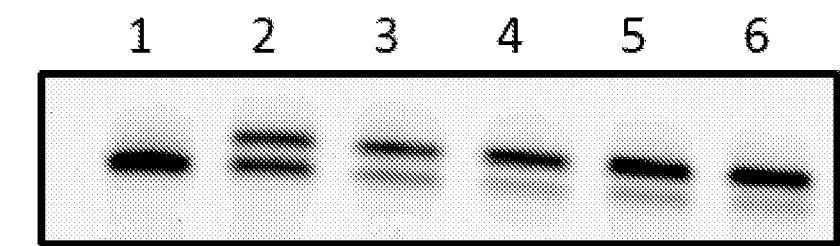


Figure 18e

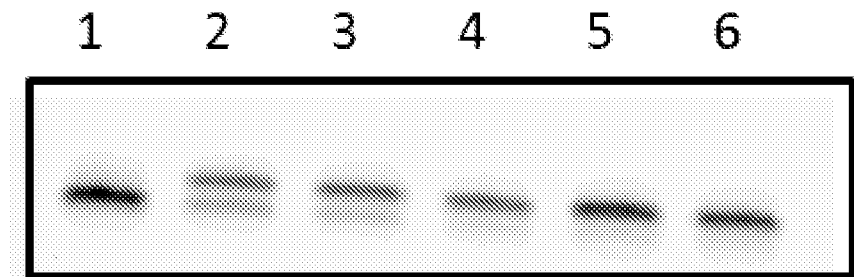


Figure 18f

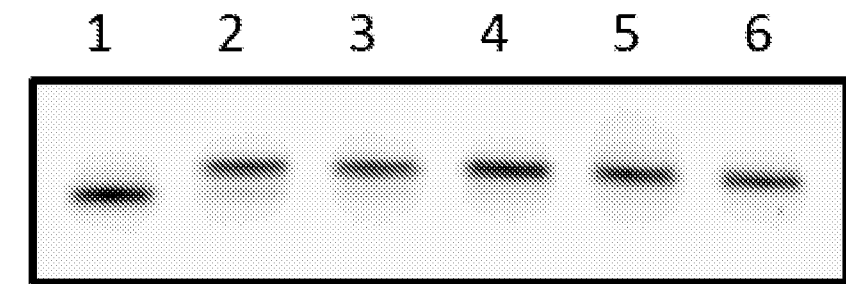
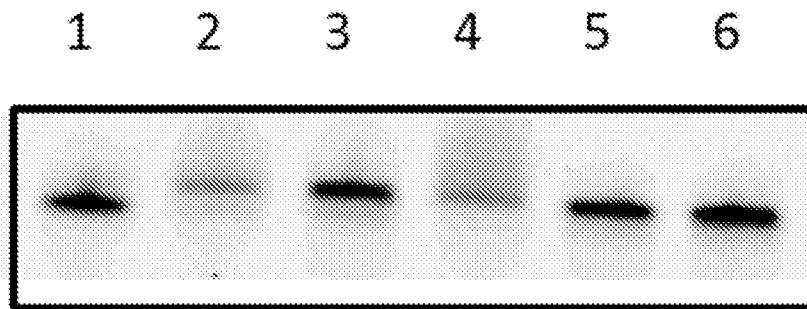


Figure 18g**Figure 18h**

	0 min	1 min	2 min	5 min	10 min	20 min
27°C without Mn ²⁺	0	5	10	20	35	40
37°C without Mn ²⁺	0	30	58	90	90	90
47°C without Mn ²⁺	0	30	65	90	90	90
27°C with Mn ²⁺	0	70	85	92	95	95
37°C with Mn ²⁺	0	85	90	>96	>96	>96
47°C with Mn ²⁺	0	85	90	>96	>96	>96

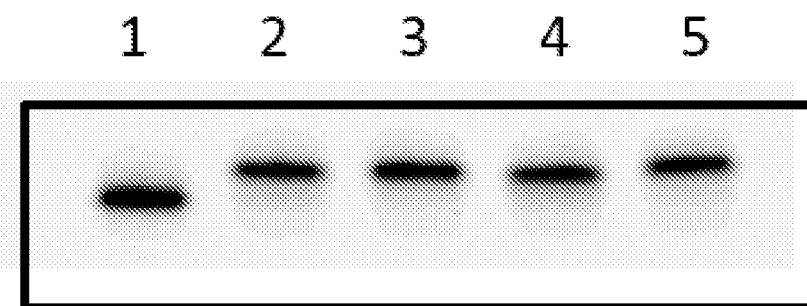
Figure 18i

Figure 18j

SEQ ID NO:	Oligonucleotide name	Sequence	Modification
22	Primer	5'-GCGACAGGTGACTGCAGC-3'	TAMRA at 5'-end (attached to terminal guanine)
23	Template-A	5'-CACATCACGTCGTAGTCXAGCTGCAG TCACCTGTCGC-3'	X = 2'- deoxyinosine
24	Template-G	5'-CACATCACGTCGTAGTCXGGCTGCAG TCACCTGTCGC-3'	X = 2'- deoxyinosine
25	Template-T	5'-CACATCACGTCGTAGTCXAGCTGCAG TCACCTGTCGC-3'	X = 2'- deoxyinosine
26	Template-C	5'-CACATCACGTCGTAGTCXAGCTGCAG TCACCTGTCGC-3'	X = 2'- deoxyinosine
27	Helper strand-T	5'-TCGACTACGACGTGATGTG-3'	None
28	Helper strand-C	5'-CCGACTACGACGTGATGTG-3'	None
29	Helper strand-A	5'-ACGACTACGACGTGATGTG-3'	None
30	Helper strand-G	5'-GCGACTACGACGTGATGTG-3'	None

Figure 19a

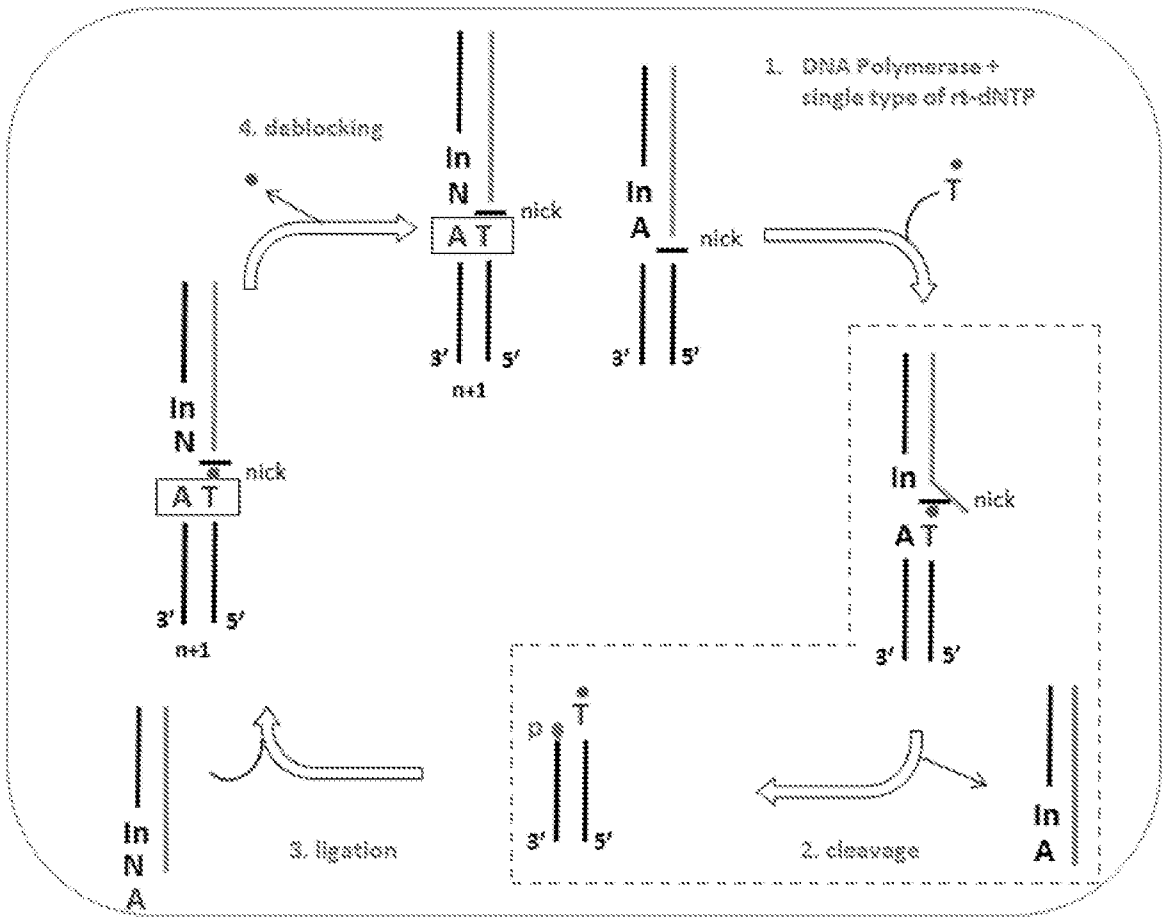


Figure 19b

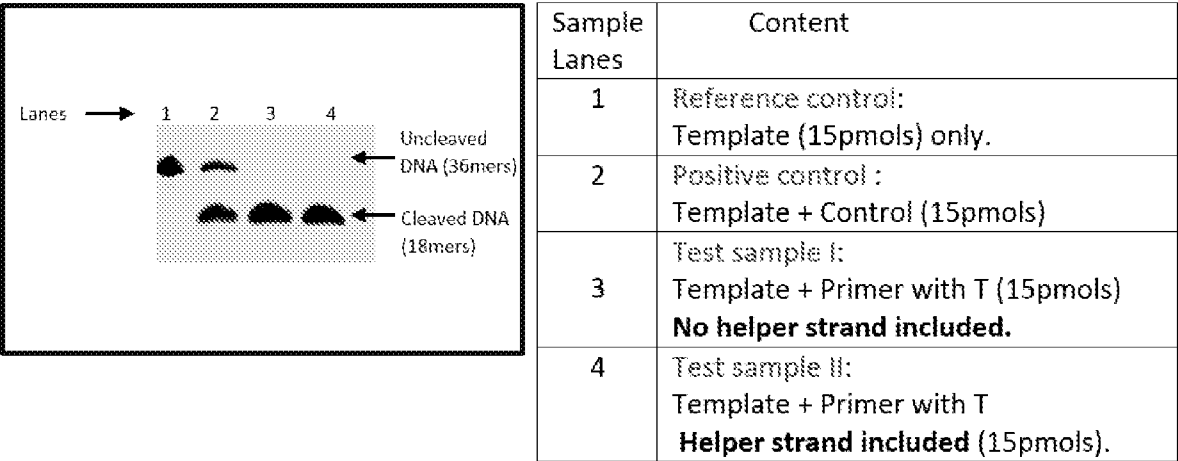


Figure 19c

Cleavage reaction combination	% Conversion of cleaved DNA
Full length positive control	80
Presence of helper strand	>99
Absence of helper strand	>99

Figure 19d

SEQ ID NO:	Oligonucleotide name	Sequence	Modification
31	Template	5'-CACATCACGTCGTAGTCXAGCT GCAGTCACCTGTCGC-3'	TAMRA at 5'-end (attached to terminal cytosine), X = deoxyinosine
32	Primer with T	5'-GCGACAGGTGACTGCAGCT-3'	None
33	Control	5'-GCGACAGGTGACTGCAGCTGA CTACGACGTGATGTG-3'	None
34	Helper strand	5'-TCGACTACGACGTGATGTG-3'	None

Figure 20a

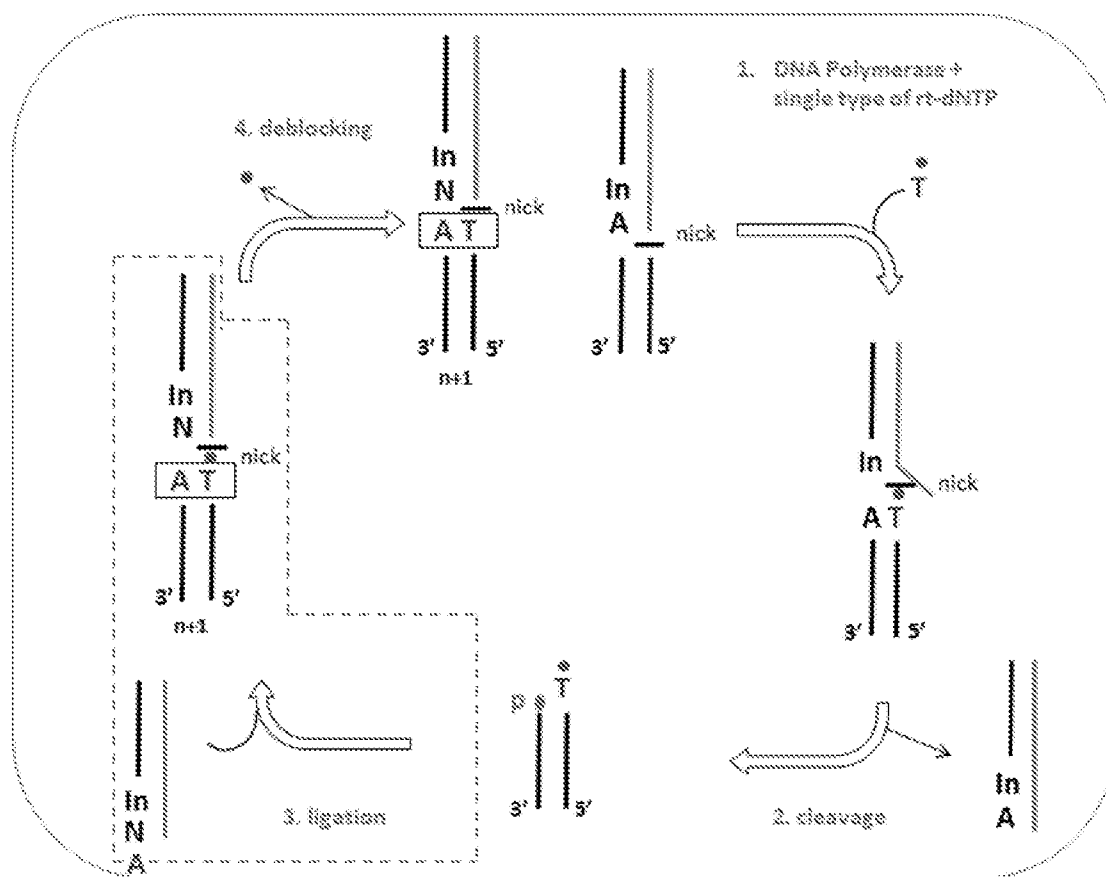


Figure 20b

SEQ ID NO:	Oligonucleotide name	Sequence	Modification
35	Phosphate strand	5'-GCTGCAGTCACCTGTCGC-3'	TAMRA at 3'-end (attached to terminal cytosine), phosphate at 5' end
36	Primer with T	5'-GCGACAGGTGACTGCAGCT-3'	None
37	Inosine strand	5'-CACATCACGTCGTAGTXGA-3'	X = 2'-deoxyinosine
38	Helper strand	5'-CCACTACGACGTGATGTG-3'	None

Figure 21a

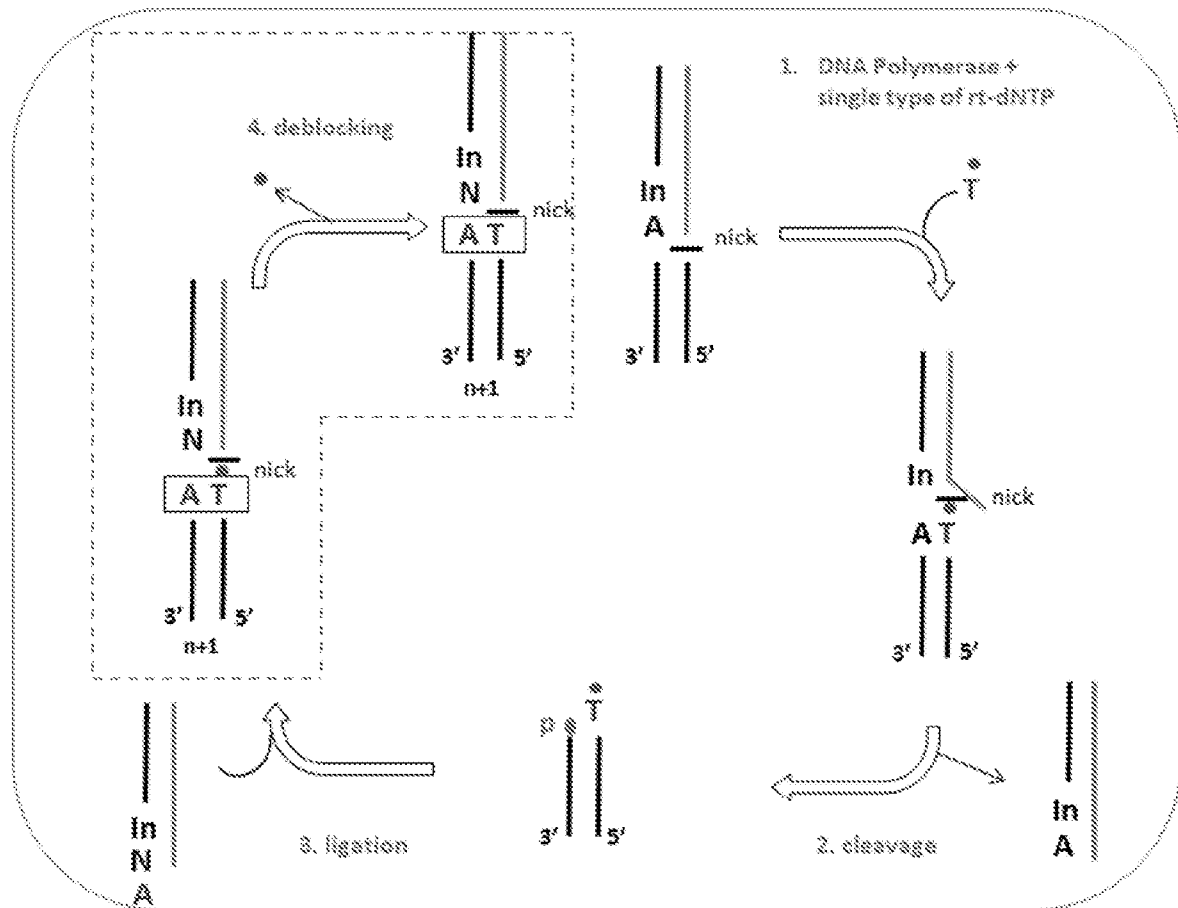


Figure 21b

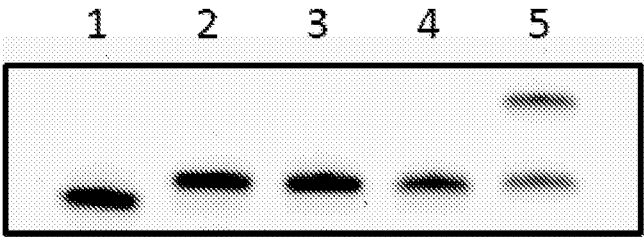


Figure 21c

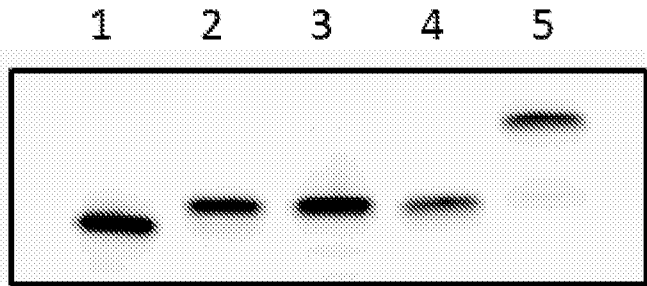


Figure 21d

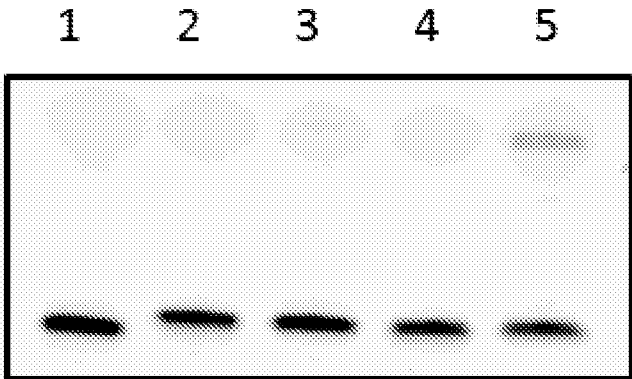


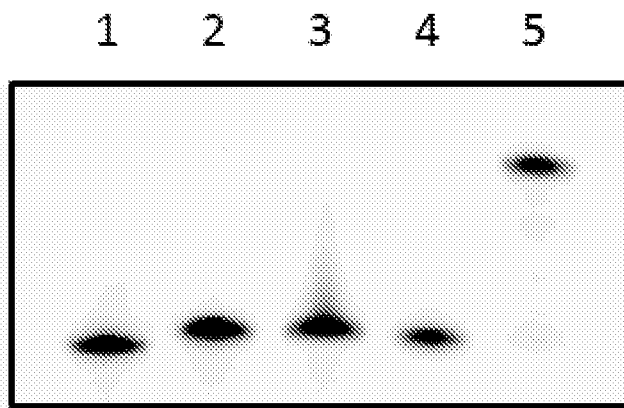
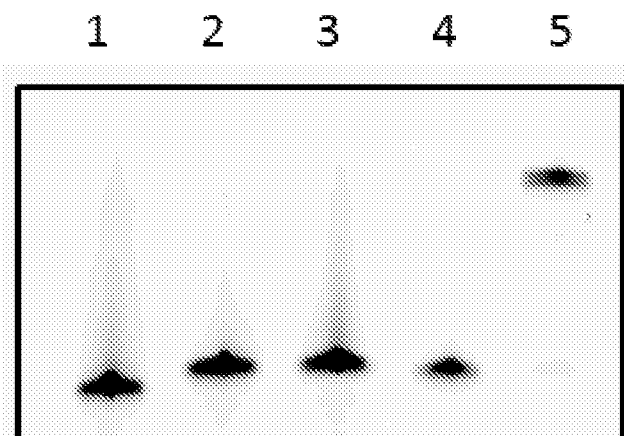
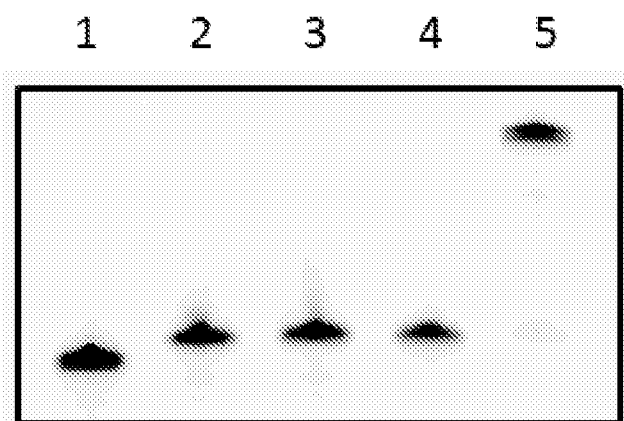
Figure 21e**Figure 21f****Figure 21g**

Figure 21h

Incorporation	Deprotection by 50 mM TCEP	Deprotection by 300 mM TCEP
3'-O-azidomethyl-dTTP	50%	95%
3'-O-azidomethyl-dCTP	20%	95%
3'-O-azidomethyl-dATP	Not tested	95%
3'-O-azidomethyl-dGTP	Not tested	95%

Figure 21i

SEQ ID NO:	Oligonucleotide name	Sequence	Modification
39	Primer	5'-GCGACAGGTGACTGCAGC-3'	TAMRA at 5'-end (attached to terminal cytosine)
40	Template-A	5'-CACATCACGTCGTCAGTCAAGCTGCAGTCACCTGTCGC-3'	None
41	Template-G	5'-CACATCACGTCGTCAGTCCGGCTGCAGTCACCTGTCGC-3'	None
42	Template-T	5'-CACATCACGTCGTCAGTCTGCTGCAGTCACCTGTCGC-3'	None
43	Template-C	5'-CACATCACGTCGTCAGTCCGCTGCAGTCACCTGTCGC-3'	None

Figure 22a

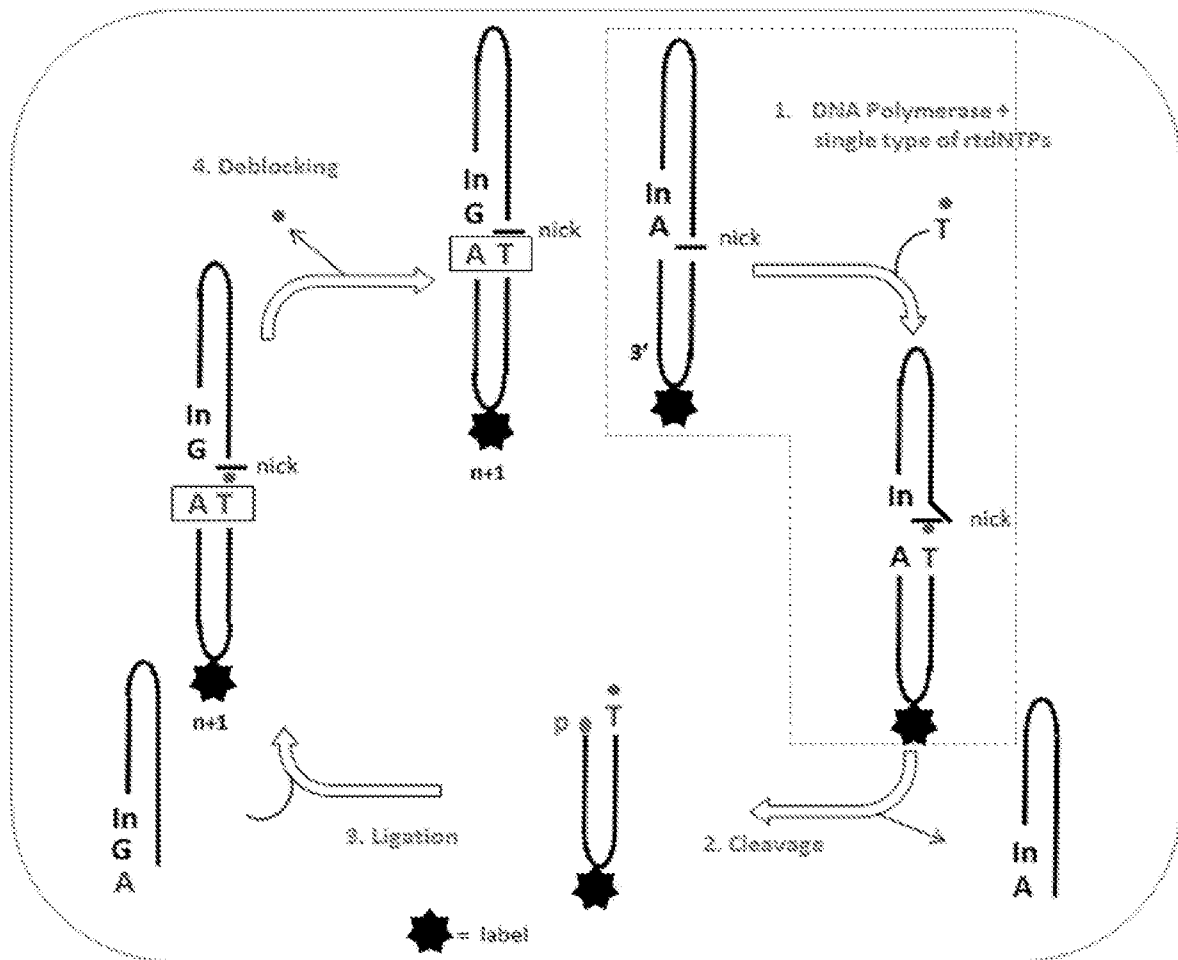
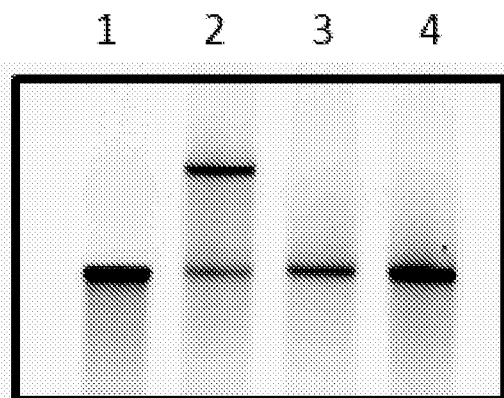


Figure 22b**Figure 22c**

SEQ ID NO:	Oligonucleotide name	Sequence	Modification
44	Dual hairpin model for incorporation	5'-TCGACTACGACGTGACTTTTAGTCACGTCGTAGTCXAGCTGCAGTCACCTGCTGCTTYTGCAGCAGGTGACTGCAGC-3'	X = 2'-deoxyinosine Y = Tamra-dT

Figure 23a

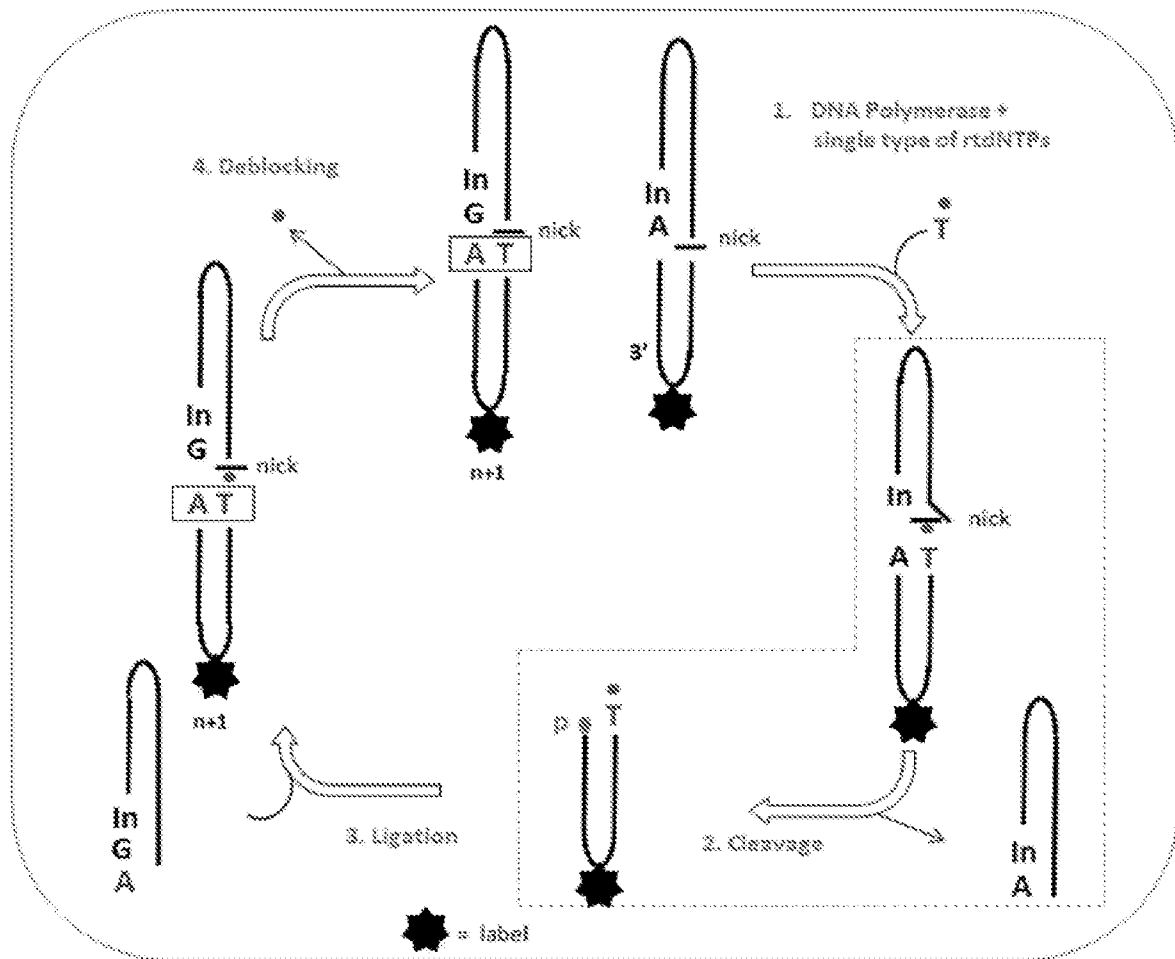
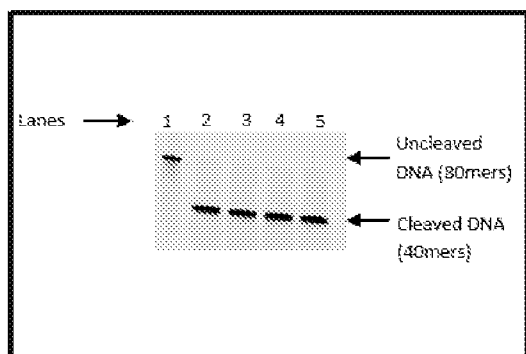


Figure 23b



Sample Lanes	Content
1	Reference control: Template (15pmols) only.
2	Test Sample i: Cleaved hairpin template after 5mins incubation (15pmols)
3	Test sample i: Cleaved hairpin template after 10mins incubation (15pmols)
4	Test sample i: Cleaved hairpin template after 30mins incubation (15pmols)
5	Test sample i: Cleaved hairpin template after 1hr incubation (15pmols)

Figure 23c

SEQ ID NO:	Oligonucleotide name	Sequence	Modification
45	Dual hairpin model for incorporation	5'- TCGACTACGACGTGACTTTTAGTCAC GTCGTAGTCXAGCTGCAGTCACCTGCTG CTTYTTGCAGCAGGTGACTGCAGCT-3'	X = 2'- deoxyinosine Y = Tamra-dT

Figure 24a

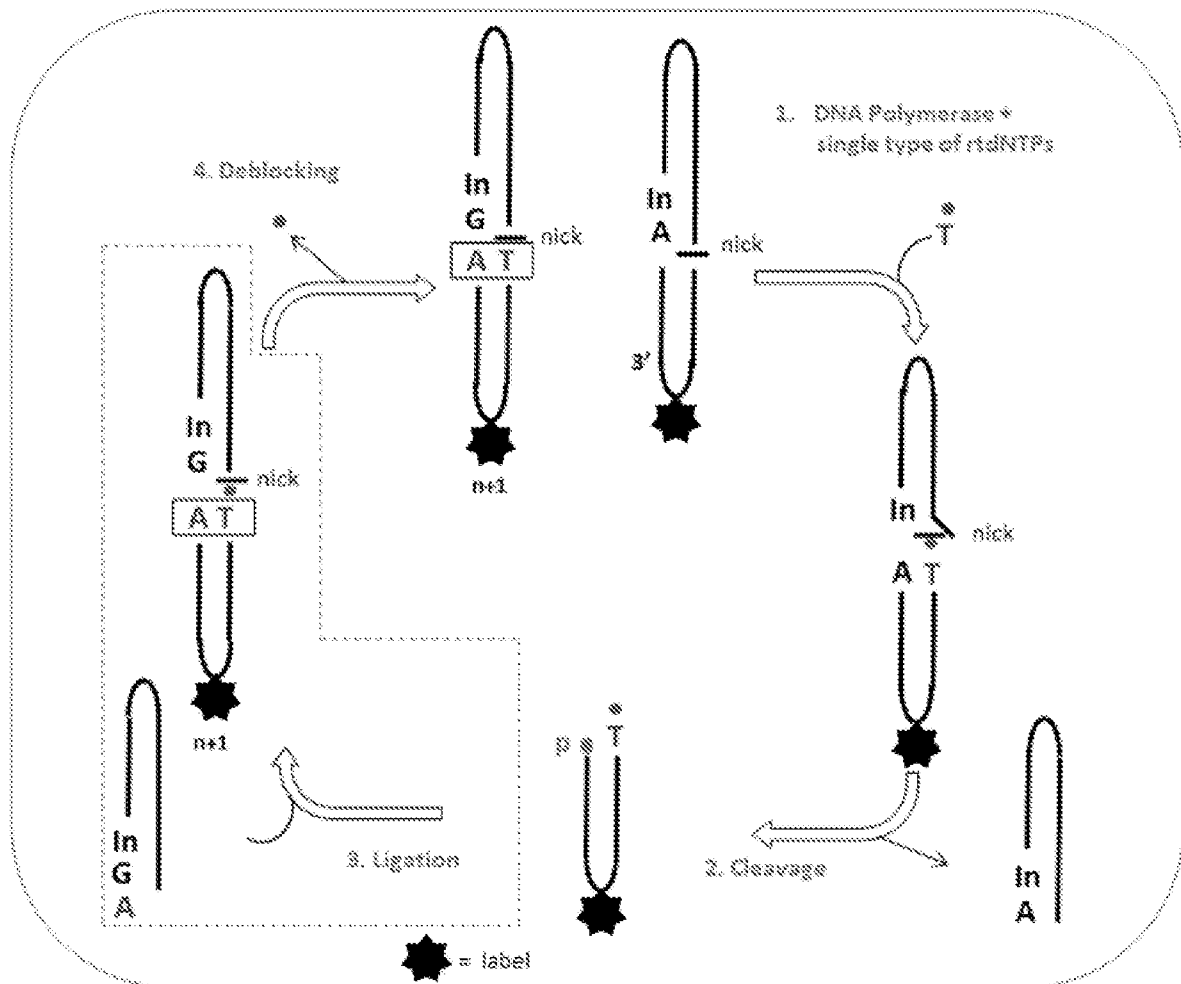


Figure 24b

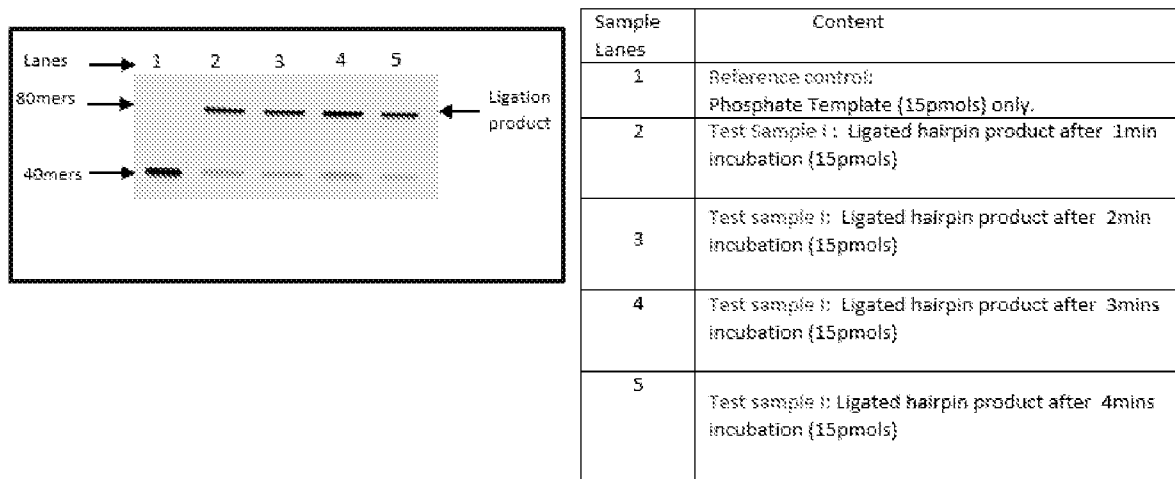


Figure 24c

SEQ ID NO:	Oligonucleotide name	Sequence	Modification
46	Phosphate hairpin	5'-GCTGCAGTCACCTGCTGCTTYT TGCAGCAGGTGACTGCAGCT-3'	Y = Tamra-dT phosphate
47	Inosine Hairpin	5'- CCGACTACGACGTGACTTTTAGT CACGTCGTAGTCXGA-3'	X = 2-deoxyinosine

Figure 25a

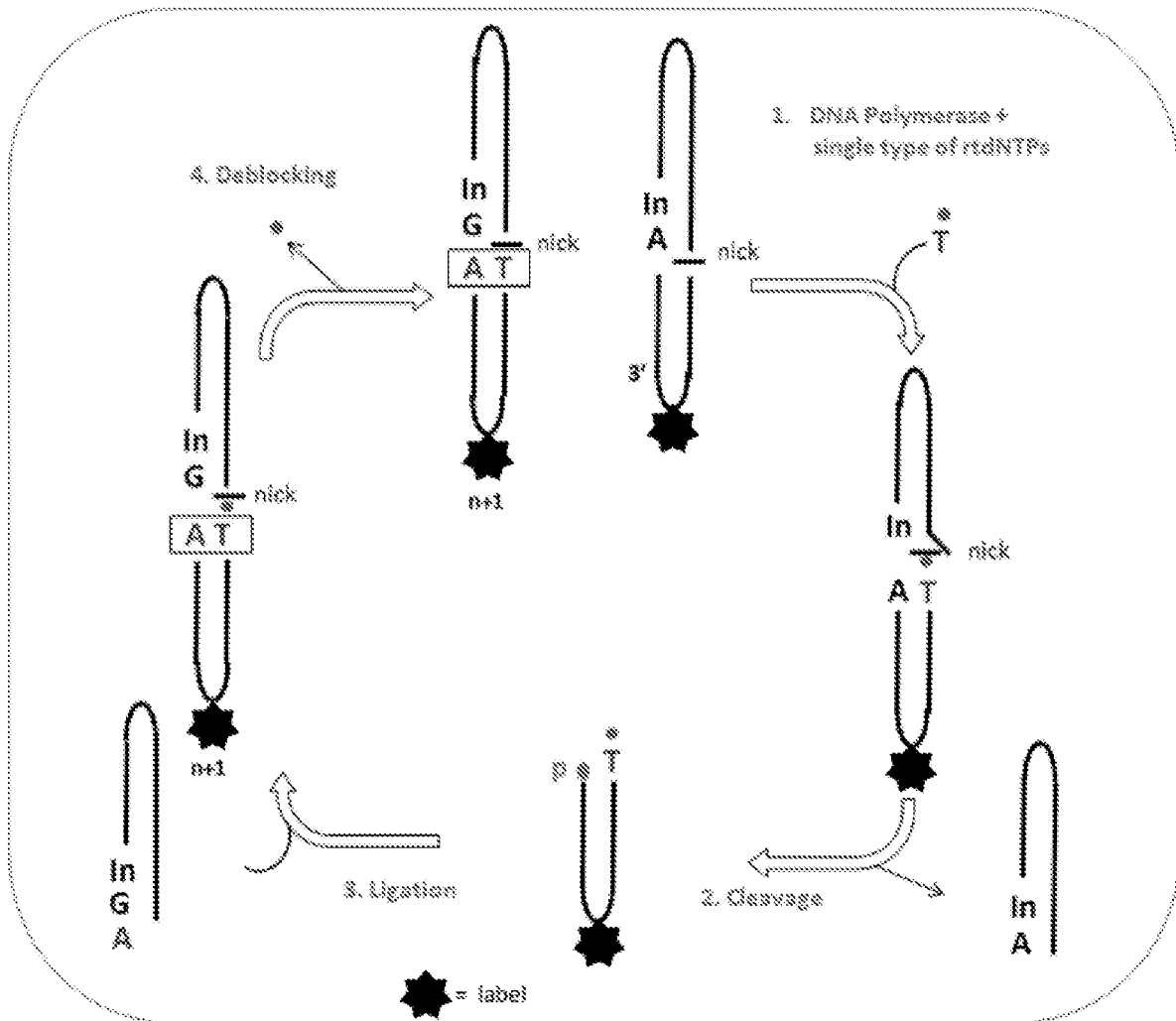


Figure 25b

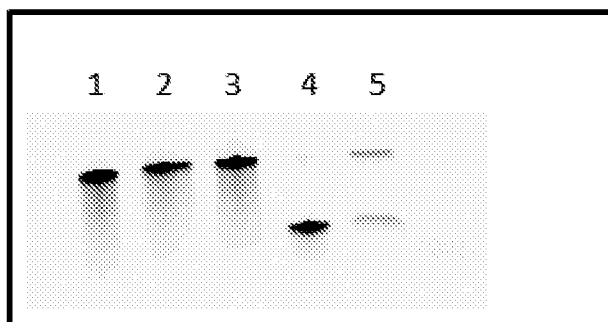


Figure 25c

SEQ ID NO:	Oligonucleotide name	Sequence	Modification
48	Double hairpin model	5'- TCGACTACGACGTGACTTTTAGTCAC GTCGTAGTCXAGCTGCAGTCACCTGCTGC TTYTTGCAGCAGGTGACTGCAGC-3'	X = 2'- deoxyinosine Y = Tamra-dT
49	Strand for ligation	5'- CCGACTACGACGTGACTTTTAGTCAC GTCGTAGTCXGA-3'	X = 2'- deoxyinosine

Figure 26a

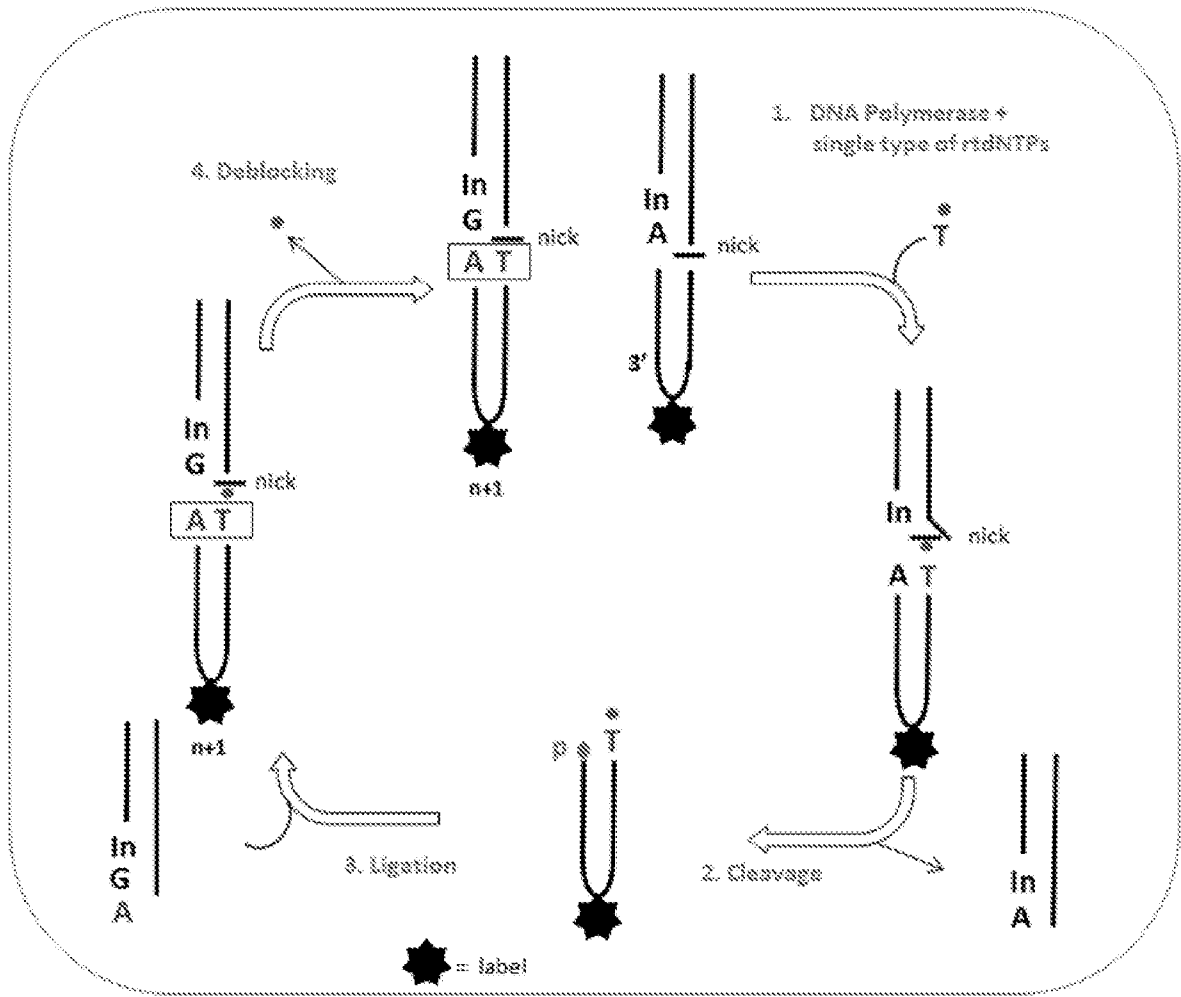


Figure 26b

SEQ ID NO:	Oligonucleotide name	Sequence	Modification
50	Single hairpin model for incorporation	5'- AGTCACGTCGTAGTCXAGCTGCAGTC ACCTGCTGCTTYTTGCAGCAGGTGACTGCAG C-3'	X = 2'-deoxyinosine Y = Tamra-dT
51	Helper strand	5'- TCGACTACGACGTGACT-3'	
52	Strand for ligation	5'- AGTCACGTCGTAGTCXGA	X = 2'-deoxyinosine
53	Helper strand for ligation	5'- CCGACTACGACGTGACT-3'	

Figure 27a

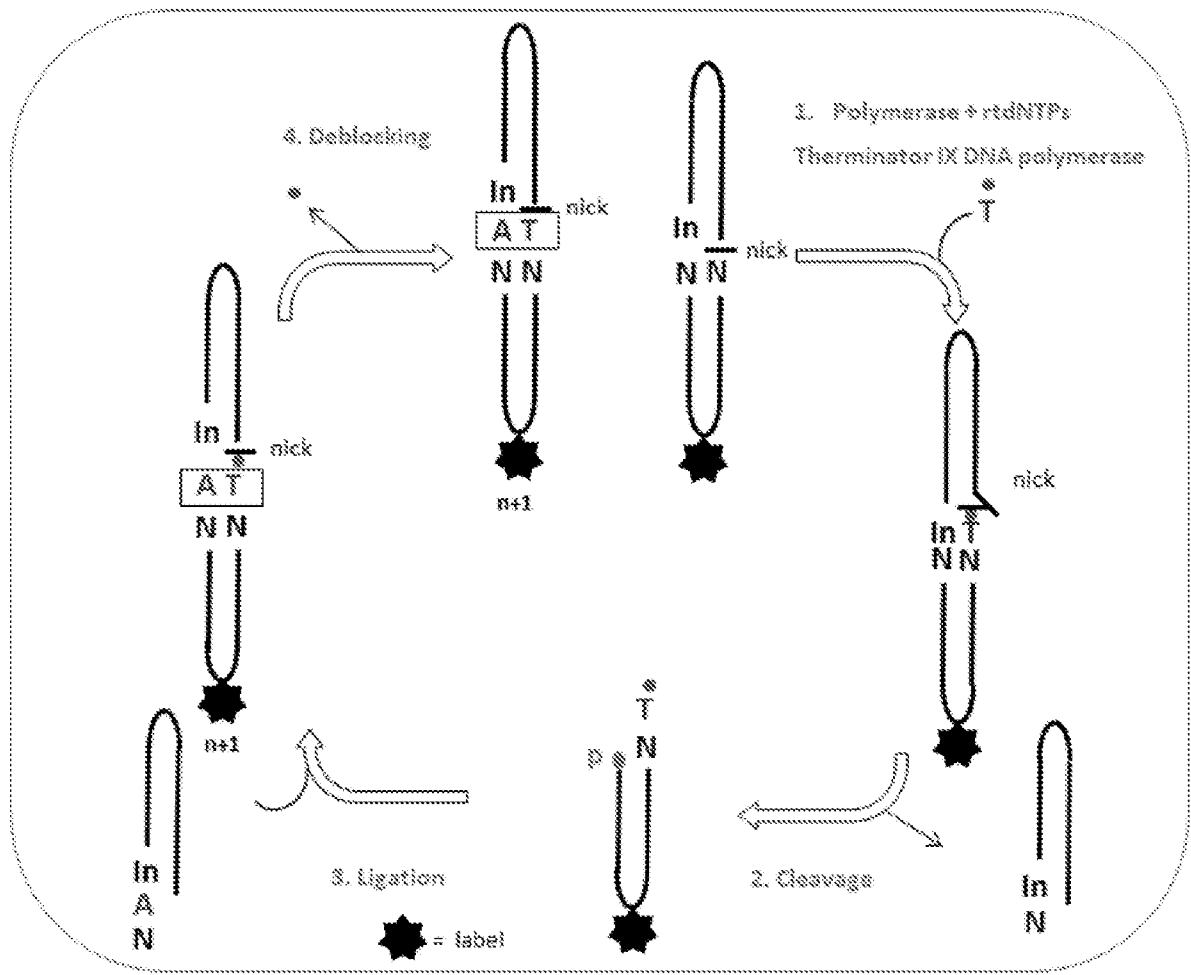


Figure 27b

SEQ ID NO:	Oligonucleotide name	Sequence	Modification
54	Double hairpin model	5'- CGACTACGACGTGACTTTTAGTCAC GTCGTAGTCXAGCTGCAGTCACCTGCTGC TTYTTGCAGCAGGTGACTGCAGCT-3'	X = 2'- deoxyinosine Y = Tamra-dT
55	Strand for ligation	5'- CGACTACGACGTGACTTTTAGTCAC GTCGTAGTCXAA-3'	X = 2'- deoxyinosine

Figure 28a

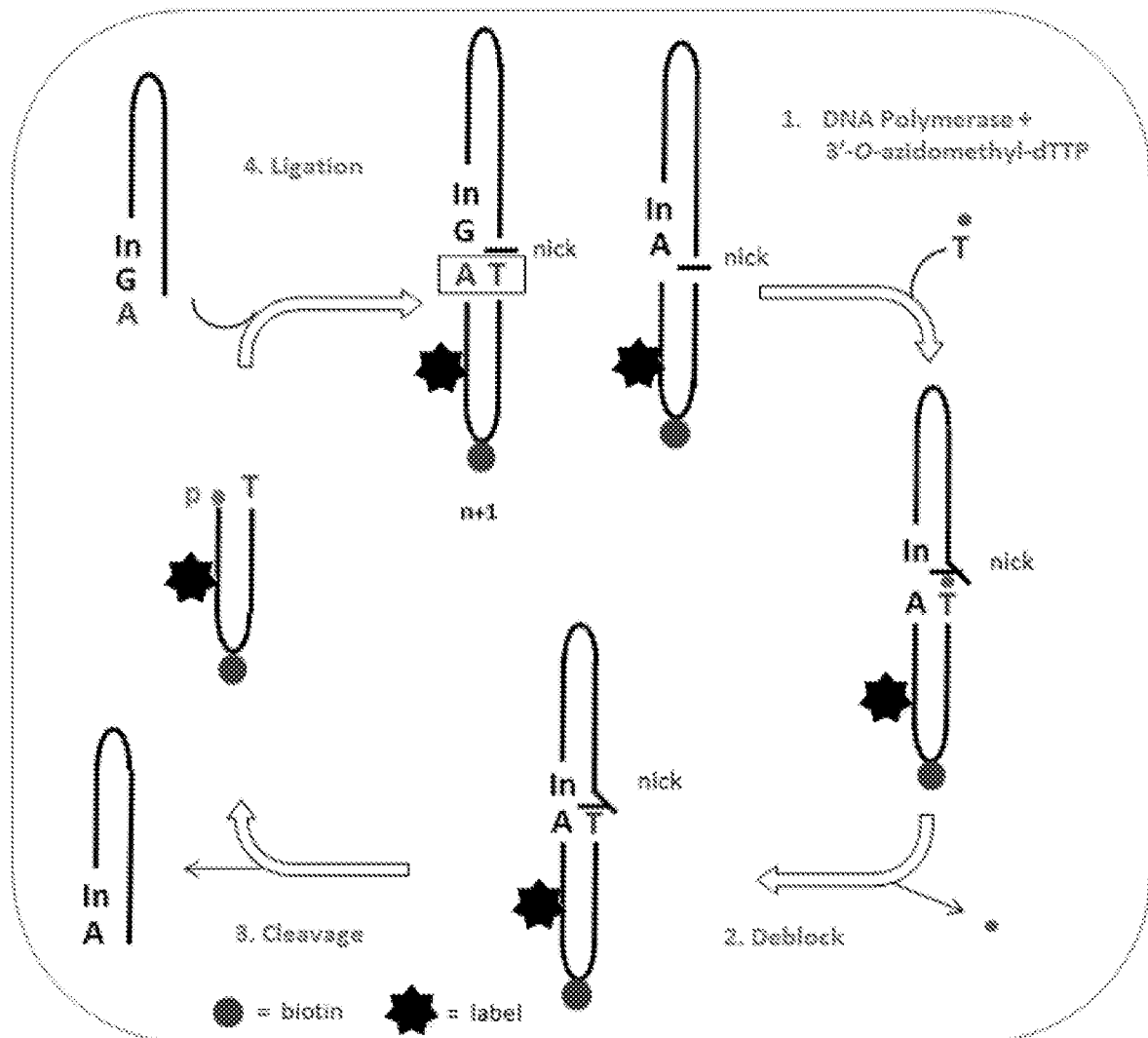


Figure 28b

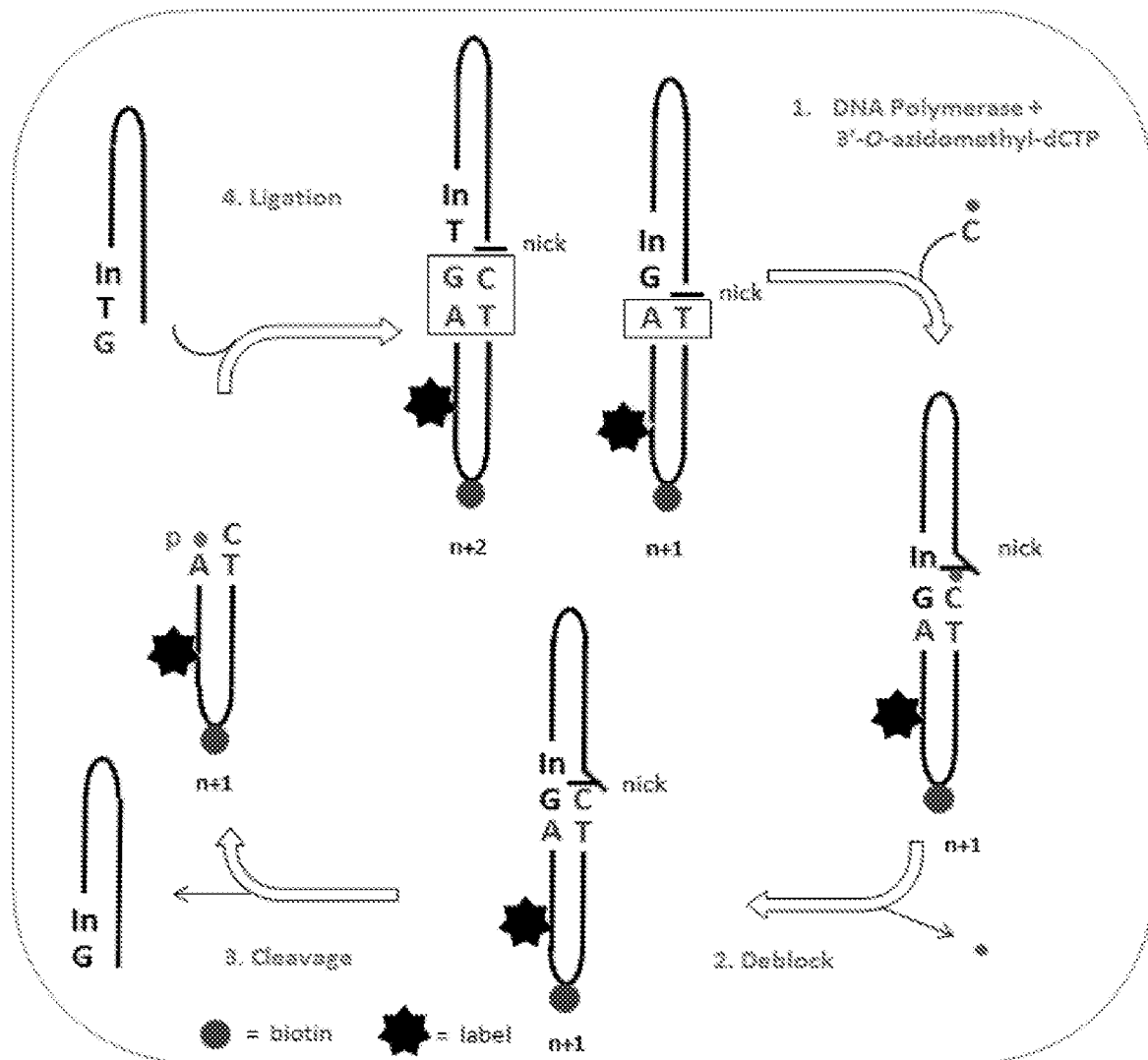


Figure 28c

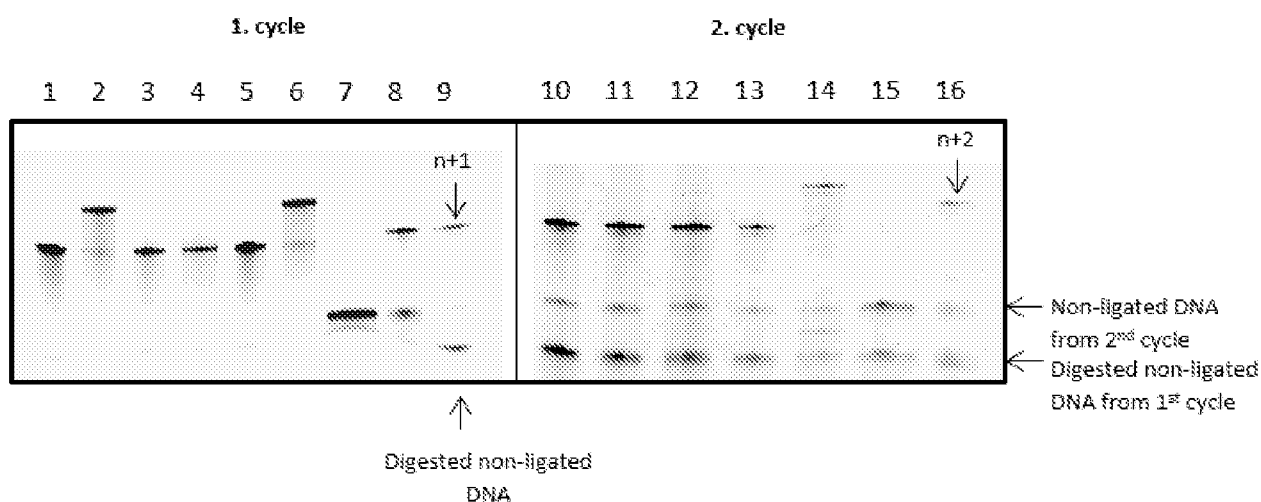


Figure 28d

SEQ ID NO:	Oligonucleotide name	Sequence	Modification
56	Double hairpin model	5'- CGACTACGACGTGACTTTTAGTCAC GTCGTAGTCXAGCTGCAGTCACCTGCYG CTTZTTGCAGCAGGTGACTGCAGCT-3'	X = 2'- deoxyinosine Y = Tamra-dT Z = Biotin-dT
57	Strand for ligation in the 1 st cycle	5'- CCGACTACGACGTGACTTTTAGTCAC GTCGTAGTCXGA-3'	X = 2'- deoxyinosine
58	Strand for ligation in the 2 nd cycle	5'- ACGAGTGACCTGGTTTTTTTTTTTTTTTT TTTTTTTTTTTTTTTTTACCAGGTCAC CXTG-3'	X = 2'- deoxyinosine

Figure 29

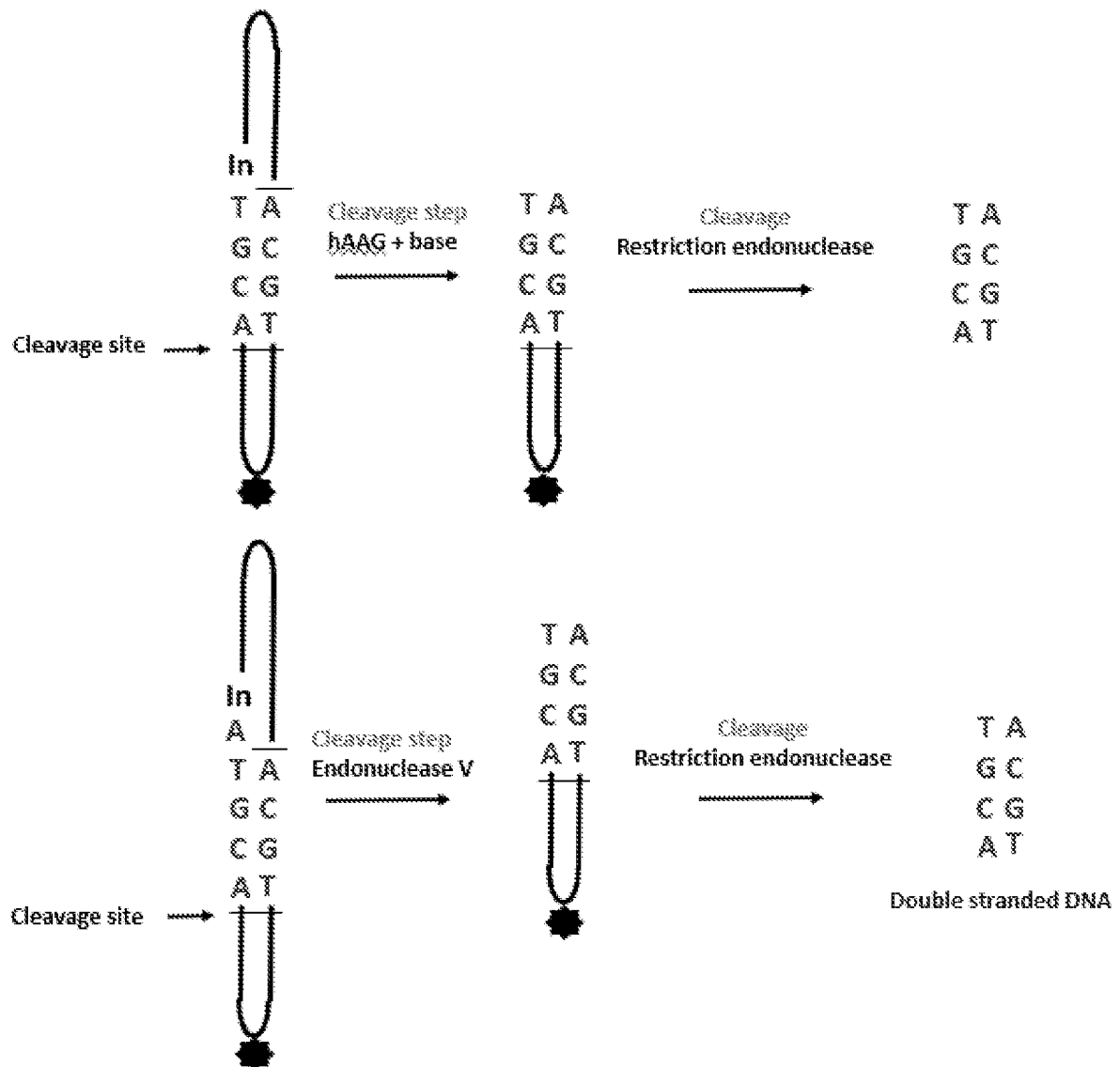


Figure 30

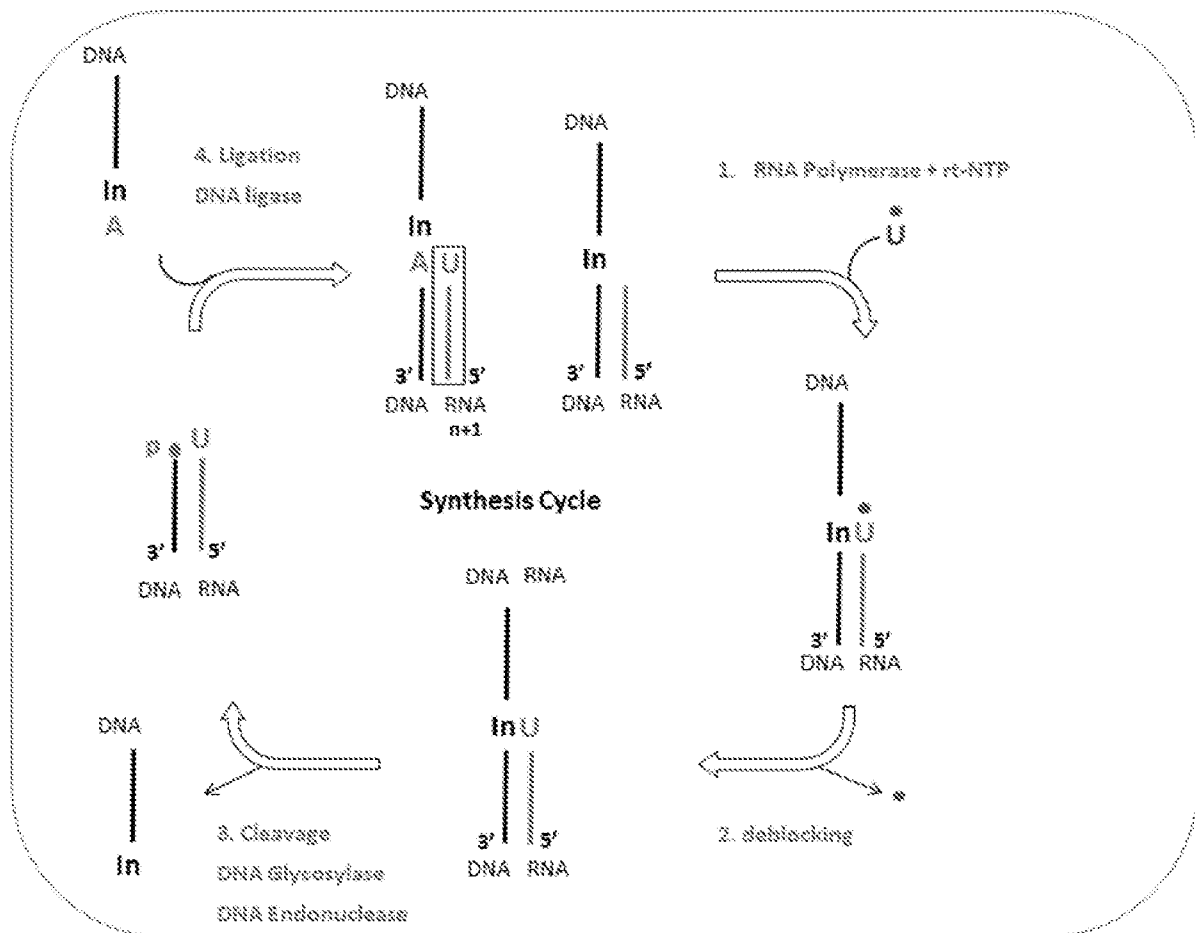


Figure 31

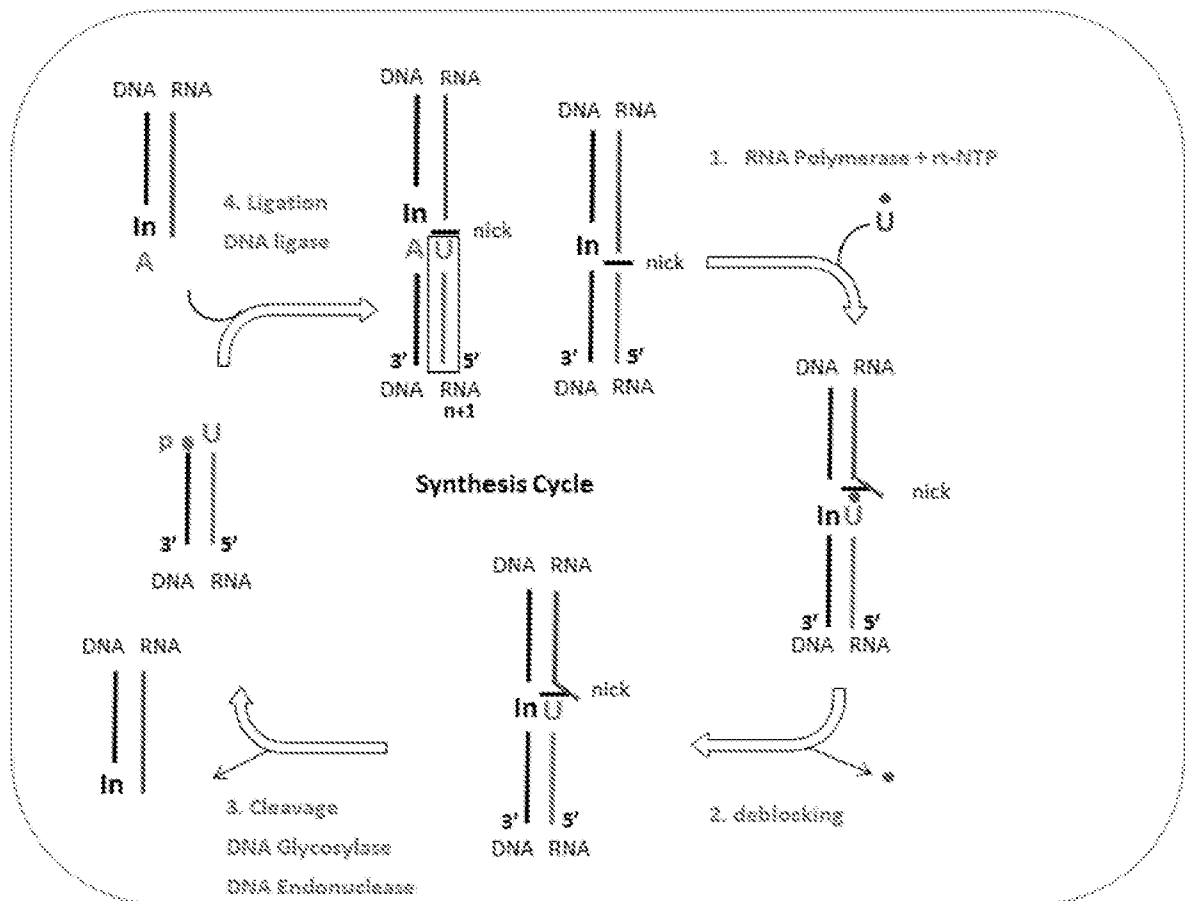


Figure 32

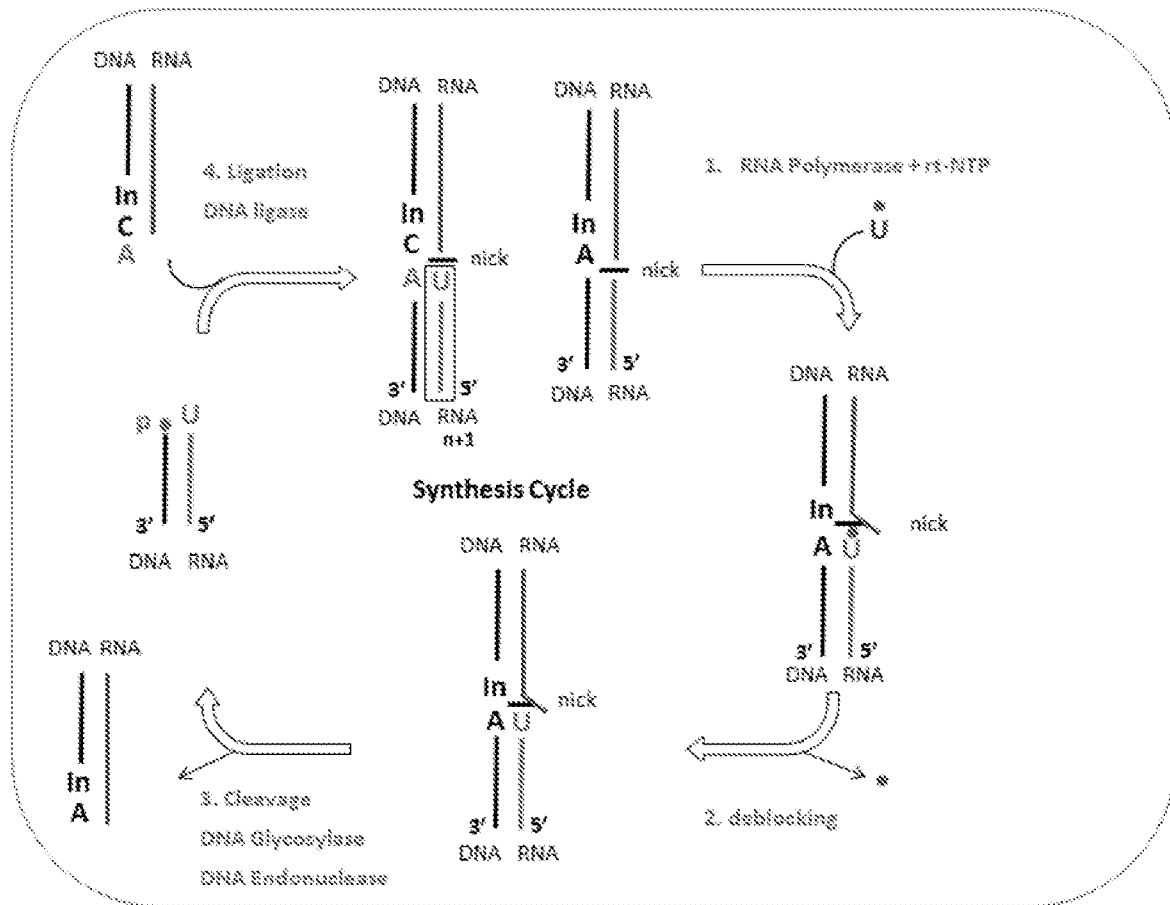


Figure 33

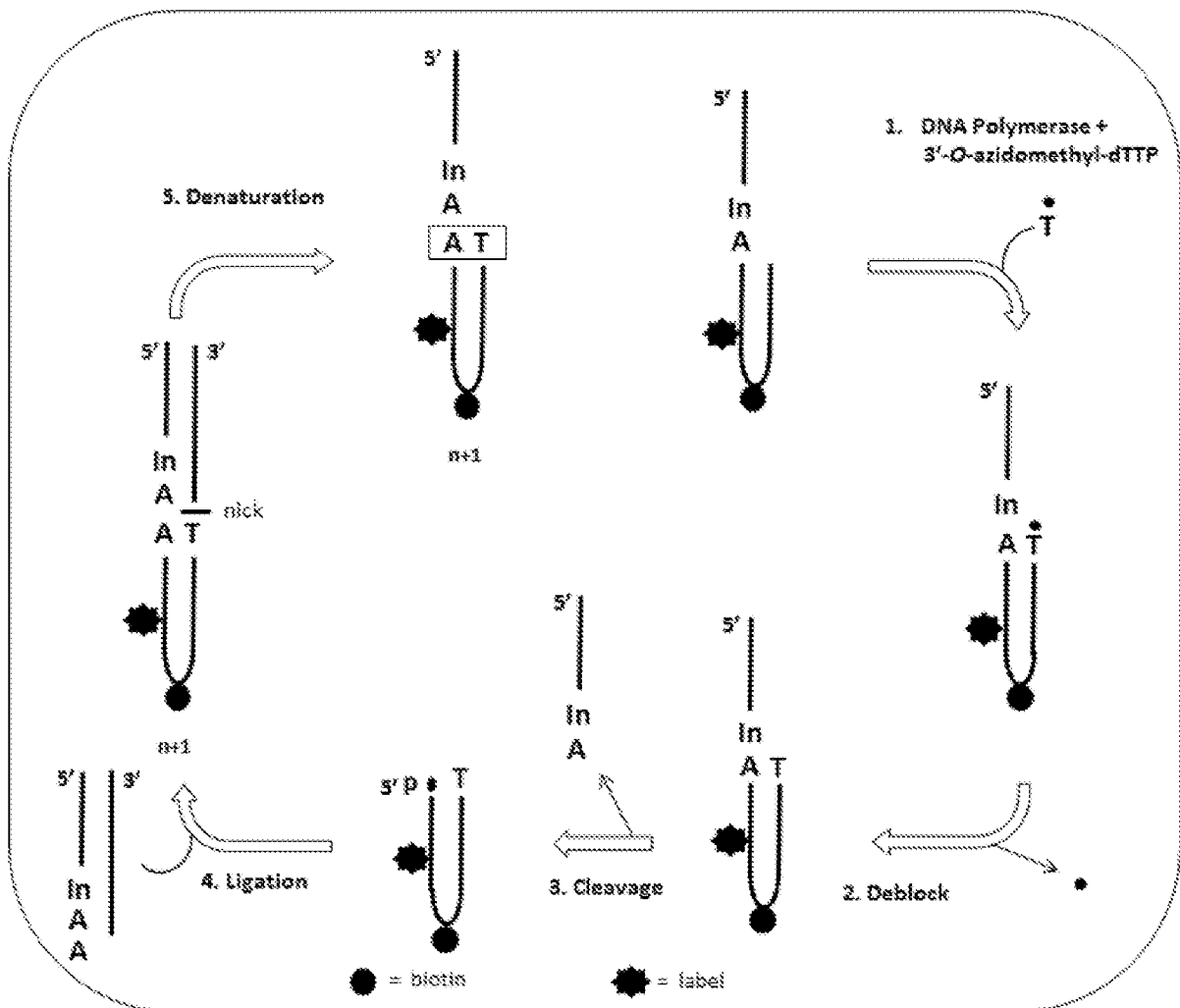


Figure 34

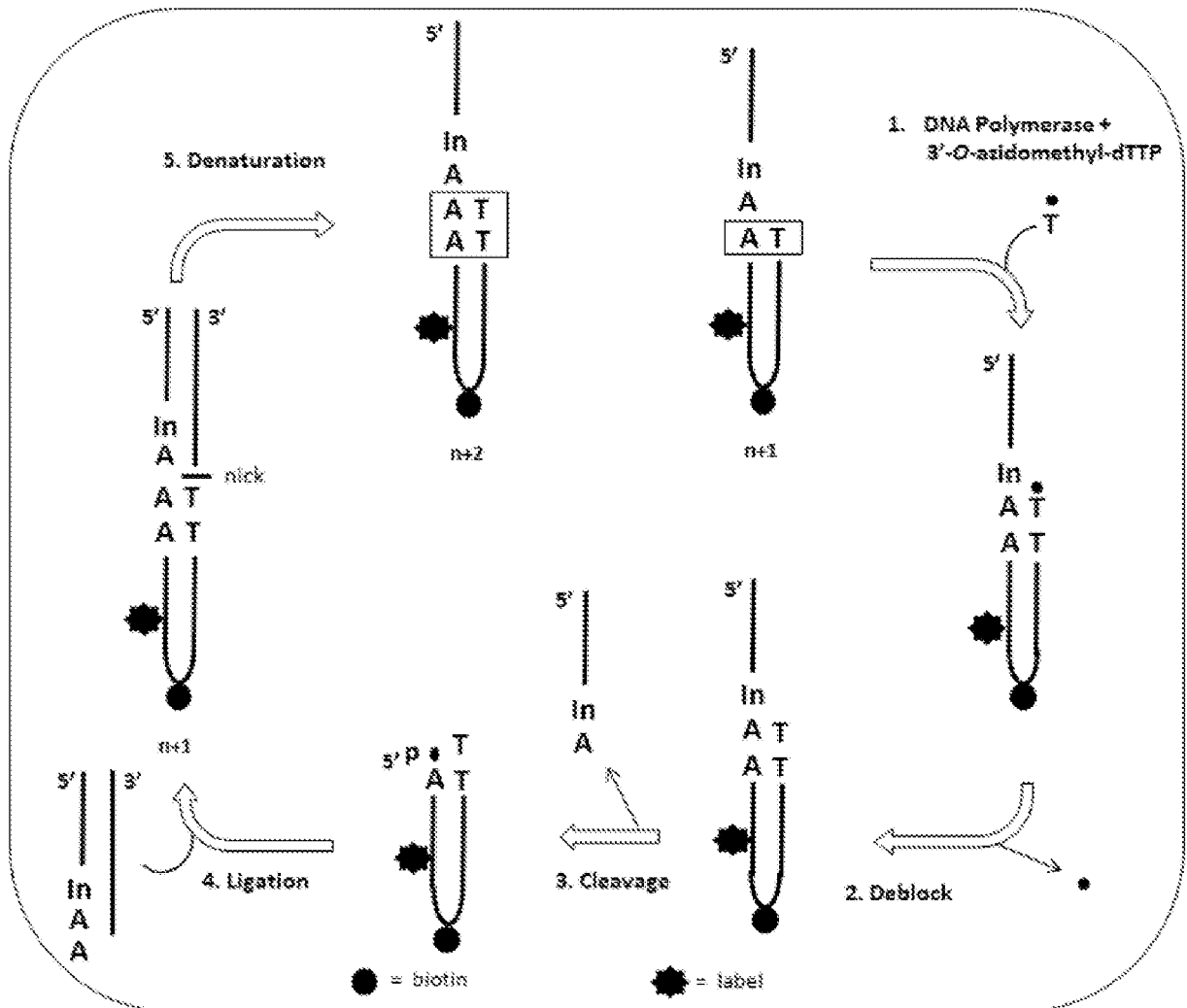


Figure 35

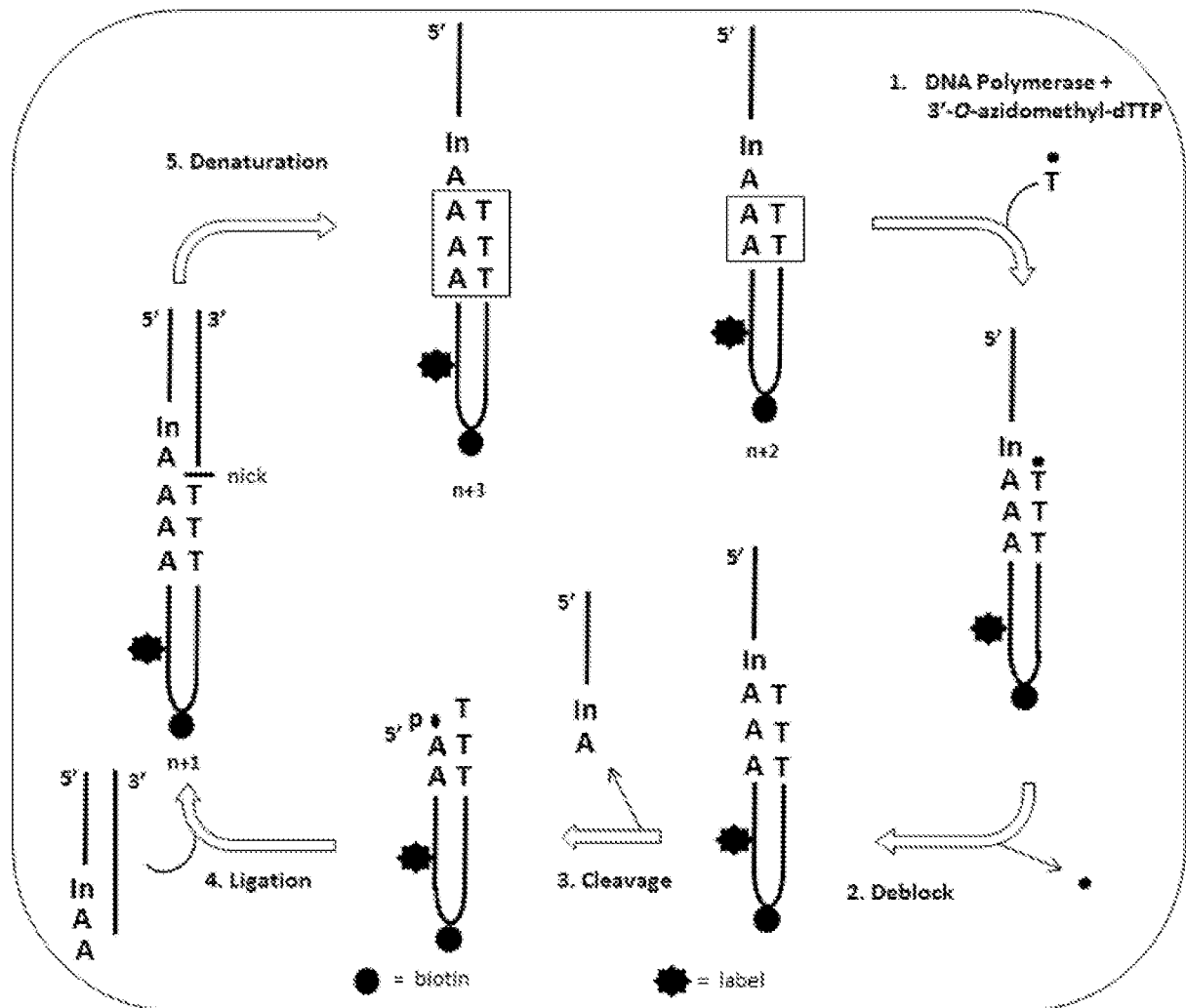


Figure 36

SEQ ID NO:	Oligonucleotide name	Sequence	Modification
59	Single hairpin model	5'- AGTCACGTCGTCAGTCXAGCTGCAGTC ACCTGCYGCTTZTTGCAGCAGGTGACTG CAGC-3'	X = 2'-deoxyinosine Y = Tamra-dT Z = Biotin-dT
60	Inosine strand for ligation	5'- AGTCACGTCGTCAGTCXAA-3'	X = 2'-deoxyinosine
51	Helper strand for ligation	5'- TCGACTACGACGTGACT-3'	

Figure 37

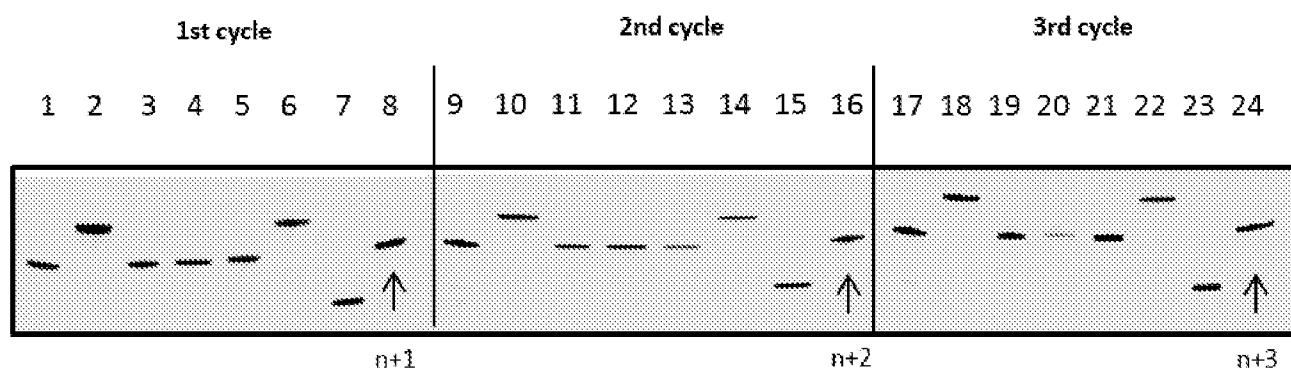


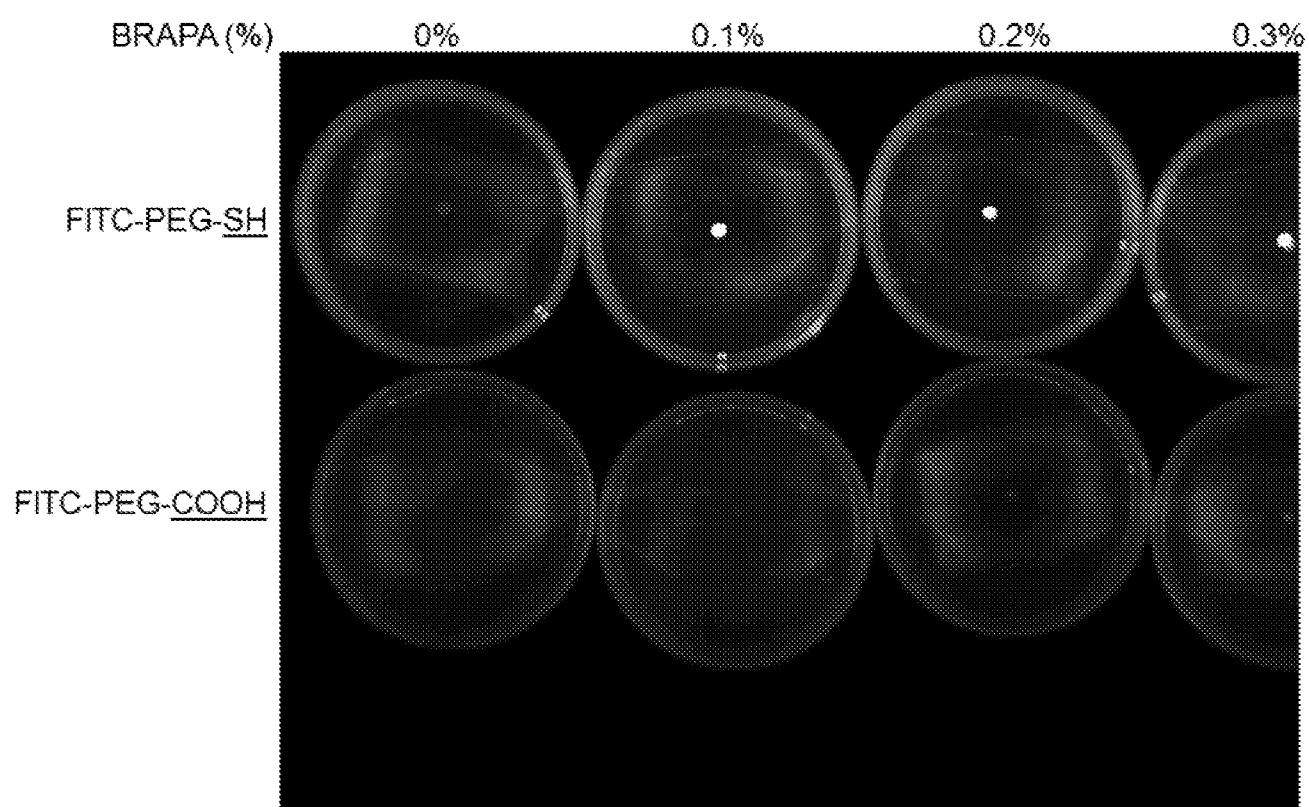
Figure 38

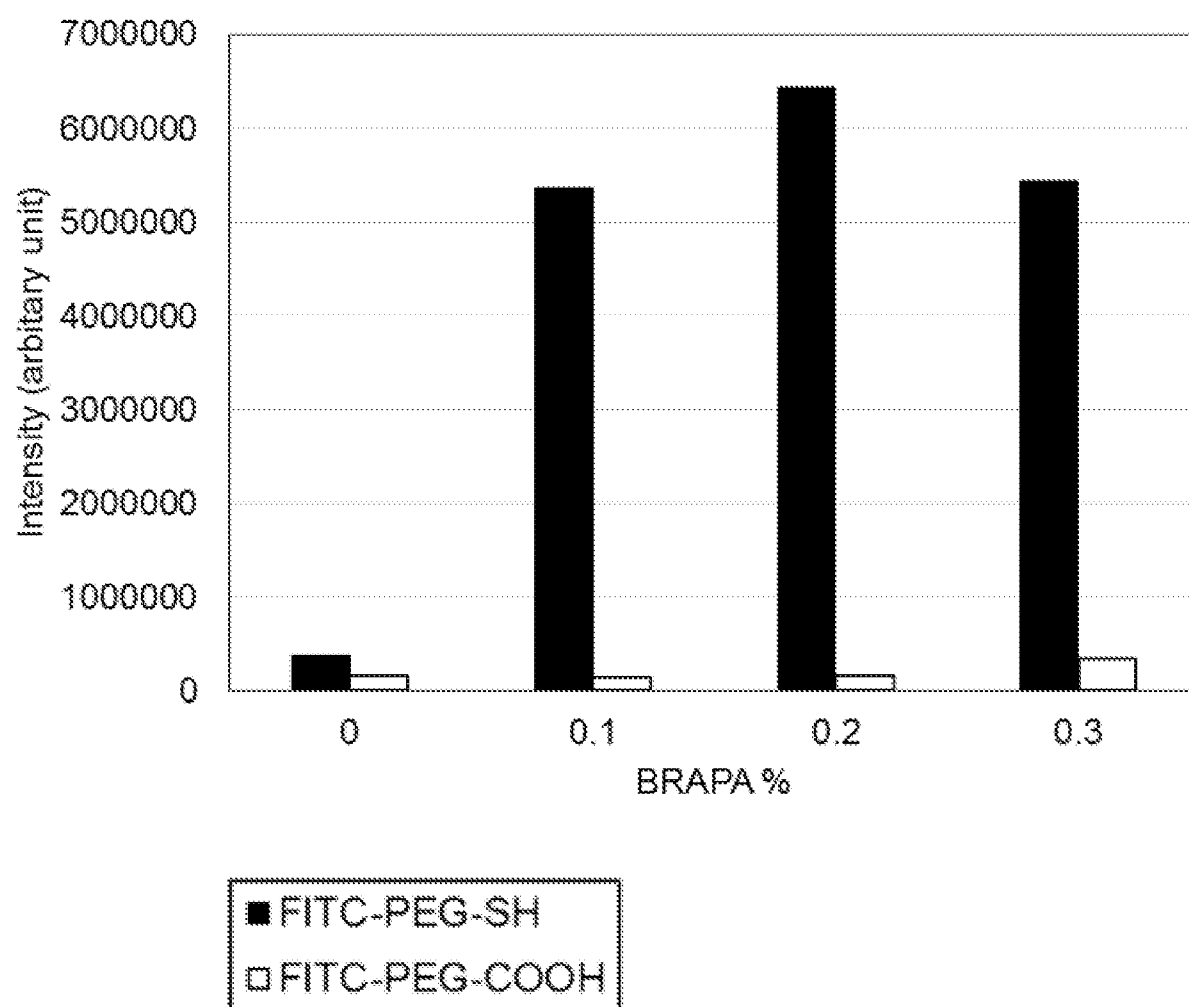
Figure 39

Figure 40a

Sample ID	Sequence	SEQ ID NO:
A (BRAPA control)	5'-Alexa647- AGTCACGTCGTAGTCIAGCTGCAGTCACCTGCTGCTTTT T*T*T*T*T*T*TTTTGCAGCAGGTGACTGCAGC	SEQ ID NO: 61
B (BRAPA modified 1)	5'-Alexa647- AGTCACGTCGTAGTCIAGCTGCAGTCACCTGCTGCTTTT T*T*T*T*T*T*TTTTGCAGCAGGTGACTGCAGC	SEQ ID NO: 61
C (BRAPA modified 2)	5'-Alexa647- AGTCACGTCGTAGTCIAGCTGCAGTCACCTGCTGCTTTT T*T*T*T*T*T*TTTTGCAGCAGGTGACTGCAGC	SEQ ID NO: 61
D (Oligomer control)	5'-Alexa647- AGTCACGTCGTAGTCIAGCTGCAGTCACCTGCTGCTTTT TTTTTTTTTGCAGCAGGTGACTGCAGC	SEQ ID NO: 62
* = phosphorothioate bond; I = 2'-deoxyinosine		

Figure 40b

Sample ID	Sequence	SEQ ID NO:
A (BRAPA control)	5'-Alexa647- AGTCACGTCGTAGTCIAGCTGCAGTCACCTGCTG CTTZZTGCAGCAGGTGACTGCAGC-3'	SEQ ID NO: 63
B (BRAPA modified 1)	5'-Alexa647- AGTCACGTCGTAGTCIAGCTGCAGTCACCTGCTG CTTZZTGCAGCAGGTGACTGCAGC-3'	SEQ ID NO: 63
C (BRAPA modified 2)	5'-Alexa647- AGTCACGTCGTAGTCIAGCTGCAGTCACCTGCTG CTTZZTGCAGCAGGTGACTGCAGC-3'	SEQ ID NO: 63
D (Oligomer control)	5'-Alexa647- AGTCACGTCGTAGTCIAGCTGCAGTCACCTGCTG CTTYTTGCAGCAGGTGACTGCAGC-3'	SEQ ID NO: 64
Z = 5''-thiophosphate-Sp9-Sp9-Sp9-5-methylC, Y = 5''-phosphate-Sp9-Sp9-Sp9-5-methylC; Sp9 = Spacer 9; I = 2'-deoxyinosine		

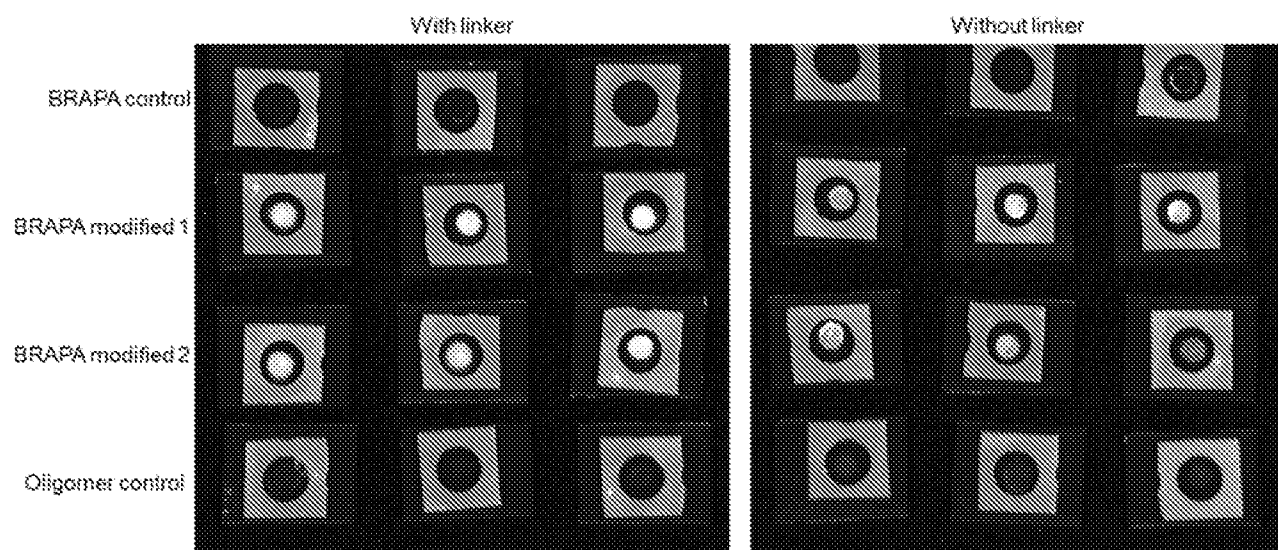
Figure 41

Figure 42

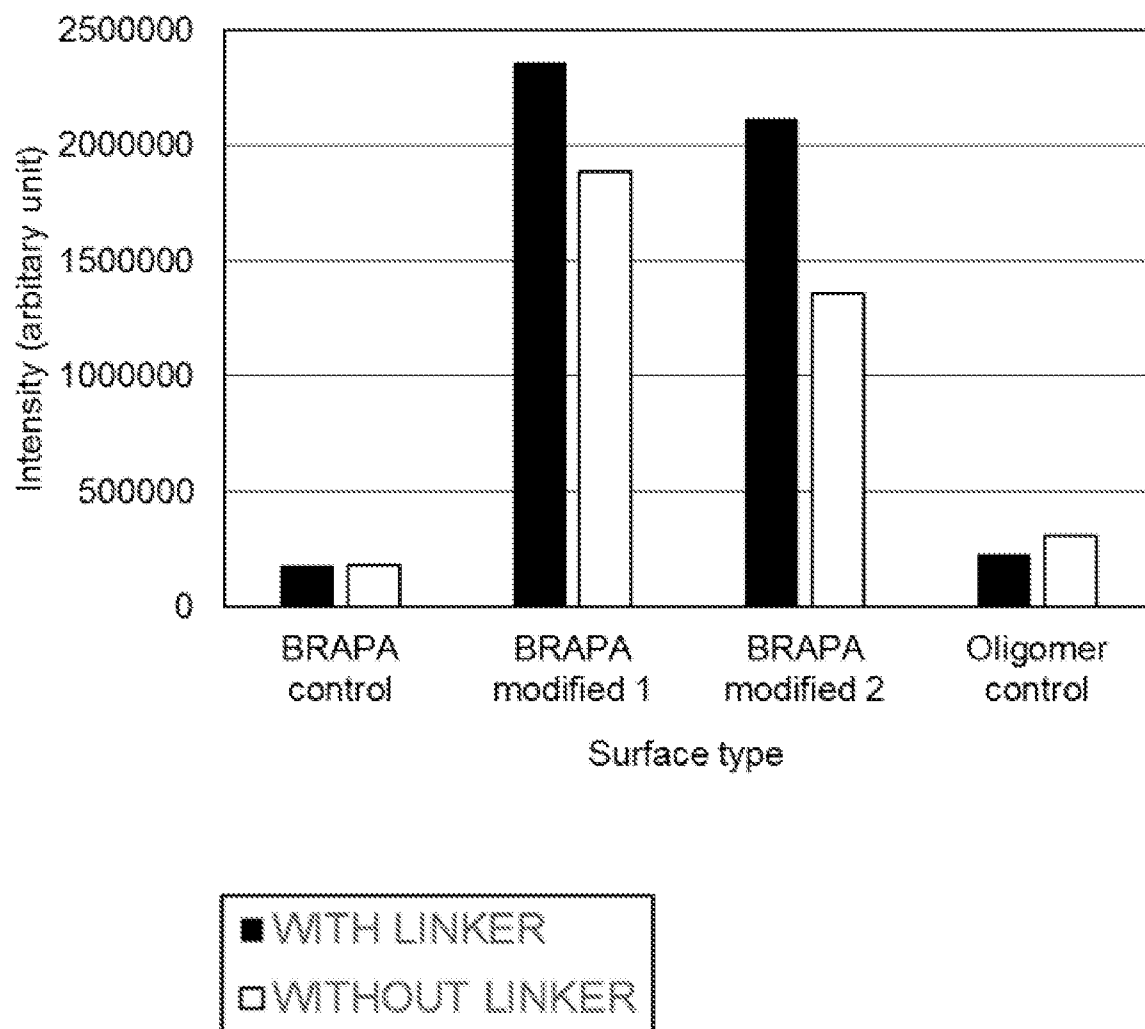


Figure 43

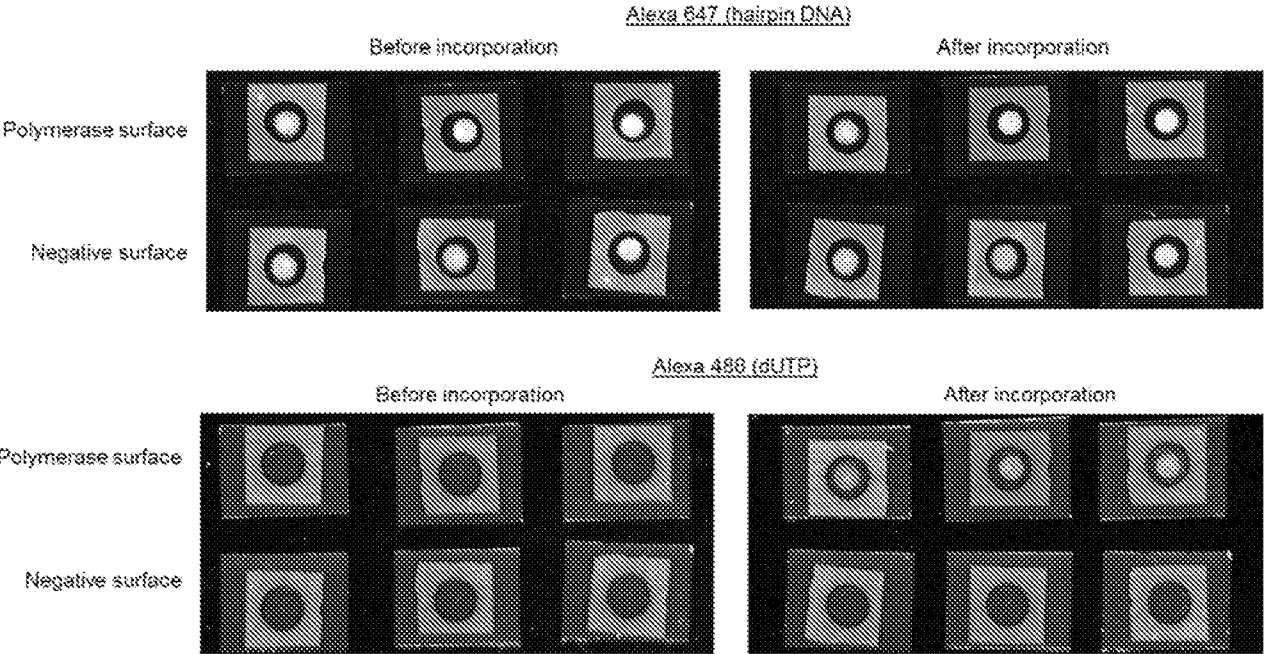


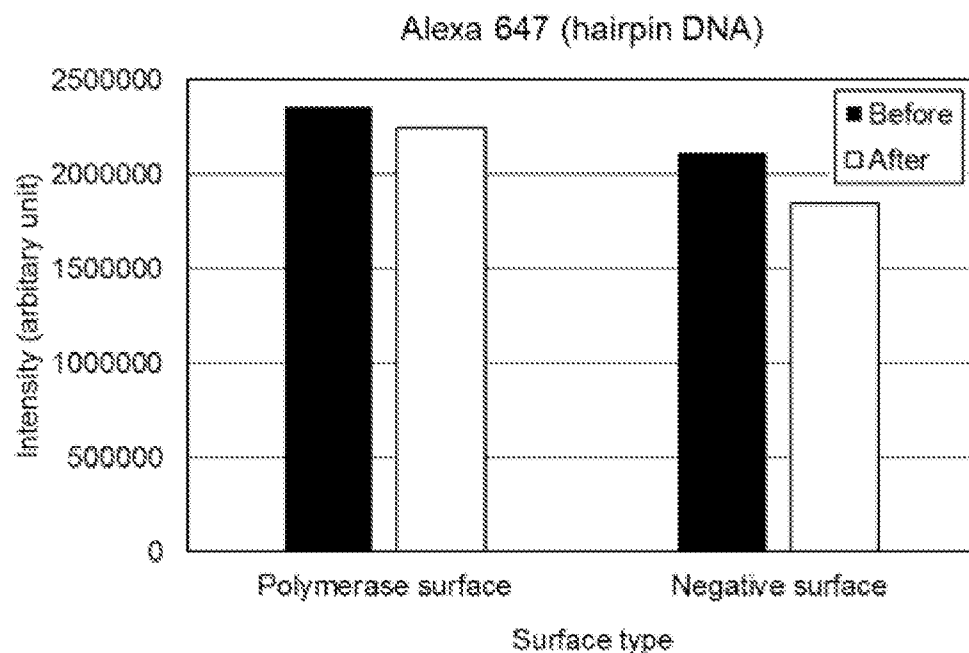
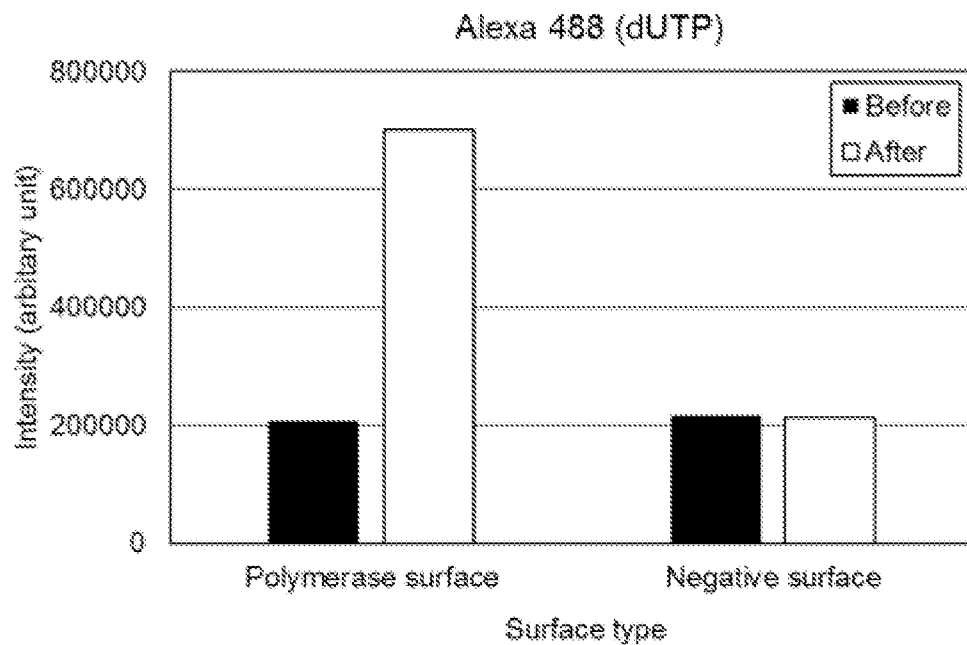
Figure 44a**Figure 44b**

Figure 45a

Sample ID	Sample description (experimental outcome)	Cleavage step	Ligation step
A (positive)	Cleavage (+) Ligation (+)	+ inosine + Endonuclease V	+ helper strand + Ligase
B (ligation negative control)	Cleavage (+) Ligation (-)	+ inosine + Endonuclease V	+ helper strand - Ligase
C (cleavage negative control 1)	Cleavage (-) Ligation (N/A)	+ inosine - Endonuclease V	N/A
D (cleavage negative control 2)	Cleavage (-) Ligation (N/A)	- inosine + Endonuclease V	N/A

Figure 45b

Sample ID	Sequence	SEQ ID NO:
A (positive samples)	5'-Alexa647-AGTCACGTCGTAGTCIAGCTGCAGTCACCTGCTG CTTZTTGCAGCAGGTGACTGCAGCT-3'	SEQ ID NO: 65
B (ligation negative control)	5'-Alexa647-AGTCACGTCGTAGTCIAGCTGCAGTCACCTGCTG CTTZTTGCAGCAGGTGACTGCAGCT -3'	SEQ ID NO: 65
C (cleavage negative control 1)	5'-Alexa647-AGTCACGTCGTAGTCIAGCTGCAGTCACCTGCTG CTTZTTGCAGCAGGTGACTGCAGCT -3'	SEQ ID NO: 65
D (cleavage negative control 2)	5'-Alexa647-AGTCACGTCGTAGTCGAGCTGCAGTCACCTGCTG CTTZTTGCAGCAGGTGACTGCAGCT-3'	SEQ ID NO: 66
Z = 5''-thiophosphate-Sp9-Sp9-Sp9-5-methylC; Sp9 = Spacer 9; I = 2'-deoxyinosine		

Figure 46

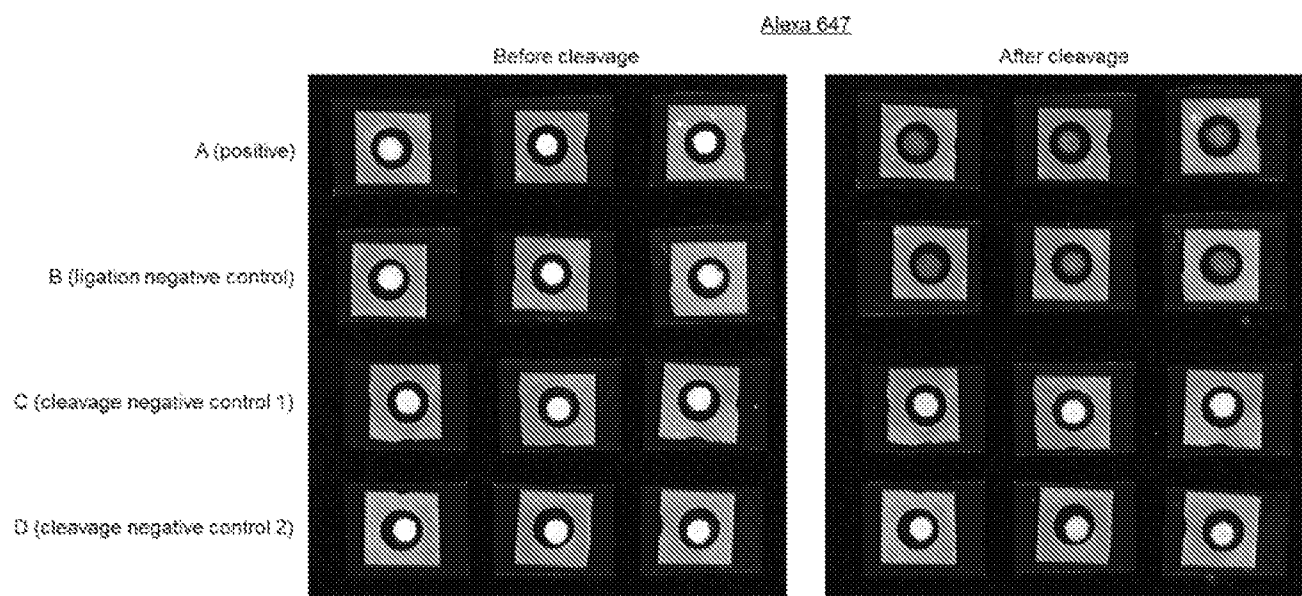
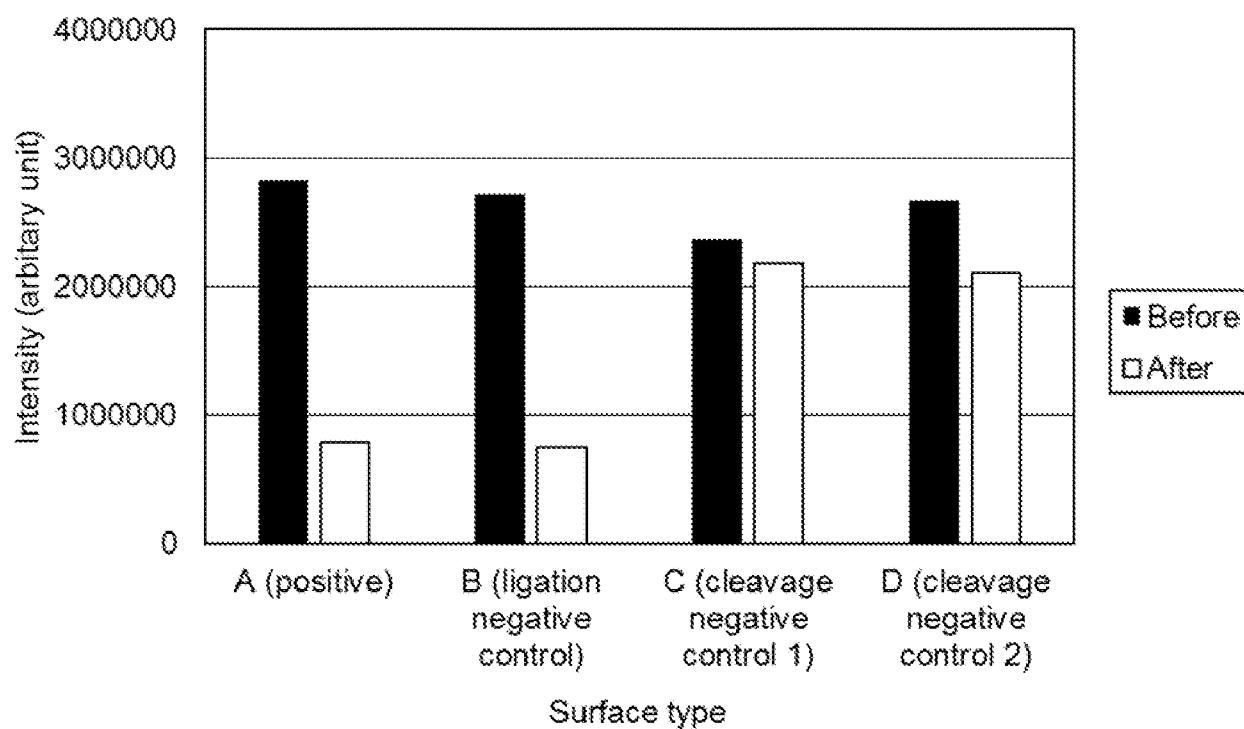


Figure 47**Figure 48**

DNA	Sequence	SEQ ID NO:
Inosine strand	5'-Alexa647-AGTCACGTCGTAGTCIAA-3'	SEQ ID NO: 67
Helper strand	5'-TCGACTACGACGTGACT-3'	SEQ ID NO: 51
I = 2'-deoxyinosine		

Figure 49

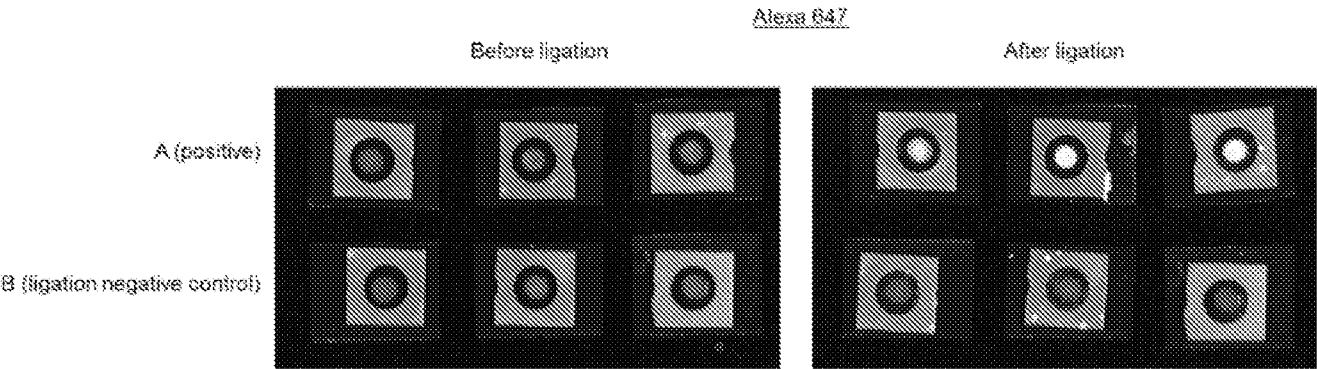


Figure 50

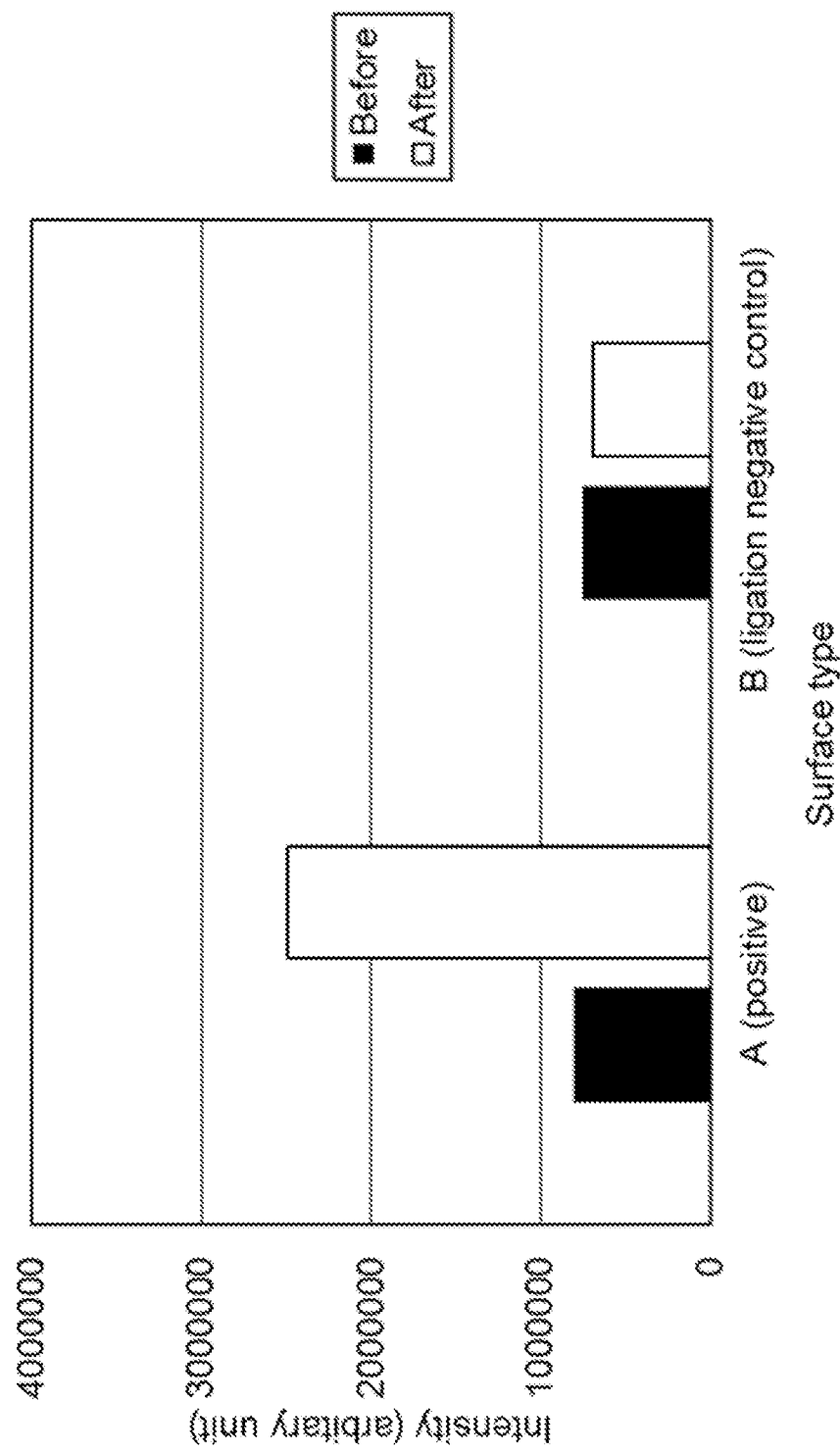
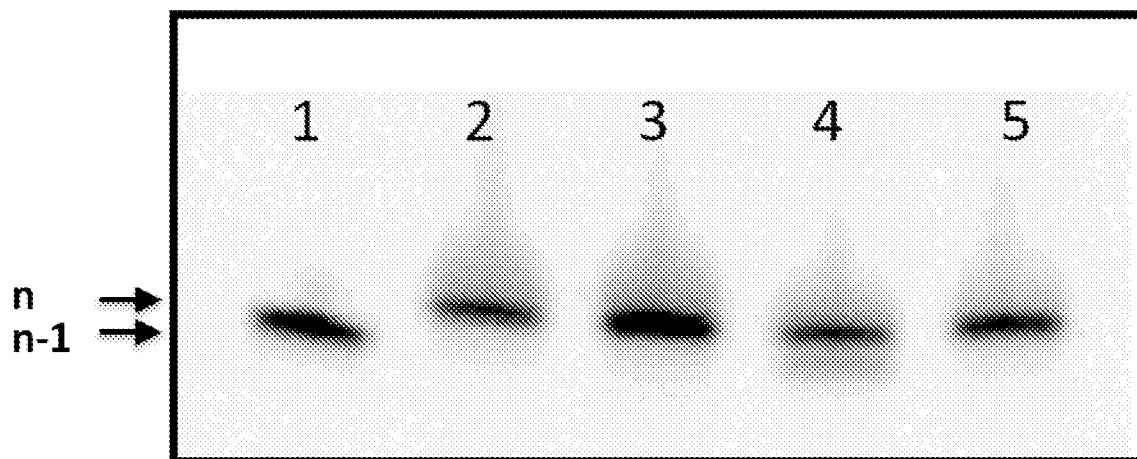


Figure 51**A**

SEQ ID NO:	Oligonucleotide name	Sequence	Modification
68	Primer	5'-GCGACAGGTGACTGCAGC-3'	TAMRA at 5'-end
69	Template	5'-GCTGCAGTCACCTGTCGC-3'	

B

SEQUENCE LISTING

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<120> POLYNUCLEOTIDE SYNTHESIS METHOD, SYSTEM AND KIT

<130> N413528W0

<140> To be assigned

<141> 2019-05-23

<150> GB1808474.9

<151> 2018-05-23

<160> 69

<170> PatentIn version 3.5

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<223> 2'-deoxyinosine

<400> 24
c a c a t c a c g t c g t a g t c n g g c t g c a g t c a c c t g t c g c
37

<210> 25
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<220>
<221> modified_base

<222> (18)..(18)
<223> 2'-deoxyinosine

<400> 25
c a c a t c a c g t c g t a g t c n a g c t g c a g t c a c c t g t c g c
37

<210> 26
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<220>
<221> modified_base
<222> (18)..(18)
<223> 2'-deoxyinosine

<400> 26
c a c a t c a c g t c g t a g t c n a g c t g c a g t c a c c t g t c g c
37

<210> 27
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<400> 27
t c g a c t a c g a c g t g a t g t g
19

<210> 28

<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<400> 28
c c g a c t a c g a c g t g a t g t g
19

<210> 29
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<400> 29
a c g a c t a c g a c g t g a t g t g
19

<210> 30
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<400> 30
g c g a c t a c g a c g t g a t g t g
19

<210> 31

<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<220>
<221> modified_base
<222> (18)..(18)
<223> Deoxyinosine

<400> 31
c a c a t c a c g t c g t a g t c n a g c t g c a g t c a c c t g t c g c
37

<210> 32
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
primer"

<400> 32
g c g a c a g g t g a c t g c a g c t
19

<210> 33
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<400> 33
g c g a c a g g t g a c t g c a g c t g a c t a c g a c g t g a t g t g
36

<210> 34
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<400> 34
t c g a c t a c g a c g t g a t g t g
19

<210> 35
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<400> 35
g c t g c a g t c a c c t g t c g c
18

<210> 36
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
primer"

<400> 36

g c g a c a g g t g
19

a c t g c a g c t

<210> 37

<211> 19

<212> DNA

<213> Artificial Sequence

<220>

<221> source

<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<220>

<221> modified_base

<222> (17)..(17)

<223> 2'-deoxyinosine

<400> 37

c a c a t c a c g t
19

c g t a g t n g a

<210> 38

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<221> source

<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<400> 38

c c a c t a c g a c
18

g t g a t g t g

<210> 39

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<221> source

<223> /note="Description of Artificial Sequence: Synthetic primer"

<400> 39

g c g a c a g g t g
18

a c t g c a g c

<210> 40

<211> 36

<212> DNA

<213> Artificial Sequence

<220>

<221> source

<223> /note="Description of Artificial Sequence: Synthetic oligonucleotide"

<400> 40

c a c a t c a c g t
36

c g t a g t c a g c

t g c a g t c a c c

t g t c g c

<210> 41

<211> 36

<212> DNA

<213> Artificial Sequence

<220>

<221> source

<223> /note="Description of Artificial Sequence: Synthetic oligonucleotide"

<400> 41

c a c a t c a c g t
36

c g t a g t c g g c

t g c a g t c a c c

t g t c g c

<210> 42

<211> 36

<212> DNA

<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<400> 42
c a c a t c a c g t c g t a g t c t g c t g c a g t c a c c t g t c g c
36

<210> 43
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<400> 43
c a c a t c a c g t c g t a g t c c g c t g c a g t c a c c t g t c g c
36

<210> 44
<211> 78
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<220>
<221> modified_base
<222> (36)..(36)
<223> 2'-deoxyinosine

<220>
<221> modified_base
<222> (58)..(58)

<223> Tamra-dT

<400> 44

tcgactacga cgtgactttt agtcacgtcg tagtcnagct gcagtcacct gctgcttntt
60

g c a g c a g g t g
78

a c t g c a g c

<210> 45

<211> 79

<212> DNA

<213> Artificial Sequence

<220>

<221> source

<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<220>

<221> modified_base

<222> (36)..(36)

<223> 2'-deoxyinosine

<220>

<221> modified_base

<222> (58)..(58)

<223> Tamra-dT

<400> 45

tcgactacga cgtgactttt agtcacgtcg tagtcnagct gcagtcacct gctgcttntt
60

g c a g c a g g t g
79

a c t g c a g c t

<210> 46

<211> 42

<212> DNA

<213> Artificial Sequence

<220>

<221> source
<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<220>
<221> modified_base
<222> (21)..(21)
<223> Tamra-dT phosphate

<400> 46
gctgcagtca cctgctgctt nttgcagcag gtgactgcag ct
42

<210> 47
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<220>
<221> modified_base
<222> (36)..(36)
<223> 2'-deoxyinosine

<400> 47
ccgactacga cgtgactttt agtcacgtcg tagtcnga
38

<210> 48
<211> 78
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<220>
<221> modified_base
<222> (36)..(36)
<223> 2'-deoxyinosine

<220>
<221> modified_base
<222> (58)..(58)
<223> Tamra-dT

<400> 48
tcgactacga cgtgactttt agtcacgtcg tagtcnagct gcagtcacct gctgcttntt
60

g c a g c a g g t g a c t g c a g c
78

<210> 49
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<220>
<221> modified_base
<222> (36)..(36)
<223> 2'-deoxyinosine

<400> 49
ccgactacga cgtgactttt agtcacgtcg tagtcnga
38

<210> 50
<211> 58
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<220>
<221> modified_base
<222> (16)..(16)
<223> 2'-deoxyinosine

<220>
<221> modified_base
<222> (38)..(38)
<223> Tamra-dT

<400> 50
agtcacgtcg tagtcnagct gcagtcacct gctgcttntt gcagcagggtg actgcagc
58

<210> 51
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<400> 51
t c g a c t a c g a c g t g a c t
17

<210> 52
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic

oligonucleotide"

<220>

<221> modified_base

<222> (16)..(16)

<223> 2'-deoxyinosine

<400> 52

a g t c a c g t c g
18

t a g t c n g a

<210> 53

<211> 17

<212> DNA

<213> Artificial Sequence

<220>

<221> source

<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<400> 53

c c g a c t a c g a
17

c g t g a c t

<210> 54

<211> 78

<212> DNA

<213> Artificial Sequence

<220>

<221> source

<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<220>

<221> modified_base

<222> (35)..(35)

<223> 2'-deoxyinosine

<220>

<221> modified_base

<222> (57)..(57)

<223> Tamra-dT

<400> 54

cgactacgac gtgactttta gtcacgtcgt agtcnagctg cagtcacctg ctgcttnttg
60

c a g c a g g t g a
78

c t g c a g c t

<210> 55

<211> 37

<212> DNA

<213> Artificial Sequence

<220>

<221> source

<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<220>

<221> modified_base

<222> (35)..(35)

<223> 2'-deoxyinosine

<400> 55

cgactacgac gtgactttta gtcacgtcgt agtcnaa
37

<210> 56

<211> 78

<212> DNA

<213> Artificial Sequence

<220>

<221> source

<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<220>

<221> modified_base
<222> (35)..(35)
<223> 2'-deoxyinosine

<220>
<221> modified_base
<222> (52)..(52)
<223> Tamra-dT

<220>
<221> modified_base
<222> (57)..(57)
<223> Biotin-dT

<400> 56
cgactacgac gtgactttta gtcacgtcgt agtcnagctg cagtcacctg cngcttnttg
60

c a g c a g g t g a c t g c a g c t
78

<210> 57
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<220>
<221> modified_base
<222> (36)..(36)
<223> 2'-deoxyinosine

<400> 57
ccgactacga cgtgactttt agtcacgtcg tagtcnga
38

<210> 58
<211> 62

<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<220>
<221> modified_base
<222> (60)..(60)
<223> 2'-deoxyinosine

<400> 58
acgagtgacc tgggtttttt tttttttttt tttttttttt tttttttacc aggtcactcn
60

t g
62

<210> 59
<211> 58
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<220>
<221> modified_base
<222> (16)..(16)
<223> 2'-deoxyinosine

<220>
<221> modified_base
<222> (33)..(33)
<223> Tamra-dT

<220>
<221> modified_base

<222> (38)..(38)

<223> Biotin-dT

<400> 59

agtcacgtcg tagtcnagct gcagtcacct gcngcttntt gcagcagggtg actgcagc
58

<210> 60

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<221> source

<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<220>

<221> modified_base

<222> (16)..(16)

<223> 2'-deoxyinosine

<400> 60

a g t c a c g t c g
18

t a g t c n a a

<210> 61

<211> 67

<212> DNA

<213> Artificial Sequence

<220>

<221> source

<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<220>

<221> modified_base

<222> (16)..(16)

<223> 2'-deoxyinosine

<400> 61

agtcacgtcg tagtcnagct gcagtcacct gctgcttttt tttttttttg cagcaggtga
60

c t g c a g c
67

<210> 62

<211> 67

<212> DNA

<213> Artificial Sequence

<220>

<221> source

<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<220>

<221> modified_base

<222> (16)..(16)

<223> 2'-deoxyinosine

<400> 62

agtcacgtcg tagtcnagct gcagtcacct gctgcttttt tttttttttg cagcaggtga
60

c t g c a g c
67

<210> 63

<211> 58

<212> DNA

<213> Artificial Sequence

<220>

<221> source

<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<220>

<221> modified_base

<222> (16)..(16)
<223> 2'-deoxyinosine

<220>
<221> modified_base
<222> (38)..(38)
<223> 5''-thiophosphate-Sp9-Sp9-Sp9-5-methylC

<400> 63
agtcacgtcg tagtcnagct gcagtcacct gctgcttctt gcagcaggtg actgcagc
58

<210> 64
<211> 58
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<220>
<221> modified_base
<222> (16)..(16)
<223> 2'-deoxyinosine

<220>
<221> modified_base
<222> (38)..(38)
<223> 5''-phosphate-Sp9-Sp9-Sp9-5-methylC

<400> 64
agtcacgtcg tagtcnagct gcagtcacct gctgcttctt gcagcaggtg actgcagc
58

<210> 65
<211> 59
<212> DNA
<213> Artificial Sequence

<220>

<221> source
<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<220>
<221> modified_base
<222> (16)..(16)
<223> 2'-deoxyinosine

<220>
<221> modified_base
<222> (38)..(38)
<223> 5''-thiophosphate-Sp9-Sp9-Sp9-5-methylC

<400> 65
agtcacgtcg tagtcnagct gcagtcacct gctgcttctt gcagcagggtg actgcagct
59

<210> 66
<211> 59
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<220>
<221> modified_base
<222> (38)..(38)
<223> 5''-thiophosphate-Sp9-Sp9-Sp9-5-methylC

<400> 66
agtcacgtcg tagtcgagct gcagtcacct gctgcttctt gcagcagggtg actgcagct
59

<210> 67
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<220>
<221> modified_base
<222> (16)..(16)
<223> 2'-deoxyinosine

<400> 67
a g t c a c g t c g t a g t c n a a
18

<210> 68
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> primer sequence - figure 51

<400> 68
g c g a c a g g t g a c t g c a g c
18

<210> 69
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> template sequence - figure 51

<400> 69
g c t g c a g t c a c c t g t c g c
18