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POLYNUCLEOTIDE SYNTHESIS METHOD, KIT AND SYSTEM

Abstract

The invention relates to new methods for synthesising polynucleotide molecules according to a predefined nucleotide N sequence. The invention also relates to methods for the assembly of synthetic poly nucleotides following synthesis, as well as systems 0 and kits for performing the synthesis and/or assembly methods.

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Background/Summary

FIELD OF THE INVENTION

[0001] 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.

BACKGROUND TO THE INVENTION

[0002] Two primary methods exist for the synthesis and assembly of poly nucleotide molecules, particularly DNA.

[0003] 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 detrimental to future steps of synthesis. Accordingly, this synthesis method is limited to the production of polynucleotides of length of approximately 100 nucleotides.

[0004] 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 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).

[0005] Thus previous methods cannot be used to synthesise double-stranded DNA de novo without the aid of a pre-existing template molecule which is copied.

[0006] 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.sup.8 improvement on current synthesis methods with nucleotide lengths of >100 mers to full genomes, providing a wide range of possibly applications in synthetic biology.

SUMMARY OF THE INVENTION

[0007] The invention provides an in vitro method of synthesising a double-stranded polynucleotide having a predefined sequence, the method comprising performing repeating cycles of synthesis, wherein in each cycle: [0008] (A) a first strand of a double-stranded polynucleotide is extended by the addition of a first nucleotide of the predefined sequence by the action of a ligase enzyme in a blunt-ended ligation reaction; [0009] (B) the second strand of the double-stranded polynucleotide

which is hybridized to the first strand is extended by the addition of a second nucleotide of the predefined sequence by a nucleotide transferase or polymerase enzyme, and [0010] (C) the double-stranded polynucleotide is then cleaved at a cleavage site; and wherein the first and second nucleotides of the predefined sequence of each cycle are retained in the double-stranded polynucleotide following cleavage.

[0011] In any of the above-described methods, in each cycle the first nucleotide may be a partner nucleotide for the second nucleotide, and wherein upon incorporation into the double-stranded polynucleotide the first and second nucleotides form a nucleotide pair.

[0012] In any of the above-described methods, the cleavage site may be defined by a polynucleotide sequence comprising a universal nucleotide.

[0013] In any of the above-described methods, in each cycle a cleavage site may be created in the double-stranded polynucleotide before extension of the second strand.

[0014] In any of the above-described methods, during step (A) by the action of the ligase enzyme a universal nucleotide may be incorporated into the first strand of the double-stranded polynucleotide to define the cleavage site.

[0015] In any of the above-described methods, the first nucleotide and the universal nucleotide may be components of a polynucleotide ligation molecule, and wherein the polynucleotide ligation molecule is ligated to the double-stranded polynucleotide during step (A) by the action of the ligase enzyme in the blunt-ended ligation reaction, and wherein upon ligation of the polynucleotide ligation molecule to the double-stranded polynucleotide the first strand of the double-stranded polynucleotide is extended and the cleavage site is created.

[0016] In any of the above-described methods, in a given cycle of synthesis the second nucleotide of that cycle which is added to the second strand of the double-stranded polynucleotide may comprise a reversible terminator group which prevents further extension by the enzyme, and wherein the reversible terminator group is removed from the incorporated second nucleotide of that cycle prior to the addition in the next cycle of synthesis of the second nucleotide of the next cycle.

[0017] In any of the above-described methods, the method may comprise performing a first cycle of synthesis comprising: [0018] (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 wherein the support strand is the first strand of the double-stranded polynucleotide and the synthesis strand is the strand is the second strand of the double-stranded polynucleotide; [0019] (2) ligating a double-stranded polynucleotide ligation molecule to the scaffold polynucleotide by the action of the ligase enzyme in a blunt-ended ligation reaction, the polynucleotide ligation molecule comprising a support strand and a helper strand hybridised thereto and further comprising a complementary ligation end, the ligation end comprising: [0020] (i) in the support strand a universal nucleotide and a first nucleotide of the predefined sequence; and [0021] (ii) in the helper strand a non-ligatable terminal nucleotide; [0022] wherein upon ligation the first strand of the double-stranded polynucleotide is extended with the first nucleotide and the cleavage site is created by the incorporation of the universal nucleotide into the first strand; [0023] (3) extending the terminal end of the primer strand portion of the synthesis strand of the double-stranded scaffold polynucleotide by the incorporation of a second nucleotide of the predefined sequence by the action of the nucleotide transferase or polymerase enzyme, the second nucleotide comprising a reversible terminator group which prevents further extension by the enzyme, wherein the second nucleotide is a partner for first nucleotide and wherein upon incorporation the second nucleotide and the first nucleotide form a nucleotide pair; [0024] (4) cleaving the ligated scaffold polynucleotide at the cleavage site, 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 the incorporated nucleotide pair; and [0025] (5) removing the reversible terminator group from the second nucleotide; the method further comprising performing a further cycle of synthesis comprising [0026] (6) ligating a further double-

stranded polynucleotide ligation molecule to the cleaved scaffold polynucleotide by the action of the ligase enzyme in the blunt-ended ligation reaction, the polynucleotide ligation molecule comprising a support strand and a helper strand hybridised thereto and further comprising a complementary ligation end, the ligation end comprising. [0027] (i) in the support strand a universal nucleotide and the first nucleotide of the further cycle of synthesis; and [0028] (ii) in the helper strand a non-ligatable terminal nucleotide, [0029] wherein upon ligation the first strand of the double-stranded polynucleotide is extended with the first nucleotide of the further cycle of synthesis and the cleavage site is created by the incorporation of the universal nucleotide into the first strand; [0030] (7) extending the terminal end of the primer strand portion of the synthesis strand of the double-stranded scaffold polynucleotide by the incorporation of the second nucleotide of the further cycle of synthesis by the action of the nucleotide transferase or polymerase enzyme, the second nucleotide comprising a reversible terminator group which prevents further extension by the enzyme, wherein the second nucleotide of the further cycle of synthesis is a partner for the first nucleotide of the further cycle of synthesis, and wherein upon incorporation the second and first nucleotides of the further cycle form a further nucleotide pair; [0031] (8) cleaving the ligated scaffold polynucleotide at the cleavage site, 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 the incorporated first and further nucleotide pairs; [0032] (9) removing the reversible terminator group from the second nucleotide; and [0033] (10) repeating steps 6 to 9 multiple times to provide the double-stranded polynucleotide having a predefined nucleotide sequence. In such a method in any one, more or all cycles of synthesis the reversible terminator group may alternatively be removed from the second nucleotide before the step of cleaving the ligated scaffold polynucleotide at the cleavage site.

[0034] In such a method described above: [0035] (a) in the ligation step of the first cycle (step 2) and in ligation steps of all further cycles the complementary ligation end of the polynucleotide ligation molecule may be structured such that: [0036] i. the first nucleotide of the predefined sequence of that cycle is the terminal nucleotide of the support strand, occupies nucleotide position n in the support strand and is paired with the terminal nucleotide of the helper strand; [0037] ii. the universal nucleotide is the penultimate nucleotide of the support strand, occupies nucleotide position n+1 in the support strand and is paired with the penultimate nucleotide of the helper strand; and [0038] iii. the terminal nucleotide of the helper strand is a non-ligatable nucleotide; [0039] wherein position n is the nucleotide position which is opposite the second nucleotide of the predefined sequence of that cycle upon its incorporation, and wherein position n+1 is the next nucleotide position in the support strand relative to position n in the direction distal to the complementary ligation end; and wherein upon ligation the terminal nucleotide of the support strand of the polynucleotide ligation molecule is ligated to the terminal nucleotide of the scaffold polynucleotide proximal to the primer strand portion of the synthesis strand and a single-strand break is created between the terminal nucleotides of the helper strand and the primer strand portion of the synthesis strand; [0040] (b) in the extension step of the first cycle (step 3) and in all further cycles the second nucleotide of that cycle is incorporated into the second strand opposite the first nucleotide in the first strand and is paired therewith; [0041] (c) in the cleavage step of the first cycle (step 4) and in all further cycles the support strand of the ligated scaffold polynucleotide is cleaved between positions n+1 and n, thereby releasing the polynucleotide ligation molecule from the scaffold polynucleotide and retaining the first nucleotide of that cycle attached to the first strand of the cleaved scaffold polynucleotide and paired with the second nucleotide of that cycle, and whereupon the position occupied by the first nucleotide of that cycle in the support strand of the cleaved scaffold polynucleotide is defined as nucleotide position n−1 in the next cycle of synthesis.

[0042] Alternatively, in such a method described above [0043] (a) in the ligation step of the first cycle (step 2) and in ligation steps of all further cycles the complementary ligation end of the

polynucleotide ligation molecule may be structured such that: [0044] the first nucleotide of the predefined sequence of that cycle is the terminal nucleotide of the support strand, occupies nucleotide position n in the support strand and is paired with the terminal nucleotide of the helper strand; [0045] ii. the universal nucleotide occupies nucleotide position n+2 in the support strand and is paired with a partner nucleotide in the helper strand; and [0046] iii. the terminal nucleotide of the helper strand is a non-ligatable nucleotide; [0047] wherein position n is the nucleotide position which is opposite the second nucleotide of the predefined sequence of that cycle upon its incorporation, and wherein position n+2 is the second nucleotide position in the support strand relative to position n in the direction distal to the complementary ligation end; and wherein upon ligation the terminal nucleotide of the support strand of the polynucleotide ligation molecule is ligated to the terminal nucleotide of the scaffold polynucleotide proximal to the primer strand portion of the synthesis strand and a single-strand break is created between the terminal nucleotides of the helper strand and the primer strand portion of the synthesis strand, [0048] (b) in the extension step of the first cycle (step 3) and in all further cycles the second nucleotide of that cycle is incorporated into the second strand opposite the first nucleotide in the first strand and is paired therewith; [0049] (c) in the cleavage step of the first cycle (step 4) and in all further cycles the support strand of the ligated scaffold polynucleotide is cleaved between positions n+1 and n, thereby releasing the polynucleotide ligation molecule from the scaffold polynucleotide and retaining the first nucleotide of that cycle attached to the first strand of the cleaved scaffold polynucleotide and paired with the second nucleotide of that cycle, and whereupon the position occupied by the first nucleotide of that cycle in the support strand of the cleaved scaffold polynucleotide is defined as nucleotide position n−1 in the next cycle of synthesis.

[0050] Alternatively, in such a method described above: [0051] (a) in the ligation step of the first cycle (step 2) and in ligation steps of all further cycles the complementary ligation end of the polynucleotide ligation molecule may be structured such that: [0052] i. the first nucleotide of the predefined sequence of that cycle is the terminal nucleotide of the support strand, occupies nucleotide position n in the support strand and is paired with the terminal nucleotide of the helper strand; [0053] ii. the universal nucleotide occupies nucleotide position n+2+x in the support strand and is paired with a partner nucleotide in the helper strand, and [0054] iii. the terminal nucleotide of the helper strand is a non-ligatable nucleotide; [0055] wherein position n is the nucleotide position which is opposite the second nucleotide of the predefined sequence of that cycle upon its incorporation, wherein position n+2 is the second nucleotide position in the support strand relative to position n in the direction distal to the complementary ligation end, and wherein x is a number of nucleotide positions relative to position n+2 in the direction distal to the complementary ligation end wherein the number is a whole number from 1 to 10 or more; and wherein upon ligation the terminal nucleotide of the support strand of the polynucleotide ligation molecule is ligated to the terminal nucleotide of the scaffold polynucleotide proximal to the primer strand portion of the synthesis strand and a single-strand break is created between the terminal nucleotides of the helper strand and the primer strand portion of the synthesis strand; [0056] (b) in the extension step of the first cycle (step 3) and in all further cycles the second nucleotide of that cycle is incorporated into the second strand opposite the first nucleotide in the first strand and is paired therewith; [0057] (c) in the cleavage step of the first cycle (step 4) and in all further cycles the support strand of the ligated scaffold polynucleotide is cleaved between positions n+1 and n, thereby releasing the polynucleotide ligation molecule from the scaffold polynucleotide and retaining the first nucleotide of that cycle attached to the first strand of the cleaved scaffold polynucleotide and paired with the second nucleotide of that cycle, and whereupon the position occupied by the first nucleotide of that cycle in the support strand of the cleaved scaffold polynucleotide is defined as nucleotide position n−1 in the next cycle of synthesis.

[0058] In any such method described above the method may be modified such that: [0059] (i) in step (2) the polynucleotide ligation molecule is provided with a complementary ligation end

comprising a first nucleotide of the predefined sequence of the first cycle and further comprising one or more further nucleotides of the predefined sequence of the first cycle; [0060] (ii) in step (3) the terminal end of the primer strand portion of the synthesis strand of the double-stranded scaffold polynucleotide is extended by the incorporation of a second nucleotide of the predefined sequence of the first cycle by the action of the nucleotide transferase or polymerase enzyme, and wherein the terminal end of the primer strand portion is further extended by the incorporation of one or more further nucleotides of the predefined sequence of the first cycle by the action of the nucleotide transferase or polymerase enzyme, wherein each one of the second and further nucleotides of the first cycle comprises a reversible terminator group which prevents further extension by the enzyme, and wherein following each further extension the reversible terminator group is removed from a nucleotide before the incorporation of the next nucleotide; [0061] (iii) in step (4) following cleavage the first, second and further nucleotides of the predefined sequence of the first cycle are retained in the cleaved scaffold polynucleotide; [0062] (iv) in step (6) the polynucleotide ligation molecule is provided with a complementary ligation end comprising a first nucleotide of the predefined sequence of the further cycle and further comprising one or more further nucleotides of the predefined sequence of the further cycle; [0063] (v) in step (6) the terminal end of the primer strand portion of the synthesis strand of the double-stranded scaffold polynucleotide is extended by the incorporation of a second nucleotide of the predefined sequence of the further cycle by the action of the nucleotide transferase or polymerase enzyme, and wherein the terminal end of the primer strand portion is further extended by the incorporation of one or more further nucleotides of the predefined sequence of the further cycle by the action of the nucleotide transferase or polymerase enzyme, wherein each one of the second and further nucleotides of the further cycle comprises a reversible terminator group which prevents further extension by the enzyme, and wherein following each further extension the reversible terminator group is removed from a nucleotide before the incorporation of the next nucleotide; [0064] (vi) in step (8) following cleavage the first, second and further nucleotides of the predefined sequence of the further cycle are retained in the cleaved scaffold polynucleotide.

[0065] In any such method the complementary ligation end of the polynucleotide ligation molecule may be structured such that in steps (4) and (8) prior to cleavage the universal nucleotide occupies a position in the support strand which is the next nucleotide position in the support strand after the nucleotide positions of the first and further nucleotides in the direction distal to the complementary ligation end, and the support strand is cleaved between the position occupied by the last further nucleotide and the position occupied by the universal nucleotide. Alternatively, in any such method the complementary ligation end of the polynucleotide ligation molecule may be structured such that in steps (4) and (8) prior to cleavage the universal nucleotide occupies a position in the support strand which is the next+1 nucleotide position in the support strand after the nucleotide positions of the first and further nucleotides in the direction distal to the complementary ligation end, and the support strand is cleaved between the position occupied by the last further nucleotide and the position occupied by the next nucleotide in the support strand. In any such method the reversible terminator group of the last further nucleotide of a further cycle to be incorporated may alternatively be removed from the last nucleotide before the step of cleaving the ligated scaffold polynucleotide at the cleavage site.

[0066] In any of the methods described above and herein, in any one, more or all cycles of synthesis a partner nucleotide which pairs with the first nucleotide of the predefined sequence may be a nucleotide which is complementary with the first nucleotide, preferably naturally complementary.

[0067] In any of the methods described above and herein, in any one, more or 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, more or all cycles of synthesis, prior to step (2) and/or

(6) the synthesis strand may be removed from the scaffold polynucleotide.

[0068] In any of the methods described above and herein, in any one, more or all cycles of synthesis, after the step of ligating the double-stranded polynucleotide ligation molecule 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 8 M 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.

[0069] In any such method described above and herein wherein the support strand of the scaffold polynucleotide is cleaved between the position occupied by the universal nucleotide and the position occupied by the next nucleotide in the support strand relative to the universal nucleotide in the direction proximal to the primer strand portion of the synthesis strand, 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′-dimethyletbylenediamine. 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.

[0070] In any such method described above and herein wherein the support strand of the scaffold polynucleotide is cleaved between the position occupied by the universal nucleotide and the position occupied by the next nucleotide in the support strand relative to the universal nucleotide in the direction proximal to the primer strand portion of the synthesis strand, 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, formamidopyrimidine DNA glycosylase (Fpg) or 8-oxoguanine DNA glycosylase (hOGG1).

[0071] In any such method described above and herein wherein the support strand of the scaffold polynucleotide is cleaved between the position occupied by the next nucleotide in the support strand relative to the universal nucleotide in the direction proximal to the primer strand portion and the position occupied by the second nucleotide the support strand relative to the universal nucleotide in the direction proximal to the primer strand portion, the cleavage step may comprise cleaving the support strand with an enzyme. Such an enzyme may be Endonuclease V.

[0072] 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.

[0073] In any of the methods described above and herein, one strand of the synthesised double-stranded polynucleotide may be a DNA strand and the other 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 an RNA or a DNA strand. In such cases nucleotides incorporated by the transferase enzyme or the polymerase enzyme 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.
[0074] 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 enzyme, preferably a DNA polymerase enzyme, more preferably a modified DNA polymerase enzyme 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.
[0075] 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 enzyme, preferably an RNA polymerase enzyme such as T3 or T7 RNA polymerase, more preferably a modified RNA polymerase enzyme having an enhanced ability to incorporate an NTP comprising a reversible terminator group compared to an unmodified polymerase.
[0076] 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.
[0077] 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 Φ29 DNA polymerase.
[0078] 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).
[0079] In any of the methods described above and herein, the step of ligating a double-stranded polynucleotide ligation molecule 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.
[0080] In any of the methods described above and herein, in any one, more or all cycles of synthesis in step (1)/(6) in the scaffold polynucleotide the synthesis strand comprising the primer strand portion and the portion of the support strand hybridized thereto may be connected by a hairpin loop.
[0081] In any of the methods described above and herein, in any one, more or all cycles of synthesis in steps (2)/(6) in the polynucleotide ligation molecule the helper strand and the portion of the support strand hybridized thereto may be connected by a hairpin loop at the end opposite the complementary ligation end.
[0082] In any of the methods described above and herein, m any one, more or all cycles of synthesis: [0083] a) in steps (1)/(6) in the scaffold polynucleotide the synthesis strand comprising the primer strand portion and the portion of the support strand hybridized thereto are connected by a hairpin loop; and [0084] b) in steps (2)/(6) in the polynucleotide ligation molecule the helper strand and the portion of the support strand hybridized thereto are connected by a hairpin loop at the end opposite the complementary ligation end.
[0085] In any of the methods described above and herein, at least one or more or all of the

polynucleotide ligation molecules 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 polynucleotide ligation molecules 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.

[0086] In any of the methods described above and herein, in steps (1) and (6) the synthesis strand of the scaffold polynucleotide comprising the primer strand portion and/or the portion of the support strand hybridized thereto may be tethered to a common surface. The synthesis strand of the scaffold polynucleotide comprising the primer strand portion and/or the portion of the support strand hybridized thereto may comprise a cleavable linker, wherein the linker may be cleaved to detach the double-stranded polynucleotide from the surface following synthesis.

[0087] In any of the methods described above and herein, in steps (1) and (6) 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.

[0088] 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.

[0089] 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.

[0090] In any of the methods described above and herein, the surface to which polynucleotides are attached may comprise a gel. The surface may comprise a polyacrylamide surface, such as about 2% polyacrylamide, preferably wherein the polyacrylamide surface is coupled to a solid support such as glass.

[0091] 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 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).

[0092] 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).

[0093] 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.

[0094] In any of the methods described above and herein, following synthesis the double-stranded polynucleotide or a region thereof is amplified, preferably by PCR.

[0095] 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 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 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.

[0096] 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 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 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, and sequentially delivering the droplets to the scaffold polynucleoside 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-dielectric system (EWOD). In any such methods the synthesis and assembly steps may be performed within the same system.

[0097] In any of the in vitro methods for synthesising a double-stranded polynucleotide having a predefined sequence as described above and herein, 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.

[0098] In a related aspect, the invention further provides an in vitro method of extending a double stranded polynucleotide to synthesise a double-stranded polynucleotide having a predefined sequence, the method comprising one or more cycles of synthesis wherein in each cycle of synthesis a universal nucleotide and a first nucleotide of the predefined sequence are added to a first strand of a double-stranded scaffold polynucleotide in a blunt ended ligation reaction, a second nucleotide of the predefined sequence is added to the opposite strand of the scaffold polynucleotide, and the scaffold polynucleotide is cleaved at a cleavage site defined by a sequence comprising the universal nucleotide, wherein upon cleavage the universal nucleotide is released from the scaffold polynucleotide and the first and second nucleotides are retained in the scaffold polynucleotide.

[0099] In a related aspect, the invention further provides the use of a universal nucleotide in an in vitro method of extending a double-stranded polynucleotide to synthesise a double-stranded polynucleotide having a predefined sequence, wherein in a cycle of synthesis the universal nucleotide is added to a double-stranded scaffold polynucleotide in a blunt ended ligation reaction to create a polynucleotide cleavage site in the scaffold polynucleotide, and wherein the scaffold polynucleotide is cleaved to provide a site in a first strand of the scaffold polynucleotide for the incorporation in the next cycle of a first nucleotide of the predefined sequence and optionally one or more further nucleotides of the predefined sequence, and to provide a site in the opposite strand of the scaffold polynucleotide for the incorporation in the next cycle of a second nucleotide of the predefined sequence and optionally one or more further nucleotides of the predefined sequence.

[0100] 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 in cycles of synthesis to create a polynucleotide cleavage site in a double-stranded scaffold polynucleotide, and wherein cleavage of the scaffold polynucleotide provides a site in each strand of the scaffold polynucleotide for the incorporation of

one or more nucleotides of the predefined sequence, wherein in each cycle of synthesis said use comprises: providing a double-stranded scaffold polynucleotide comprising a synthesis strand and a support strand hybridized thereto, wherein the synthesis strand comprises a primer strand portion; providing a double-stranded polynucleotide ligation molecule comprising a support strand and a helper strand hybridized thereto and further comprising a complementary ligation end, wherein the terminal nucleotide of the helper strand at the complementary ligation end comprises a non-ligatable nucleotide, wherein the terminal nucleotide of the support strand at the complementary ligation end comprises a ligatable first nucleotide of the predefined sequence, and wherein the support strand comprises a universal nucleotide for use in creating a polynucleotide cleavage site; ligating the support strand of the polynucleotide ligation molecule to the support strand of the scaffold polynucleotide in a blunt ended ligation reaction, whereupon the support strand of the scaffold polynucleotide is extended with the first nucleotide of the predefined sequence and a single-strand break is created between the helper strand and the primer strand portion of the synthesis strand, and then optionally removing the helper strand, adding to the terminal end of the primer strand portion of the synthesis strand of the scaffold polynucleotide by a polymerase enzyme or a transferase enzyme a second nucleotide of the predefined sequence comprising a reversible terminator group; and cleaving the support strand of the scaffold polynucleotide at a cleavage site defined by a sequence comprising the universal nucleotide, whereupon the polynucleotide ligation molecule comprising the universal nucleotide is removed from the scaffold polynucleotide and the first and second nucleotides of the predefined sequence are retained in the cleaved scaffold polynucleotide, wherein the reversible terminator group is removed from the second nucleotide before or after the cleavage step.

[0101] In any such method of synthesising a double-stranded polynucleotide having a predefined sequence, the support strand of the polynucleotide ligation molecule may further comprise one or more further nucleotides of the predefined sequence next to the first nucleotide of the predefined sequence, wherein the first nucleotide of the predefined sequence is the terminal nucleotide of the support strand of the polynucleotide ligation molecule and is ligated to the terminal nucleotide of the support strand of the scaffold polynucleotide; and wherein following the addition of the second nucleotide of the predefined sequence the method further comprises adding to the terminal end of the primer strand portion of the synthesis strand by the action of a polymerase enzyme or a transferase enzyme one or more further nucleotides of the predefined sequence by performing one or more cycles of adding a further nucleotide comprising a reversible terminator group and then removing the reversible terminator group, and wherein upon cleavage the first, second and further nucleotides of the predefined sequence are retained in the cleaved scaffold polynucleotide.

[0102] 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 herein.

[0103] In a related aspect, the invention further provides an in vitro method of extending with a predefined nucleotide each strand of a double-stranded polynucleotide molecule at the same terminal end, the method comprising: providing a double-stranded scaffold polynucleotide comprising a synthesis strand and a support strand hybridized thereto, wherein the synthesis strand comprises a primer strand portion; adding to the terminal end of the support strand of the scaffold polynucleotide by the action of a ligase enzyme in a blunt ended ligation reaction a first nucleotide of the predefined sequence, wherein the first nucleotide is the terminal nucleotide in a support strand of a double-stranded polynucleotide ligation molecule, the support strand further comprising a universal nucleotide, wherein the first nucleotide is ligated to the terminal nucleotide of the support strand of the scaffold polynucleotide and a single-strand break is created between the opposite/helper strand of the polynucleotide ligation molecule and the primer strand portion of the synthesis strand, and optionally removing the helper strand of the polynucleotide ligation molecule, adding to the terminal end of the primer strand portion of the synthesis strand by the action of a

polymerase enzyme or a transferase enzyme a second nucleotide of the predefined sequence comprising a reversible terminator group; cleaving the support strand of the scaffold polynucleotide at a cleavage site defined by a sequence comprising the universal nucleotide whereupon the polynucleotide ligation molecule comprising the universal nucleotide is removed from the scaffold polynucleotide, wherein following cleavage the first nucleotide is retained in the support strand and the second nucleotide is retained in the primer strand portion; and removing the reversible terminator group from the second nucleotide, wherein said removing is performed before or after cleavage.

[0104] In any such method of extending each strand of a double-stranded polynucleotide molecule, the support strand of the polynucleotide ligation molecule may further comprise one or more further nucleotides of the predefined sequence next to the first nucleotide of the predefined sequence, wherein the first nucleotide of the predefined sequence is the terminal nucleotide of the support strand of the polynucleotide ligation molecule and is ligated to the terminal nucleotide of the support strand of the scaffold polynucleotide, and wherein following the addition of the second nucleotide of the predefined sequence the method further comprises adding to the terminal end of the primer strand portion of the synthesis strand by the action of a polymerase enzyme or a transferase enzyme one or more further nucleotides of the predefined sequence by performing one or more cycles of adding a further nucleotide comprising a reversible terminator group and then removing the reversible terminator group, and wherein upon cleavage the first, second and further nucleotides of the predefined sequence are retained in the cleaved scaffold polynucleotide.

[0105] The invention provides an in vitro method of synthesising a double-stranded polynucleotide having a predefined sequence, the method comprising preforming one or more extension cycles according to the aforementioned extension method.

[0106] In any such method of extending with a predefined nucleotide each strand of a double-stranded polynucleotide molecule, or 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.

[0107] In a related aspect, the invention further provides an in vitro method of ligating a polynucleotide ligation molecule comprising a universal nucleotide to a double-stranded scaffold polynucleotide during a cycle of extending with a predefined nucleotide each strand of the double-stranded scaffold polynucleotide at the same terminal end, the method comprising: providing a double-stranded scaffold polynucleotide comprising a support strand and a synthesis strand hybridized thereto, wherein the synthesis strand comprises a primer strand portion; and ligating a double-stranded polynucleotide ligation molecule to the double-stranded scaffold polynucleotide, wherein the polynucleotide ligation molecule comprises a support strand and a helper strand hybridized thereto and further comprises a complementary ligation end, wherein the terminal nucleotide of the helper strand at the complementary ligation end comprises a non-ligatable nucleotide, wherein the terminal nucleotide of the support strand at the complementary ligation end comprises a ligatable first nucleotide of the predefined sequence, and wherein the support strand comprises a universal nucleotide for use in creating a polynucleotide cleavage site, wherein the ligation reaction comprises ligating the support strand of the polynucleotide ligation molecule to the support strand of the double-stranded scaffold polynucleotide in a blunt ended ligation reaction, whereupon the support strand of the scaffold polynucleotide is extended with the first nucleotide of the predefined sequence and a single-strand break is created between the helper strand and the primer strand portion of the synthesis strand, and then optionally removing the helper strand; the method further comprising: adding to the terminal end of the primer strand portion of the synthesis strand of the scaffold polynucleotide by a polymerase enzyme or a transferase enzyme a second nucleotide of the predefined sequence comprising a reversible terminator group; and cleaving the support strand of the scaffold polynucleotide at a cleavage site defined by a sequence comprising the universal nucleotide, whereupon the polynucleotide ligation molecule comprising the universal

nucleotide is removed from the scaffold polynucleotide and the first and second nucleotides of the predefined sequence are retained in the cleaved scaffold polynucleotide; wherein the reversible terminator group is removed from the second nucleotide before or after the cleavage step.

[0108] In any such method of ligating a polynucleotide ligation molecule comprising a universal nucleotide to a double-stranded scaffold polynucleotide, the support strand of the polynucleotide ligation molecule may further comprise one or more further nucleotides of the predefined sequence next to the first nucleotide of the predefined sequence, wherein the first nucleotide of the predefined sequence is the terminal nucleotide of the support strand of the polynucleotide ligation molecule and is ligated to the terminal nucleotide of the support strand of the scaffold polynucleotide, and wherein following the addition of the second nucleotide of the predefined sequence the method further comprises adding to the terminal end of the primer strand portion of the synthesis strand by the action of a polymerase enzyme or a transferase enzyme one or more further nucleotides of the predefined sequence by performing one or more cycles of adding a further nucleotide comprising a reversible terminator group and then removing the reversible terminator group, and wherein upon cleavage the first, second and further nucleotides of the predefined sequence are retained in the cleaved scaffold polynucleotide.

[0109] The invention provides an in vitro method of synthesising a double-stranded polynucleotide having a predefined sequence, the method comprising preforming one or more extension cycles according to the aforementioned ligation method.

[0110] 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.

[0111] 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.

[0112] 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.

[0113] 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: [0114] a) providing a surface comprising a plurality of reaction areas, each area comprising one or more double-stranded anchor or scaffold polynucleotides, and [0115] 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.

[0116] 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

[0117] 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 all associated methods, reagents and protocols are presented for illustration only and are not to be construed as limiting.

[0118] Relevant Figures, such as e.g. FIGS. **6**, **7**, **8**, **9**, **10**, **13***a*, **14***a*, **15***a* 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 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 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 FIGS. **1** to **5**, as well as in FIG. **52**, are methods of the invention.

FIG. **1**. Scheme of Exemplary Method Version 1 of the Invention.

[0119] Scheme showing a first synthesis cycle according to exemplary method version 1 of the invention.

[0120] The method comprises a cycle of provision of a scaffold polynucleotide, ligation of a polynucleotide ligation molecule to the scaffold polynucleotide, incorporation of a nucleotide comprising a reversible terminator group or blocking group, deprotection and cleavage.

[0121] The scheme shows the provision of a scaffold polynucleotide (**101**, **106**) comprising a support strand (labelled “a”) and a synthesis strand (labelled “b”) hybridised thereto. The synthesis strand comprises a primer strand portion (dotted line). The terminal nucleotide of the support strand proximal to the primer strand portion comprises a ligatable group, preferably a terminal phosphate group as depicted in the Figure. The terminal nucleotide of the primer strand portion and the nucleotide paired therewith are depicted as “X”. These two nucleotides can be any two nucleotides or analogs or derivatives thereof, and are not limited to being a naturally complementary pair of nucleotides.

[0122] The scheme shows the provision of a polynucleotide ligation molecule (**102**, **107**; structure in the top right of the Figure). The polynucleotide ligation molecule comprises a helper strand (dashed line), a support strand hybridised thereto and a complementary ligation end. The terminal nucleotide of the support strand of the complementary ligation end is a first nucleotide of the predefined sequence and is depicted as “A” (adenosine). The terminal nucleotide of the helper strand of the complementary ligation end is depicted as “T” (thymine). The terminal nucleotide of the helper strand of the complementary ligation end comprises a non-ligatable nucleotide. The complementary ligation end comprises a universal nucleotide (depicted as “Un”) in the support strand and which is paired with a partner nucleotide in the helper strand (depicted as “X”). A and T are depicted purely for illustration and can be any nucleotides or analogs or derivatives thereof. X can be any nucleotide or analog or derivative thereof. It is not necessary for paired nucleotides to comprise naturally complementary nucleotides.

[0123] The scheme shows the ligation of the support strand of the polynucleotide ligation molecule (**102**, **107**) to the support strand of the scaffold polynucleotide and the creation of single-stranded break (“nick”) in the synthesis strand between the helper strand and primer strand portion.

[0124] The scheme shows the incorporation (**103**, **108**) of a second nucleotide of the predefined sequence. This nucleotide comprises a reversible terminator group (triangle) and is depicted as “T” (thymine) purely for illustration, it can be any nucleotide or analog or derivative thereof.

[0125] The scheme shows a deprotection step (**104**, **109**) comprising removal of the reversible terminator group from the second nucleotide of the predefined sequence.

[0126] The scheme shows a cleavage step (**105**, **110**) comprising cleaving the support strand (jagged arrowhead) at a cleavage site defined by a sequence comprising the universal nucleotide Cleavage releases the polynucleotide ligation molecule comprising the universal nucleotide and leads to the retention of the first and second nucleotides in the scaffold polynucleotide. In synthesis

method of the invention version 1 the support strand is cleaved between the position occupied by the universal nucleotide and the nucleotide which occupies the next nucleotide position in the support strand in the direction proximal to the primer strand portion/distal to the helper strand portion.

[0127] In synthesis method of the invention version 1 in each cycle the first nucleotide of the predefined sequence and the second nucleotide of the predefined sequence form a nucleotide pair.

FIG. **2**. Scheme of Exemplary Method Version 2 of the Invention.

[0128] Scheme showing a first synthesis cycle according to exemplary method version 2 of the invention.

[0129] The method comprises a cycle of provision of a scaffold polynucleotide, ligation of a polynucleotide ligation molecule to the scaffold polynucleotide, incorporation of a nucleotide comprising a reversible terminator group or blocking group, deprotection and cleavage.

[0130] The scheme shows the provision of a scaffold polynucleotide (**201**, **206**) comprising a support strand (labelled "a") and a synthesis strand (labelled "b") hybridised thereto. The synthesis strand comprises a primer strand portion (dotted line). The terminal nucleotide of the support strand proximal to the primer strand portion comprises a ligatable group, preferably a terminal phosphate group as depicted in the Figure. The terminal nucleotide of the primer strand portion and the nucleotide paired therewith are depicted as "X". These two nucleotides can be any two nucleotides or analogs or derivatives thereof, and are not limited to being a naturally complementary pair of nucleotides.

[0131] The scheme shows the provision of a polynucleotide ligation molecule (**202**, **207**; structure in the top right of the Figure). The polynucleotide ligation molecule comprises a helper strand (dashed line), a support strand hybridised thereto and a complementary ligation end. The terminal nucleotide of the support strand of the complementary ligation end is a first nucleotide of the predefined sequence and is depicted as "A" (adenosine). The terminal nucleotide of the helper strand of the complementary ligation end is depicted as "T" (thymine). The terminal nucleotide of the helper strand of the complementary ligation end comprises a non-ligatable nucleotide. The complementary ligation end comprises a universal nucleotide (depicted as "Un") in the support strand and which is paired with a partner nucleotide in the helper strand (depicted as "X"). The penultimate nucleotides of both the support strand and helper strand at the complementary ligation end are depicted as "X". A and T are depicted purely for illustration and can be any nucleotides or analogs or derivatives thereof. The nucleotides depicted as "X" can be any nucleotides or analogs or derivatives thereof. It is not necessary for paired nucleotides to comprise naturally complementary nucleotides.

[0132] The scheme shows the ligation of the support strand of the polynucleotide ligation molecule (**202**, **207**) to the support strand of the scaffold polynucleotide and the creation of single-stranded break ("nick") in the synthesis strand between the helper strand and primer strand portion.

[0133] The scheme shows the incorporation (**203**, **208**) of a second nucleotide of the predefined sequence. This nucleotide comprises a reversible terminator group (triangle) and is depicted as "T" (thymine) purely for illustration, it can be any nucleotide or analog or derivative thereof.

[0134] The scheme shows a deprotection step (**204**, **209**) comprising removal of the reversible terminator group from the second nucleotide of the predefined sequence.

[0135] The scheme shows a cleavage step (**205**, **210**) comprising cleaving the support strand (jagged arrowhead) at a cleavage site defined by a sequence comprising the universal nucleotide. Cleavage releases the polynucleotide ligation molecule comprising the universal nucleotide and leads to the retention of the first and second nucleotides in the scaffold polynucleotide. In synthesis method of the invention version 2 the support strand is cleaved between the nucleotide which occupies the next nucleotide position relative to the universal nucleotide in the direction proximal to the primer strand portion/distal to the helper strand portion and the nucleotide which occupies the second nucleotide position relative to the universal nucleotide in the direction proximal to the

primer strand portion/distal to the helper strand portion.
[0136] In synthesis method of the invention version 2 in each cycle the first nucleotide of the predefined sequence and the second nucleotide of the predefined sequence form a nucleotide pair
FIG. **3**. Scheme Showing Variants of Exemplary Method Version 2 of the Invention.
[0137] Scheme showing a first synthesis cycle according to variants of exemplary method version 2 of the invention.
[0138] The method comprises a cycle of provision of a scaffold polynucleotide, ligation of a polynucleotide ligation molecule to the scaffold polynucleotide, incorporation of a nucleotide comprising a reversible terminator group or blocking group, deprotection and cleavage.
[0139] The scheme shows the provision of a scaffold polynucleotide (**301**, **306**) comprising a support strand (labelled "a") and a synthesis strand (labelled "b") hybridised thereto. The synthesis strand comprises a primer strand portion (dotted line). The terminal nucleotide of the support strand proximal to the primer strand portion comprises a ligatable group, preferably a terminal phosphate group as depicted in the Figure. The terminal nucleotide of the primer strand portion and the nucleotide paired therewith are depicted as "X" These two nucleotides can be any two nucleotides or analogs or derivatives thereof, and are not limited to being a naturally complementary pair of nucleotides.
[0140] The scheme shows the provision of a polynucleotide ligation molecule (**302**, **307**; structure in the top right of the Figure). The polynucleotide ligation molecule comprises a helper strand (dashed line), a support strand hybridised thereto and a complementary ligation end. The terminal nucleotide of the support strand of the complementary ligation end is a first nucleotide of the predefined sequence and is depicted as "A" (adenosine). The terminal nucleotide of the helper strand of the complementary ligation end is depicted as "T" (thymine). The terminal nucleotide of the helper strand of the complementary ligation end comprises a non-ligatable nucleotide. The complementary ligation end comprises a universal nucleotide (depicted as "Un") in the support strand and which is paired with a partner nucleotide in the helper strand (depicted as "X"). At the complementary ligation end two nucleotides, depicted as "X", are positioned between the universal nucleotide and the terminal nucleotide of the support strand and are paired with partner nucleotides in the helper strand, also depicted as "X". A and T are depicted purely for illustration and can be any nucleotides or analogs or derivatives thereof. The nucleotides depicted as "X" can be any nucleotides or analogs or derivatives thereof. It is not necessary for paired nucleotides to comprise naturally complementary nucleotides.
[0141] The scheme shows the ligation of the support strand of the polynucleotide ligation molecule (**302**, **307**) to the support strand of the scaffold polynucleotide and the creation of single-stranded break ("nick") in the synthesis strand between the helper strand and primer strand portion.
[0142] The scheme shows the incorporation (**303**, **308**) of a second nucleotide of the predefined sequence. This nucleotide comprises a reversible terminator group (triangle) and is depicted as "T" (thymine) purely for illustration, it can be any nucleotide or analog or derivative thereof.
[0143] The scheme shows a deprotection step (**304**, **309**) comprising removal of the reversible terminator group from the second nucleotide of the predefined sequence.
[0144] The scheme shows a cleavage step (**305**, **310**) comprising cleaving the support strand (jagged arrowhead) at a cleavage site defined by a sequence comprising the universal nucleotide Cleavage releases the polynucleotide ligation molecule comprising the universal nucleotide and leads to the retention of the first and second nucleotides in the scaffold polynucleotide. In these variants of synthesis method of the invention version 2 the support strand is always cleaved between the position occupied by the first nucleotide of the predefined sequence and the position occupied by the next nucleotide in the support strand in the direction proximal to the helper strand/distal to the primer strand portion.
[0145] In all of these particular variants of synthesis method of the invention version 2, in each cycle the first nucleotide of the predefined sequence and the second nucleotide of the predefined

sequence form a nucleotide pair.

FIG. **4**. Scheme Showing Variants of Exemplary Method Version 1 of the Invention Involving Incorporation of More Than Two Nucleotides Per Cycle.

[0146] Scheme showing a first synthesis cycle according to variants of exemplary method version 1 of the invention involving the incorporation of multiple nucleotides in both steps of ligation and incorporation.

[0147] The method comprises a cycle of provision of a scaffold polynucleotide, ligation of a polynucleotide ligation molecule to the scaffold polynucleotide, multiple steps of (a) incorporation of a nucleotide comprising a reversible terminator group or blocking group followed by (b) deprotection, and then finally cleavage.

[0148] The scheme shows the provision of a scaffold polynucleotide (**401**, **406**) comprising a support strand (labelled "a") and a synthesis strand (labelled "b") hybridised thereto. The synthesis strand comprises a primer strand portion (dotted line). The terminal nucleotide of the support strand proximal to the primer strand portion comprises a ligatable group, preferably a terminal phosphate group as depicted in the Figure. The terminal nucleotide of the primer strand portion and the nucleotide paired therewith are depicted as "X". These two nucleotides can be any two nucleotides or analogs or derivatives thereof, and are not limited to being a naturally complementary pair of nucleotides.

[0149] The scheme shows the provision of a polynucleotide ligation molecule (**402**, **407**; structure in the top right of the Figure). The polynucleotide ligation molecule comprises a helper strand (dashed line), a support strand hybridised thereto and a complementary ligation end. The terminal nucleotide of the support strand of the complementary ligation end is a first nucleotide of the predefined sequence and is depicted as "A" (adenosine). The terminal nucleotide of the helper strand of the complementary ligation end is depicted as "T" (thymine). The terminal nucleotide of the helper strand of the complementary ligation end comprises a non-ligatable nucleotide. The penultimate nucleotide of the support strand is a further nucleotide of the predefined sequence and is depicted as "G" (guanine) and is paired with the penultimate nucleotide of the helper strand which is depicted as "C" (cytosine). The complementary ligation end comprises a universal nucleotide (depicted as "Un") in the support strand and which is paired with a partner nucleotide in the helper strand (depicted as "X"). A. T, G and C are depicted purely for Illustration and can be any nucleotides or analogs or derivatives thereof. X can be any nucleotide or analog or derivative thereof. It is not necessary for paired nucleotides to comprise naturally complementary nucleotides.

[0150] The scheme shows the ligation of the support strand of the polynucleotide ligation molecule (**402**, **407**) to the support strand of the scaffold polynucleotide and the creation of single-stranded break ("nick") in the synthesis strand between the helper strand and primer strand portion.

[0151] The scheme shows the incorporation (**403**, **408**) of a second nucleotide of the predefined sequence. This nucleotide comprises a reversible terminator group (triangle) and is depicted as "T" (thymine) purely for illustration, it can be any nucleotide or analog or derivative thereof. Upon incorporation the second nucleotide forms a nucleotide pair with the first nucleotide.

[0152] The scheme shows a deprotection step following incorporation of the second nucleotide (**404**, **409**) comprising removal of the reversible terminator group from the second nucleotide of the predefined sequence.

[0153] The scheme shows the incorporation (**403′**, **408′**) of a further nucleotide of the predefined sequence. This nucleotide comprises a reversible terminator group (triangle) and is depicted as "C" (cytosine). Upon incorporation the further nucleotide forms a nucleotide pair with the further nucleotide provided by the polynucleotide ligation molecule in step (2), depicted as "G" (guanine). Cytosine and guanine are depicted purely for illustration, these nucleotides can be any nucleotides or analogs or derivatives thereof and are not limited to being a naturally complementary pair of nucleotides.

[0154] The scheme shows a second deprotection step following incorporation of the further

nucleotide (**404′**, **409′**) comprising removal of the reversible terminator group from the further nucleotide of the predefined sequence.

[0155] The scheme shows a cleavage step (**405**, **410**) comprising cleaving the support strand (jagged arrowhead) at a cleavage site defined by a sequence comprising the universal nucleotide. Cleavage releases the polynucleotide ligation molecule comprising the universal nucleotide and leads to the retention of the first, second and further nucleotides in the scaffold polynucleotide. In this particular variant of synthesis method of the invention version 1 the support strand is cleaved between the position occupied by the universal nucleotide and the nucleotide which occupies the next nucleotide position in the support strand in the direction proximal to the primer strand portion/distal to the helper strand portion.

[0156] In all of these particular variants of synthesis method of the invention version 1, in each cycle the first nucleotide of the predefined sequence and the second nucleotide of the predefined sequence form a nucleotide pair, the first further nucleotide provided by the polynucleotide ligation molecule in step (2) and the first further nucleotide incorporated in step (3′) form a nucleotide pair, and so on and so forth.

FIG. **5**. Scheme Showing Variants of Exemplary Method Version 2 of the Invention Involving Incorporation of More Than Two Nucleotides Per Cycle.

[0157] Scheme showing a first synthesis cycle according to variants of exemplary method version 2 of the invention involving the incorporation of multiple nucleotides in both steps of ligation and incorporation.

[0158] The method comprises a cycle of provision of a scaffold polynucleotide, ligation of a polynucleotide ligation molecule to the scaffold polynucleotide, multiple steps of (a) incorporation of a nucleotide comprising a reversible terminator group or blocking group followed by (b) deprotection, and then finally cleavage.

[0159] The scheme shows the provision of a scaffold polynucleotide (**501**, **506**) comprising a support strand (labelled “a”) and a synthesis strand (labelled “b”) hybridised thereto. The synthesis strand comprises a primer strand portion (dotted line). The terminal nucleotide of the support strand proximal to the primer strand portion comprises a ligatable group, preferably a terminal phosphate group as depicted in the Figure. The terminal nucleotide of the primer strand portion and the nucleotide paired therewith are depicted as “X”. These two nucleotides can be any two nucleotides or analogs or derivatives thereof, and are not limited to being a naturally complementary pair of nucleotides.

[0160] The scheme shows the provision of a polynucleotide ligation molecule (**502**, **507**, structure in the top right of the Figure). The polynucleotide ligation molecule comprises a helper strand (dashed line), a support strand hybridised thereto and a complementary ligation end. The terminal nucleotide of the support strand of the complementary ligation end is a first nucleotide of the predefined sequence and is depicted as “A” (adenosine). The terminal nucleotide of the helper strand of the complementary ligation end is depicted as “T” (thymine). The terminal nucleotide of the helper strand of the complementary ligation end comprises a non-ligatable nucleotide. The penultimate nucleotide of the support strand is a further nucleotide of the predefined sequence and is depicted as “G” (guanine) and is paired with the penultimate nucleotide of the helper strand which is depicted as “C” (cytosine). The complementary ligation end comprises a universal nucleotide (depicted as “Un”) in the support strand and which is paired with a partner nucleotide in the helper strand (depicted as “X”). An additional nucleotide, depicted as “X”, is positioned between the universal nucleotide and the further nucleotide of the predefined sequence in the support strand. This additional nucleotide is paired with a partner nucleotide in the helper strand which is also depicted as “X”. A. T. G and Care depicted purely for illustration and can be any nucleotides or analogs or derivatives thereof. X can be any nucleotide or analog or derivative thereof. It is not necessary for paired nucleotides to comprise naturally complementary nucleotides.

[0161] The scheme shows the ligation of the support strand of the polynucleotide ligation molecule

(**502**, **507**) to the support strand of the scaffold polynucleotide and the creation of single-stranded break ("nick") in the synthesis strand between the helper strand and primer strand portion.
[0162] The scheme shows the incorporation (**503**, **505**) of a second nucleotide of the predefined sequence. This nucleotide comprises a reversible terminator group (triangle) and is depicted as "T" (thymine) purely for illustration, it can be any nucleotide or analog or derivative thereof. Upon incorporation the second nucleotide forms a nucleotide pair with the first nucleotide.
[0163] The scheme shows a deprotection step following incorporation of the second nucleotide (**504**, **509**) comprising removal of the reversible terminator group from the second nucleotide of the predefined sequence.
[0164] The scheme shows the incorporation (**503**′, **508**′) of a further nucleotide of the predefined sequence. This nucleotide comprises a reversible terminator group (triangle) and is depicted as "C" (cytosine). Upon incorporation the further nucleotide forms a nucleotide pair with the further nucleotide provided by the polynucleotide ligation molecule in step (2), depicted as "(" (guanine). Cytosine and guanine are depicted purely for illustration, these nucleotides can be any nucleotides or analogs or derivatives thereof and are not limited to being a naturally complementary pair of nucleotides.
[0165] The scheme shows a second deprotection step following incorporation of the further nucleotide (**504**′, **509**′) comprising removal of the reversible terminator group from the further nucleotide of the predefined sequence.
[0166] The scheme shows a cleavage step (**505**, **510**) comprising cleaving the support strand (jagged arrowhead) at a cleavage site defined by a sequence comprising the universal nucleotide Cleavage releases the polynucleotide ligation molecule comprising the universal nucleotide and leads to the retention of the first, second and further nucleotides in the scaffold polynucleotide. In this particular variant of synthesis method of the invention version 2 the support strand is cleaved between the nucleotide which occupies the next nucleotide position relative to the universal nucleotide in the direction proximal to the primer strand portion/distal to the helper strand portion and the nucleotide which occupies the second nucleotide position relative to the universal nucleotide in the direction proximal to the primer strand portion/distal to the helper strand portion.
[0167] In all of these particular variants of synthesis method of the invention version 2, in each cycle the first nucleotide of the predefined sequence and the second nucleotide of the predefined sequence form a nucleotide pair, the first further nucleotide provided by the polynucleotide ligation molecule in step (2) and the first further nucleotide incorporated in step (3′) form a nucleotide pair, and so on and so forth.
FIG. **6**. Scheme of Exemplary Method Version 1.
[0168] 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.
FIG. **7**. Scheme of Exemplary Method Version 2.
[0169] 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.

FIG. **8**. Scheme of Exemplary Method Version 3.

[0170] 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.

FIG. **9**. Scheme of Exemplary Method Version 4.

[0171] 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.

FIG. **10**. Scheme of Exemplary Method Version 5.

[0172] 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.

FIG. **11**. Scheme Showing Surface Immobilization of Scaffold Polynucleotides.

[0173] Schemes show (a to h) possible example hairpin loop configurations of scaffold polynucleotides and their immobilisation to surfaces.

[0174] 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.

FIG. **12**. Absence of Helper Strand-Incorporation.

[0175] a) Scheme showing incorporation step highlighted in dashed box. [0176] 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-), Therminator I and Therminator IX) in presence of Ma.sup.2+ 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-d′TTPs using Therminator I DNA polymerase. Lane 6: Incorporation of 3′-O-azidomethyl-dTTPs using Therminator I DNA polymerase. Lane 7. Incorporation of 3′-O-allyl-dTTPs using Therminator IX DNA polymerase Lane 8. Incorporation of 3′-O-azidomethyl-dTTPs using Therminator IX DNA polymerase [0177] c) Evaluation of DNA polymerases for incorporation of 3′-O-modified-dTTPs opposite inosine. Results of incorporation using various DNA polymerases. [0178] d) Evaluation of the temperature on the incorporation using Therminator IX DNA polymerase. The Figure depicts a gel showing results of incorporation of 3′-modified-dTTP opposite inosine in presence of Mn.sup.2+ ions using Therminator 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. [0179] e) Evaluation of the temperature on the incorporation using Therminator IX DNA polymerase. Results of incorporation performed at different temperatures. [0180] f) Evaluation of the presence of Mn.sup.2+ on the incorporation using Therminator 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.sup.2+ ions. Lane 2: Incorporation of 3′-O-azidomethyl-dttps without Mn.sup.2+ ions. Lane 3: Incorporation of 3′-O-allyl-dTTPs in presence of Mn.sup.2+ ions. Lane 4: Incorporation of 3′-O-azidomethyl-dTTPs in presence of Mn.sup.2+ ions. [0181] g) Evaluation of the presence of Mn.sup.2+ on the incorporation using Therminator IX DNA polymerase. Results of incorporation in presence and absence of Mn.sup.2+ ions. [0182] h) Oligonucleotides used for study of the incorporation step.

FIG. **13**. Absence of Helper Strand-Cleavage.

[0183] a) Scheme showing cleavage of hybridized polynucleotide strands in the absence of a helper strand. Cleavage step is highlighted in dashed box. [0184] 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%. [0185] 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%. [0186] d) A summary of cleavage of oligonucleotide with bAAG/Endo VIII and HAAG/Chemical base. [0187] e) Oligonucleotides used for study of the cleavage step.

FIG. **14**. Absence of Helper Strand-Ligation.

[0188] a) Scheme showing ligation of hybridized polynucleotide strands in the absence of a helper strand. Ligation step highlighted in dashed box. [0189] 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 36 mers TAMRA single stranded oligos and 18 mers TAMRA single stranded oligos. These oligos served reference bands. [0190] c) Oligonucleotides used for study of the ligation step.

FIG. **15**. Version 1 Chemistry with Helper Strand-Incorporation. [0191] a) Scheme showing incorporation step highlighted in dashed box. [0192] b) Oligonucleotides applicable for study of the incorporation step.

FIG. **16**. Version 1 Chemistry with Helper Strand-Cleavage. [0193] a) Scheme showing cleavage of hybridized polynucleotide strands in the absence of a helper strand. Cleavage step is highlighted in dashed box. [0194] 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%. 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%. [0195] 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 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%.Math.90%. [0196] d) Evaluation of N,N′-dimethylethylenediamine for cleavage of abasic sites. Gel shows cleavage of oligonucleotide with hAAG and 100 mM N,N′-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%. [0197] e) A summary of cleavage of oligonucleotide with hAAG/Endo VIII, HAAG/chemical base and hAAG/alternative chemical base. [0198] f) Oligonucleotides used for study of the cleavage step.

FIG. **17**. Version 1 Chemistry with Helper Strand-Ligation. [0199] a) Scheme showing ligation of hybridized polynucleotide strands in the presence of a helper strand. Ligation step highlighted in dashed box. [0200] 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 36 mers TAMRA single stranded oligos and 18 mers TAMRA single stranded oligos. These oligos served reference bands. In lane 2 there was an observable ligation product of expected band size 36 mers after 20 minutes. [0201] 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 36 mers TAMRA single stranded oligos and 18 mers 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 36 mers. [0202] d) Oligonucleotides used for study of the ligation step.

FIG. **18**. Version 2 Chemistry with Helper Strand-Incorporation. [0203] a) Scheme showing incorporation step highlighted in orange dashed box [0204] b) Gel showing results of incorporation of 3′-O-modified-dTTPs by Therminator IX DNA polymerase at 27° C. Lane 1: Starting material. Lane 2: Incorporation after 1 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%. [0205] c) The Figure depicts a gel showing results of incorporation of 3′-O-modified-dTTPs by Therminator IX DNA polymerase at 37° C. Lane 1: Starting material. Lane 2: 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%. [0206] d) Gel showing results of incorporation of 3′-O-modified-dTTPs by Therminator IX DNA polymerase at 47° C. Lane 1: Starting material Lane 2: Incorporation after | 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% [0207] e) Gel showing results of incorporation of 3′-O-modified-dTTPs by

Therminator 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%. [0208] f) Gel showing results of incorporation of 3′-O-modified-dTTPs by Therminator IX 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%. [0209] g) Gel showing results of incorporation of 3′-O-modified-dTTPs by Therminator 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.Math. Incorporation after 10 minutes, conversion 96%. Lane 6: Incorporation after 20 minutes, conversion 96%. [0210] h) Summary of incorporation of 3′-O-azidomethyl-dTTP at various temperatures and presence of Mn.sup.2+ ions [0211] i) Gel showing results of incorporation of 3′-O-modified-dNTPs opposite complementary base by Therminator IX DNA polymerase in presence of Mn.sup.2+ 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 [0212] j) Oligonucleotides used for study of the incorporation step.

FIG. **19**. Version 2 Chemistry with Helper Strand-Cleavage [0213] a) Scheme showing cleavage of hybridized polynucleotide strand in the presence of a helper strand. Cleavage step is highlighted in orange dashed box. [0214] 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%. [0215] c) A summary of cleavage study with Endonuclease V. [0216] d) Oligonucleotides used for study of the cleavage step.

FIG. **20**. Version 2 Chemistry with Helper Strand—Ligation. [0217] a) Scheme showing ligation of hybridized polynucleotide strands in the absence of a helper strand. Ligation step highlighted in orange dashed box. [0218] b) Oligonucleotides for study of the ligation step.

FIG. **21**. Version 2 Chemistry with Helper Strand-Deprotection. [0219] a) Scheme showing deprotection step highlighted in orange dashed box. [0220] b) The Figure depicts a gel showing results of 3′-O-azidomethyl group deprotection by 50 mM TCEP after incorporation of 3′-O-azidomethyl-dTTP. Lane 1: Starting primer Lane 2: Incorporation of 3′-O-azidomethyl-dTTPs in presence Mn.sup.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 50 mM TCEP. Lane 5: Extension of the product in lane 4 by addition of all natural dNTPs. [0221] c) The Figure depicts a gel showing results of 3′-O-azidomethyl group deprotection by 300 mM TCEP after incorporation of 3′-O-azidomethyl-dTTP. Lane 1: Starting primer. Lane 2: Incorporation of 3-O-azidomethyl-dTTPs in presence Mn.sup.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 300 mM TCEP. Lane 5: Extension of the product in lane 4 by addition of all natural dNTPs. [0222] d) The Figure depicts a gel showing results of 3′-O-azidomethyl group deprotection by 50 mM TCEP after incorporation of 3′-O-azidomethyl-dCTP. Lane 1: Starting primer. Lane 2: Incorporation of 3-O-azidomethyl-dCTPs in presence Mn.sup.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 300 mM TCEP. Lane 5 Extension of the product in lane 4 by addition of all natural dNTPs. [0223] e) The Figure depicts a gel showing results of 3′-O-azidomethyl group deprotection by 300 mM TCEP after incorporation of 3′-O-

azidomethyl-dCTP. Lane 1. Starting primer Lane 2: Incorporation of 3-O-azidomethyl-dCTPs in presence Mn.sup.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 300 mM TCEP. Lane 5: Extension of the product in lane 3 by addition of all natural dNTPs. [0224] f). The Figure depicts a gel showing results of 3′-O-azidomethyl group deprotection by 300 mM TCEP after incorporation of 3′-O-azidomethyl-dATP. [0225] Lane 1: Starting primer [0226] Lane 2: Incorporation of 3-O-azidomethyl-dATPs in presence Mn.sup.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 300 mM TCEP. Lane 5. Extension of the product in lane 4 by addition of all natural dNTPs. [0227] g) The Figure depicts a gel showing results of 3′-O-azidomethyl group deprotection by 300 mM TCEP after incorporation of 3′-O-azidomethyl-dGTP. Lane 1: Starting primer. Lane 2: Incorporation of 3-O-azidomethyl-dGTPs in presence Mn.sup.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 300 mM TCEP. Lane 5: Extension of the product in lane 4 by addition of all natural dNTPs. [0228] h) Efficiency of deprotection by TCEP on 0.2 μM DNA. [0229] i) Oligonucleotides used for study of the cleavage step.

FIG. **22**. Version 2 Chemistry with Double Hairpin Model-Incorporation. [0230] a) Scheme showing incorporation step highlighted in dashed box. [0231] 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 Therminator 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 Therminator IX DNA polymerase. Lane 4: Extension of the product in lane 3 by addition of all natural dNTPs. [0232] c) Evaluation of DNA polymerases for incorporation of 3-O-modified-dTTPs opposite its natural counterpart. Oligonucleotides applicable for study of the incorporation step.

FIG. **23**. Version 2 Chemistry with Double Hairpin Model—Cleavage. [0233] a) Scheme showing cleavage of a hairpin Oligonucleotide. Cleavage step is highlighted in dashed box. [0234] 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 1 hr showed a high yield of digested DNA with a ratio of ˜99%. [0235] c) Oligonucleotides used for study of the cleavage step.

FIG. **24**. Version 2 Chemistry with Double Hairpin Model—Ligation. [0236] a) Scheme showing ligation of hybridized hairpins. Ligation step highlighted in dashed box [0237] 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 ˜>85%. [0238] c) Hairpin Oligonucleotides used for study of the Ligation step.

FIG. **25**. Version 2 Chemistry—Complete Cycle on Double Hairpin Model.

[0239] a) Scheme showing full cycle involving enzymatic incorporation, cleavage, ligation and deprotection steps. [0240] 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 Therminator IX DNA polymerase at 37° C. Lane 1: Starting material Lane 2: Incorporation of 3′-O-azidomethyl-dTTP by Therminator 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 product in lane 4 by blunt TA ligase kit. [0241] c) Oligonucleotides applicable for study of the incorporation step.

FIG. **26**. Version 2 Chemistry—Complete Cycle on Single Hairpin Model using Helper Strand.

[0242] a) Scheme showing full cycle involving enzymatic incorporation, cleavage, ligation and deprotection steps. [0243] b) Oligonucleotides applicable for study of the incorporation step.

FIG. **27**. Version 3 Chemistry—Complete Cycle on Double-Hairpin Model

[0244] a) Scheme showing full cycle involving enzymatic incorporation, cleavage, ligation and deprotection steps. [0245] b) Oligonucleotides applicable for study of the incorporation step.

FIG. **28**. Version 2 Chemistry—Complete Two-Cycle on Double-Hairpin Model.

[0246] a) Scheme showing the first full cycle involving enzymatic incorporation, deprotection, cleavage and ligation steps. [0247] b) Scheme showing the second full cycle, following the first full cycle, involving enzymatic incorporation, deprotection, cleavage and ligation steps. [0248] c) The Figure depicts a gel showing full two-cycle experiment comprising: incorporation, deprotection, cleavage and ligation steps.

Lane 1. Starting material.
Lane 2. Extension of starting material with natural dNTPs.
Lane 3. Incorporation of 3′-O-azidomethyl-dTTP by Therminator 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.
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.
Lane 11. Incorporation of 3′-O-azidomethyl-dTTP by Therminator 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.
Lane 16. Ligation of the product in lane 15 by blunt TA ligase kit. [0249] d) Oligonucleotides used for study.

FIG. **29**.

[0250] Example showing a mechanism of release from a scaffold polynucleotide of a polynucleotide of predefined sequence, as synthesised in accordance with the methods described herein.

FIG. **30**.

[0251] Schematic of an exemplary method for the synthesis of RNA according to the invention. The exemplary method shows synthesis in the absence of a helper strand

FIG. **31**.

[0252] Schematic of an exemplary method for the synthesis of RNA according to the invention. The exemplary method shows synthesis in the presence of a helper strand.

FIG. **32**.

[0253] Schematic of an exemplary method for the synthesis of RNA according to the invention. The exemplary method shows synthesis in the presence of a helper strand.

FIG. **33**.

[0254] Schematic of the 1st 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 denaturing the helper

strand prior to the incorporation step.
FIG. **34**.
[0255] 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 denaturing the helper strand prior to the incorporation step.
FIG. **35**.
[0256] Schematic of the 3rd 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 denaturing the helper strand prior to the incorporation step.
FIG. **36**.
[0257] Oligonucleotides used in the experiments detailed in Example 9.
FIG. **37**.
[0258] Gel showing reaction products corresponding to a full three-cycle experiment as detailed in Example 9
[0259] The Figure depicts a gel showing the results of a full three-cycle experiment comprising: incorporation, deblock, cleavage and ligation steps.
Lane 1: Starting material.
Lane 2. Extension of starting material with natural dNTPs
Lane 3: Incorporation of 3′-O-azidomethyl-dTTP by Therminator 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
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.
Lane 11: Incorporation of 3-O-azidomethyl-dTTP by Therminator 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
Lane 16: Ligation of the product in lane IS 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 Therminator X DNA polymerase.
Lane 20: Extension of the product in lane 19 by addition of all natural dNTPs.
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 Endomuclease V
Lane 24: Ligation of the product in lane 23 by T3 DNA ligase
FIG. **38**.
[0260] Fluorescence signals from polyacrylamide gel surfaces spiked with different amount of BRAPA exposed to FITC-PEG-SH and FITC-PEG-COOH.
FIG. **39**
[0261] 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.
FIG. **40**.
[0262] (a) Shows sequences of hairpin DNA without linker immobilised on different samples.
[0263] (b) Shows sequences of hairpin DNA with linker immobilised on different samples.
FIG. **41**.

[0264] Fluorescence signals from hairpin DNA oligomers with and without linker immobilised onto bromoacetyl functionalised polyacrylamide surfaces.
FIG. **42**.
[0265] Measured fluorescence from hairpin DNA oligomers with and without linker immobilised onto bromoacetyl functionalised polyacrylamide surfaces.
FIG. **43**.
[0266] Fluorescence signals from hairpin DNA oligomers with and without linker immobilised onto bromoacetyl functionalised polyacrylamide surfaces following incorporation of triphosphates.
FIG. **44**.
[0267] Measured fluorescence from hairpin DNA oligomers with and without linker immobilised onto bromoacetyl functionalised polyacrylamide surfaces following incorporation of triphosphates.
FIG. **45**.
[0268] (a) Experimental overview and outcome for each reaction step as detailed in Example 12.
[0269] (b) Oligonucleotides used in the experiments detailed in Example 12.
FIG. **46**.
[0270] Shows fluorescence signals from hairpin DNA oligomers before and after cleavage reactions (Example 12).
FIG. **47**.
[0271] Shows measured fluorescence signals from hairpin DNA oligomers before and after cleavage reactions (Example 12).
FIG. **48**.
[0272] Shows the sequences for the inosine-containing strand and the complimentary ‘helper’ strand for ligation reactions (Example 12).
FIG. **49**.
[0273] Results relating to fluorescence signals from hairpin DNA oligomers corresponding to the monitoring of ligation reactions (Example 12).
FIG. **50**.
[0274] Results relating to measured fluorescence from hairpin DNA oligomers corresponding to the monitoring of ligation reactions (Example 12).
FIG. **51**.
[0275] Results relating to incorporation of 3′-O-modified-dNTPs by Therminator X DNA polymerase using an incorporation step according to methods of the invention, e.g. synthesis method versions of the invention 1 and 2 and variants thereof (FIGS. **1** to **5** and **20** Example 13).
[0276] FIG. **51***a* 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 NO: 69).
[0277] FIG. **51***b* depicts a gel showing the results of incorporation of 3′-O-modified-dNTPs by Therminator X DNA polymerase in presence of Mn2+ ions at 37° C. [0278] Lane 1: Starting oligonucleotide. [0279] Lane 2: Incorporation of 3′-O-azidomethyl-dTTP (>99% efficiency) [0280] Lane 3: Incorporation of 3′-O-azidomethyl-dATP (>99% efficiency). [0281] Lane 4: Incorporation of 3′-O-azidomethyl-dCTP (>90% efficiency). [0282] Lane 5 Incorporation of 3′-O-azidomethyl-dGTP (>99% efficiency).
[0283] 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 designated n−1
FIG. **52**.
[0284] The figure shows a scheme depicting a DNA synthesis reaction cycle as described in Example 14.
FIG. **53**.
[0285] The figure shows oligonucleotides used in the experiments described in Example 14.
FIG. **54**. Results of ligation experiments as described in Example 14
[0286] The figure shows a photograph of a gel demonstrating the results of the steps of cleaving a

hairpin scaffold polynucleotide at a cleavage site defined by 2-deoxyuridine, used as a universal nucleotide, followed by the ligation of a polynucleotide ligation molecule comprising 2-deoxyuridine to the cleaved scaffold polynucleotide. The lanes of the gel are as follows: [0287] Lane 1: Starting hairpin scaffold polynucleotide. [0288] Lane 2: Hairpin scaffold polynucleotide cleaved using a mixture of uracil DNA glycosylase and Endonuclease VIII. [0289] Lane 3: Cleaved hairpin scaffold polynucleotide ligated to polynucleotide ligation molecule.

INTERPRETATION OF FIGURES

[0290] The structures depicted in FIGS. **11**, **12***a*, **13***a*, **14***a*, **15***a*, **16***a*, **17***a*, **18***a*, **19***a*, **20***a*, **21***a*, **22***a*, **23***a*, **24***a*, **25***a*, **26***a*, **27***a*, **28***a*, **286**, **29**, **30**, **31**, **32**, **33**, **34**, and **35** are to be interpreted consistently with those depicted in FIGS. **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 FIGS. **6** to **10**), each right hand strand of a double-stranded scaffold polynucleotide molecule relates to the synthesis strand (corresponding to strand "b" in FIGS. **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 FIGS. **6** to **10**); certain scaffold polynucleotide molecules (e.g. in FIGS. **15***a* and **23***a*) 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 FIGS. **6** to **10**); certain scaffold polynucleotide molecules (e.g. in FIGS. **12***a*, **13***a* and **14***a*) are shown with no helper strand portion (corresponding to an absence of the dashed line of strand "b" in FIGS. **6** to **10**); and certain scaffold polynucleotide molecules (e.g. in FIGS. **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 FIGS. **6** to **10**) and wherein the helper strand portion is removed prior to incorporation of the new nucleotide in the next synthesis cycle.

[0291] 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 FIGS. **6** to **10**) and terminal phosphate groups are labelled "p" and depicted as a small elliptical structure.

[0292] FIGS. **11***c*, **11***d*, **11***g*, **11***h*, **22***a*, **23***a*, **24***a*, **25***a*, **27***a*, **28***a*, **28***b*, and **29** show scaffold polynucleotide molecules wherein strands comprising a helper strand portion and support strands are connected by a hairpin loop. FIGS. **11***b*, **22***a*, **23***a*, **24***a*, **25***a*, **26***a*, **27***a*, **28***a*, **28***b*, **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.

[0293] Figures such as FIGS. **27***a* and **28***a* 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 & 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.

Description

DETAILED DESCRIPTION OF THE INVENTION

[0294] 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.
[0295] The present inventors have determined that the use of a universal nucleotide, as defined herein, 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.
[0296] Certain embodiments of the synthesis methods of the invention will be described in more general detail herein by reference to exemplary methods including two exemplary method versions of the invention and certain variants thereof (FIGS. **1** to **5**). It is to be understood that all exemplary methods, including the exemplary method versions of the invention and variants thereof, 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 first 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 second nucleotide of the predefined sequence Preferably, the methods are for synthesising DNA. Specific methods described herein are provided as embodiments of the invention.
Reaction Conditions
[0297] In one aspect the invention provides a method for synthesising a double-stranded polynucleotide having a predefined sequence.
[0298] 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 manual, 3rd edition, Cold Spring Harbour Laboratory Press, and Current Protocols in Molecular Biology, Greene Publishing and Wiley-Interscience, New York (1995)).
[0299] Incorporation of nucleotides into polynucleotides can be carried out under suitable conditions, for example using a polymerase (e.g., Therminator IX polymerase) or a terminal deoxynucleotidyl transferase (TdT) enzyme or functional variant thereof to 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 (NH.sub.4).sub.2SO.sub.4, 1 mM KCl, 0.2 mM MgSO.sub.4 and 0.015% Triton® X-100.
[0300] Cleavage of polynucleotides can be carried out under suitable conditions, for 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.
[0301] Ligation of polynucleotides can be carried out under suitable conditions, for 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, 7 mM MgCl.sub.2, 0.7 mM dithiothreitol, 0.7 mM ATP, 5% polyethylene glycol (PEG6000).
[0302] 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 300 mM).
Anchor Polynucleotides and Scaffold Polynucleotides
[0303] 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.

[0304] In one embodiment a scaffold polynucleotide comprises a synthesis strand hybridized to a complementary support strand. The synthesis strand comprises a primer strand portion (e.g. see FIGS. **1** to **5**). The synthesis strand may be provided hybridized to the complementary support strand. Alternatively, the support strand and the synthesis strand may be provided separately and then allowed to hybridise.

[0305] 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.

[0306] Scaffold polynucleotides with or without hairpins may be immobilized to a solid support or surface as described in more detail herein (see FIG. **11**).

[0307] 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 FIG. **11**.

[0308] In certain methods described herein new synthesis is initiated by incorporating into the scaffold polynucleotide a first nucleotide of the predefined sequence by the action of a ligase enzyme. Thus the first nucleotide of the predefined sequence is ligated to the terminal nucleotide of the support strand of the scaffold polynucleotide as described further herein. The first nucleotide of the predefined sequence is provided by a polynucleotide ligation molecule which comprises a support strand, a helper strand and a complementary ligation end. The first nucleotide of the predefined sequence is provided as the terminal nucleotide of the support strand of the complementary ligation end.

[0309] The terminal nucleotide of the helper strand at the complementary ligation end is a non-ligatable nucleotide, and is typically provided lacking a phosphate group. This prevents the terminal nucleotide of the helper strand ligating with the terminal nucleotide of the primer strand portion of the scaffold polynucleotide and creates a single-strand break site between the helper strand and the primer strand portion following ligation. Creation and maintenance of the single-strand break could be effected by other means. For example, the terminal nucleotide of the helper strand may be provided with a suitable blocking group which prevents ligation with the primer strand portion.

[0310] In certain methods described herein a second nucleotide of the predefined sequence is incorporated into the scaffold polynucleotide by the action of a polymerase or transferase enzyme. Thus the polymerase enzyme or transferase enzyme will act to extend the terminal nucleotide of the primer strand portion.

[0311] Further details of the general method schemes of exemplary methods are provided further herein.

Nucleotides and Universal Nucleotides

[0312] Nucleotides which can be incorporated into synthetic polynucleotides by any of the methods described herein may be nucleotides, nucleotide analogues and modified nucleotides.
[0313] 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.
[0314] 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.
[0315] 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).
[0316] 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.
[0317] A non-natural nucleotide may comprise a modified sugar and/or a modified 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 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.
[0318] Nucleotides of the predefined sequence may be incorporated opposite partner 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.
[0319] Typically, a nucleotide of the predefined sequence is incorporated into a polynucleotide opposite a naturally complementary partner nucleobase. Thus adenosine 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.
[0320] Alternatively a partner nucleotide may be a non-complementary nucleotide. A 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.
[0321] The term “opposite” is to be understood as relating to the normal use of the term in the Geld 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.

[0322] 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 second or further nucleotide of the predefined sequence upon its addition to the terminal end of the primer strand portion in that cycle or in incorporation steps of subsequent cycles. Position “n” also refers to the position in the support strand of a polynucleotide ligation molecule prior to the ligation step which position is the nucleotide position which will be opposite the second or further nucleotide of the predefined sequence upon ligation of the polynucleotide ligation molecule scaffold polynucleotide and incorporation of the second or further nucleotide of the predefined sequence by the action of the polymerase enzyme or transferase enzyme.
[0323] Both the position in the support strand and the opposite position in the synthesis strand may be referred to as positon n.
[0324] Further details concerning the definition of position “n” are provided with reference to FIGS. **1** to **5** and the descriptions thereof in relation to the exemplary synthesis method versions of the invention and variants described in more detail herein.
[0325] 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:
##STR00001##
[0326] 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.
[0327] 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.
[0328] 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 of phenyl C-2′-deoxyribosyl nucleoside.

[0329] Some examples of universal bases are shown below:

##STR00002## ##STR00003##

[0330] Universal nucleotides incorporating cleavable bases may also be used, including photo- and enzymatically-cleavable bases, some examples of which are shown below

Photocleavable Bases:

##STR00004##

Base Analogues Cleavable by Endonuclease III:

##STR00005## ##STR00006##

Base Analogues Cleavable by Formamidopyrimidine DNA Glycosylase (Fpg):

##STR00007##

Base Analogues Cleavable by 8-Oxoguanine DNA Glycosylase (hOGG1):

##STR00008##

Base Analogues Cleavable by hNeil1:

##STR00009##

Base Analogues Cleavable by Thymine DNA Glycosylase (TDG):

##STR00010##

Base Analogues Cleavable by Human Alkyladenine DNA Glycosylase (HAAG):

##STR00011## ##STR00012##

Bases Cleavable by Uracil DNA Glycosylase:

##STR00013##

Bases Cleavable by Human Single-Strand-Selective Monofunctional Uracil-DNA Glycosylase (SMUG1):

##STR00014##

Bases Cleavable by 5-Methylcytosine DNA Glycosylase (ROS1):

##STR00015##

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

[0331] In any of the methods involving scaffold polynucleotides, the universal nucleotide most preferably comprises 2′-deoxyinosine.

[0332] Examples of epigenetic bases which may be incorporated using any of the synthesis methods described herein include the following:

##STR00016##

[0333] Examples of modified bases which may be incorporated using any of the synthesis methods described herein include the following:

##STR00017##

[0334] Examples of halogenated bases which may be incorporated using any of the synthesis methods described herein include the following:

##STR00018##

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

[0335] 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:

##STR00019##

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

[0336] 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:

##STR00020##
[0337] Examples of biotin-modified bases which may be incorporated using any of the synthesis methods described herein include the following:
##STR00021##
where base-A, T. G or C with alkyne or alkene linker.
[0338] Examples of bases bearing fluorophores and quenchers which may be incorporated using any of the synthesis methods described herein include the following:
##STR00022##
Nucleotide-Incorporating Enzymes
[0339] 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.
[0340] Thus in any of the methods described herein the enzyme which is used for the addition of a second nucleotide of the predefined sequence and/or a further nucleotide of the predefined sequence to the terminal end of the primer strand portion of the synthesis strand of a scaffold polynucleotide, has template-independent enzyme activity, such as template-independent polymerase or template-independent transferase activity.
[0341] 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.
[0342] 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 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.
[0343] Thus preferably the polymerase is a modified polymerase having an enhanced 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 Therminator IX DNA polymerase and Therminator X DNA polymerase available from New England BioLabs. This enzyme has an enhanced ability to incorporate 3′-O-modified dNTPs.
[0344] 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, Therminator DNA polymerase, Therminator IX DNA polymerase, Therminator X DNA polymerase, Klenow fragment (Exo-), Bst DNA polymerase, Bsu DNA polymerase, Sulfolobus DNA polymerase I, and Taq Polymerase.
[0345] 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 Φ29 DNA polymerase.
[0346] For the extension of such a polynucleotide synthesis molecule comprising DNA, a DNA polymerase may be used. Any suitable DNA polymerase may be used.
[0347] 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-Mul. V reverse transcriptase, phi29 DNA polymerase, Sulfolobus DNA polymerase IV, Taq DNA polymerase, T4 DNA polymerase, T7 DNA polymerase

and enzymes having reverse transcriptase activity, for example M-MuLV reverse transcriptase.
[0348] 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′.fwdarw.5′ exo-), M-MuL. V reverse transcriptase, Sulfolobus DNA polymerase IV, Taq DNA polymerase.
[0349] 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 DNA polymerase large fragment, Bsu DNA polymerase large fragment, DNA Pol I large (Klenow) fragment (3′.fwdarw.5′ exo-), M-MuL V reverse transcriptase, phi29 DNA polymerase.
[0350] The DNA polymerase may lack 3′ to 5′ exonuclease activity and may posess 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 polymerase large fragment, *E. coli* DNA polymerase DNA Pol I large (Klenow) fragment, M-MOLL V reverse transcriptase.
[0351] 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 large (Klenow) fragment, DNA. Pol I large (Klenow) fragment (3′.fwdarw.5′ exo-), M-Mul.V reverse transcriptase, phi29 DNA polymerase, Sulfolobus DNA polymerase IV, T4 DNA polymerase, T7 DNA polymerase.
[0352] 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 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′.fwdarw.5′ exo-), M-Mul. V reverse transcriptase.
[0353] 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 polymerase from *Thermococcus* species 9°N, such as species 9°N-7. One such example of a modified polymerase is Therminator IX DNA polymerase of Therminator X DNA polymerase available from New England BioLabs. Other engineered or variant DNA polymerases include Deep Vent (exo-), Vent (Exo-), 9°N DNA polymerase, Therminator DNA polymerase, Klenow fragment (Exo-), Bst DNA polymerase, Bsu DNA polymerase, Sulfolobus DNA polymerase 1, and Taq Polymerase.
[0354] 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.
[0355] The RNA polymerase may be T3 RNA polymerase, T7 RNA polymerase, SP6 RNA polymerase, *E. coli* RNA polymerase holoenzyme.
[0356] 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.
[0357] 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 29 DNA polymerase.

[0358] 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 function is preserved in the enzyme.

[0359] 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 molecule comprising DNA or RNA, or one strand of a blunt-ended molecule with a nucleotide without the use of a template may be used.

[0360] 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-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 (TOT), or an enzyme fragment, derivative, analogue or functional equivalent thereof. A polynucleotide synthesis molecule extended by the action of such an enzyme comprises DNA.

[0361] 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 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) 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, 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 Terminator Groups

[0362] In any of the synthesis methods of the invention defined and described herein, nucleotides which are incorporated into the synthesis strand by the action of a polymerase enzyme or a transferase enzyme are preferably incorporated as nucleotides comprising one or more reversible blocking groups, also referred to as a reversible terminator group as described herein.

[0363] Such groups act to prevent further extension by the enzyme in a given synthesis cycle so that only one 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 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.

[0364] Any suitable reversible blocking group may be attached to a nucleotide to prevent further extension by the enzyme following the incorporation of a nucleotide into the synthesis strand in a given cycle and to limit incorporation into the synthesis strand to one nucleotide per step. 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.

Propargyl Reversible Terminators:

##STR00023##

Allyl Reversible Terminators:

##STR00024##

Cyclooctene Reversible Terminators;

##STR00025##

Cyanoethyl Reversible Terminators:

##STR00026##

Nitrobenzyl Reversible Terminators:

##STR00027##

Disulfide Reversible Terminators.

##STR00028##

Azidomethyl Reversible Therminators:

##STR00029##

Aminoalkoxy Reversible Therminators:

##STR00030##

[0365] 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.

##STR00031##

[0366] 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.

[0367] Preferred modified dNTPs are modified dNTPs which are 3′-O-allyl-dNTPs and 3′-O-azidomethyl-dNTPs.

3′-O-allyl-dNTPs are shown below.

##STR00032##

3′-O-azidomethyl-dNTPs are shown below.

##STR00033##

[0368] 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.

[0369] Any suitable reagent may be used to remove the reversible terminator group at the deprotection step.

[0370] 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 Pd.sup.0) and 3′-O-azidomethyl-nucleotides following incorporation.

[0371] Examples of deprotecting reagents are provided below.

Propargyl Reversible Terminators:

[0372] Treatment by Pd catalysts—Na.sub.2PdCl.sub.4, PdCl.sub.2. [0373] Ligands can be used e. g.: Triphenylphosphine-3,3′,3″-trisulfonic acid trisodium salt.

Allyl Reversible Terminators:

[0374] Treatment by Pd catalysts—Na.sub.2PdCl.sub.4, PdCl.sub.2. [0375] Ligands can be used e. g.: Triphenylphosphine-3,3′,3″-trisulfonic acid trisodium salt.

Azidomethyl Reversible Terminators:

[0376] Treatment by thiol (mercaptoethanol or dithiothreitol), or Tris (2-carboxyethyl) phosphine-TCEP.

Cyanoethyl Reversible Terminators:

[0377] Treatment by fluoride—ammonium fluoride, tetrabutylammonium fluoride (TBAF).

Nitrobenzyl Reversible Terminators:

[0378] Exposure to UV light.

Disulfide Reversible Terminators:

[0379] Treatment by thiol (mercaptoethanol or dithiothreitol), or Tris (2-carboxyethyl) phosphine-TCEP.

Aminoalkoxy Reversible Terminators:

[0380] Treatment by nitrite (NO.sub.2.sup.−, HNO.sub.2) pH=5.5

[0381] A reversible blocking group (e.g., a reversible terminator group) can be removed by a step performed immediately after the incorporation step, provided that unwanted reagent from the incorporation step is removed to prevent further incorporation following removal of the reversible terminator group.

Polynucleotide Ligation Molecule

[0382] In methods requiring the presence of scaffold polynucleotides and steps of ligation, the selection of the configuration and structure of the polynucleotide ligation molecule will also depend upon the particular method employed. The polynucleotide ligation molecule generally comprises a support strand as described herein and a helper strand as described herein. The polynucleotide ligation molecule comprises a complementary ligation end at one end of the molecule. The complementary ligation end of the polynucleotide ligation molecule will be ligated to a terminal end of the scaffold polynucleotide.

[0383] The complementary ligation end of the polynucleotide ligation molecule is provided with a non-ligatable terminal nucleotide in the helper strand, typically a non-phosphorylated terminal nucleotide. This prevents ligation of the helper strand portion of the synthesis strand to the primer strand portion of the synthesis strand and thus creates a single-strand break in the synthesis strand following ligation. 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, as described further herein. The complementary ligation end of the polynucleotide ligation molecule is provided with a ligatable terminal nucleotide in the support strand adjacent the non-ligatable terminal nucleotide in the helper strand. The ligatable terminal nucleotide of the support strand is the first nucleotide of the predefined sequence to be incorporated into the scaffold molecule by the action of a ligase enzyme. The complementary ligation end of the polynucleotide ligation molecule is also provided with a universal nucleotide in the support strand. The exact positioning of the universal nucleotide in the support strand relative to the ligatable terminal nucleotide of the support strand will depend upon the specific reaction chemistry employed as will be apparent from the descriptions of the specific method versions and variants thereof.

[0384] The appropriate structure of a polynucleotide ligation molecule can readily be ascertained by reference to the exemplary methods described herein and depictions of the same in the Figures.

Ligation

[0385] 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 enzyme. The ligase may be a modified ligase. The ligase may be a T3 DNA ligase or a T4 DNA ligase. The ligase may 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 transformation of blunt-ended substrates. Molecules, enzymes, chemicals and methods for ligating (joining) single- and double-stranded polynucleotides are well known to the skilled person.

Cleavage of Scaffold Polynucleotide

[0386] In methods requiring the presence of scaffold polynucleotides and steps of cleavage, 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. 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.

[0387] Some examples of DNA cleaving enzymes recognizing modified bases are shown in the Table below.

TABLE-US-00001 DNA Termini glycosylase/ Main created from the cleavage Endonuclease substrate Cleavage site 5’-end 3’-end APE 1 AP site 1.sup.st phosphodiester Deoxyribose- OH bond 5’ to the 5’-phosphate lesion Endonuclease III AP site, 1.sup.st phosphodiester phosphate 3′-phospho-α, thymine glycol bond 3’ to the β-unsaturated lesion aldehyde Endonuclease IV AP site 1.sup.st phosphodiester Deoxyribose- OH bond 5’ to the 5’-phosphate lesion Endonuclease V Inosine 2.sup.nd phosphate OH phosphodiester bond 3’ to the lesion Endonuclease VIII AP site, 1.sup.st phosphodiester phosphate phosphate thymine glycol bond 5’ and 3’ to the lesion FpG 8-oxoguanine 1.sup.st phosphodiester phosphate phosphate bond 5’ and 3’ to the lesion hOGG1 8-oxoguanine 1.sup.st phosphodiester phosphate 3′-phospho-α, bond 3’ to the β-unsaturated lesion aldehyde hNeil1 Oxidized 1.sup.st phosphodiester phosphate phosphate purines bond 5’ and 3’ to the lesion ROS1 5- 1.sup.st phosphodiester phosphate phosphate methylcytosine bond 5’ and 3’ to the lesion Uracil DNA Uracil N-glycosidic AP site (no break) glycosylase bond hSMUG Uracil N-glycosidic AP site (no break) bond hAAG Inosine N-glycosidic AP site (no break) bond

Synthesis Strand

[0388] In methods of synthesising a polynucleotide or oligonucleotide described herein including, but not limited to, synthesis method versions 1 and 2 of the invention and variants thereof as described in FIGS. **1** to **5** and further herein, the scaffold polynucleotide is provided with a synthesis strand. The synthesis strand comprises a primer strand portion. During cycles of synthesis each new second nucleotide of the predefined sequence is incorporated into the synthesis strand by extension of the primer strand portion, the first nucleotide of the predefined sequence being incorporated into the support strand. An enzyme, such as a polymerase enzyme or enzyme having terminal transferase activity, can be used to catalyse incorporation/addition of each new second nucleotide. Each newly-incorporated second nucleotide of the predefined sequence will act as the terminal nucleotide of the primer strand portion for use in priming incorporation in the next incorporation step. Thus in any given cycle of synthesis the primer strand portion of the synthesis strand will comprise sufficient polynucleotide sequence to allow priming by the appropriate enzyme. In certain embodiments, described further herein, in a given cycle of synthesis a second nucleotide of the predefined sequence is incorporated into the synthesis strand followed by incorporation into the synthesis strand of one or more further nucleotides. In such embodiments the second nucleotide of the predefined sequence and further nucleotides comprise a reversible terminator group and the methods additionally comprise steps of removing the reversible terminator group from the nucleotide following incorporation and prior to incorporation of the next

nucleotide.
[0389] The terms “incorporation”, “extension” and “addition” of a nucleotide are intended to have the same meaning herein.
Helper Strand
[0390] A helper strand may be provided in the polynucleotide ligation molecule to facilitate ligation of the polynucleotide ligation molecule to the scaffold polynucleotide at the ligation step. A helper strand may also facilitate binding of cleavage enzyme(s) at the cleavage 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 of the invention the polynucleotide ligation molecule is provided with a helper strand.
[0391] 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 of ligase and cleavage enzyme(s) as necessary.
[0392] The helper strand may comprise nucleotides, nucleotide analogues/derivatives and/or non-nucleotides.
[0393] 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 addition regions of secondary structure such as hairpins or bulges should be avoided.
[0394] 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.
[0395] 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 ligase enzyme at the ligation step and/or binding of cleavage enzyme(s) at the cleavage 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.
[0396] The helper strand may be 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 portion 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.
[0397] 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.
Removal of Helper Strand
[0398] In any of the synthesis methods of the invention described herein, prior to the step of incorporation of the second nucleotide of the predefined sequence the helper strand provided by the polynucleotide ligation molecule may be removed from the scaffold polynucleotide.
[0399] 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) beating 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 area 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.

[0400] In methods wherein the helper strand portion is removed from the scaffold polynucleotide after the step of ligating the double-stranded polynucleotide ligation molecule 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

[0401] 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 the addition of a new nucleotide at the terminal end of the primer strand portion.

[0402] 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 FIGS. **1** to **5**). The primer strand may consist of a region of sequence which can act to 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.

[0403] 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.

[0404] The primer strand may comprise nucleotides, nucleotide analogues/derivatives and/or non-nucleotides.

[0405] 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 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.

[0406] 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, 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.

[0407] 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 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.

[0408] The primer strand may be connected to the corresponding region of the support strand, e.g. via a hairpin.

Support Strand

[0409] In methods of the invention including, but not limited to, synthesis method versions of the invention 1 and 2 and variants thereof as described above, the scaffold polynucleotide is provided with a support strand. The support strand is hybridized to the synthesis strand. There are no 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 as described herein and, if present, the helper strand portion of the synthesis strand, as described above.

Synthetic Polynucleotide

[0410] 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.

[0411] Hybridisation may be defined by moderately stringent or stringent hybridisation conditions. A moderately stringent hybridisation condition uses a prewashing solution containing 5× sodium chloride/sodium citrate (SSC), 0.5% SDS, 1.0 mM EDTA (pH 8.0), hybridisation buffer of about 50% formamide, 6×SSC, 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 0.5×SSC, 0.1% SDS. A stringent hybridisation condition hybridises in 6×SSC at 45° C., followed by one or more washes in 0.1×SSC, 0.2% SDS at 68° C.

[0412] 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 poly nucleotide 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)).

[0413] 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).

[0414] 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 op 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.

RNA Synthesis

[0415] 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 and 2 and variants thereof may be adapted. Thus in each of synthesis method versions 1 and 2 and variants thereof the support strand of the scaffold polynucleotide is a DNA strand, as described above. The primer strand portion of the synthesis strand of the scaffold polynucleotide is an RNA strand. The helper strand, if present, may be an RNA strand. The helper strand, if present, may be a DNA strand.

[0416] 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.

[0417] 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.

[0418] FIGS. **31** and **32** describe reaction schemes for RNA synthesis which are adaptations of DNA synthesis method versions 1 and 2 of the Examples as depicted in FIGS. **6** and **7** respectively. Method versions of the invention 1 and 2 and variants thereof, as depicted in FIGS. **1** to **5** respectively may be adapted in the same way.

[0419] In any of the adapted methods for RNA synthesis, the above descriptions of support strand, primer strand, helper strand, polynucleotide ligation molecule and universal nucleotide may be applied mutatis muicindis 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

Solid Phase Synthesis

[0420] 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.

[0421] 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.

[0422] 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.

[0423] 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 FIG. **11**).

[0424] 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 separately attached to a surface, as depicted in FIGS. **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 FIGS. **11** (*b*) and (*d*). One or other of the synthesis strand comprising the primer strand portion and the portion of the support strand hybridized thereto may be attached separately to a surface, as depicted in FIG. **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.

Solid Phase Synthesis on Planar Surfaces

[0425] 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.

[0426] 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 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 to those used in inkjet and bubblejet printing.

[0427] 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.

[0428] 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 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).

[0429] 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-bromoacetamidylpentyl) acrylamide (BRAPA), preferably the polyacrylamide surface is coupled to a solid support, such as glass.

Reversible Immobilization

[0430] 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 for the Isolation of PCR Products, Nucleic Acids Research, 23 (22): 4742-4743.).

[0431] 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.

[0432] 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.

[0433] 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

[0434] 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.

[0435] Polynucleotides having a predefined sequence may be synthesised in solid phase by methods described herein, wherein polynucleotides are immobilized on an EWOD surface 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 transported under the influence of an electric field via electrowetting techniques.

[0436] 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 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 on components comprising elastomeric materials.

[0437] 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 the range from about 150 nm to about 5 mm. Droplet diameters below 1 μm 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, pp 737-742)

Sequencing of Intermediate or Final Synthesis Products.

[0438] 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 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 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 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 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 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

[0439] 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.

[0440] 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, 6×His-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.

[0441] Some examples of chemistries suitable for attaching polynucleotides to surfaces are shown in FIG. **11***i* and FIG. **11***j*.

[0442] 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).
[0443] 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.
[0444] A linker may be a linear linker or a branched linker.
[0445] 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.sub.2).sub.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. —C(O)—O—) or one or more amide groups (i.e. —C(O)—N(H)—).
[0446] Any linker may be used selected from the group comprising PEG, polyacrylamide, poly(2-hydroxyethyl methacrylate), Poly-2-methyl-2-oxazoline (PMOXA), zwitterionic polymers, e.g. poly(carboxybetaine methacrylate) (PCBMA), poly [N-(3-sulfopropyl)-N-methacryloxyethyl-N, N dimethyl ammonium betaine] (PSBMA), glycopolymers, and polypeptides.
[0447] A linker may comprise a polyethylene glycol (PEG) having a general formula of —(CH.sub.2—CH.sub.2—O)n—, wherein n is from 1 to about 600 or more.
[0448] A linker may comprise oligoethylene glycol-phosphate units having a general formula of ˜[(CH.sub.2—CH.sub.2—O).sub.n—PO.sub.2.sup.−—O].sub.m— where n is from 1 to about 600 or more and m could be 1-200 or more.
[0449] 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 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 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 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.
[0450] In any of the methods of the invention a scaffold polynucleotide may optionally be 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.
[0451] 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.
[0452] 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.

[0453] 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.
[0454] Some non-limiting exemplary branching nucleotides are shown below.
##STR00034##
[0455] 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.
[0456] 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.
[0457] 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.
##STR00035##
[0458] A linker may comprise one or more ethylene glycol units.
[0459] A linker may comprise an oligonucleotide, wherein multiple units are nucleotides.
[0460] 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 depending on the nature of the desired branching moiety, as depicted in the structures above.
Microarrays
[0461] Any of the polynucleotide synthesis methods described herein may be used to manufacture a polynucleotide microarray (Trevino, V. et al., Mol. Med. 2007 13, pp 527-541). 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.
[0462] 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.
[0463] Other than the method of synthesising the polynucleotides of predefined sequence, microarray manufacture may be performed using techniques commonly used in this 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.
[0464] Following synthesis of the polynucleotides of predefined sequence, there may be provided a final cleavage step to remove any unwanted polynucleotide sequence from untethered ends.
[0465] 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 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.
Assembly of Synthetic Polynucleotides

[0466] A polynucleotide having a predefined sequence synthesised by methods described herein, and optionally amplified by methods described herein, may be joined to one or more other such polynucleotides to create larger synthetic polynucleotides.
[0467] 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 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 sequence.
[0468] 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 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 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.
[0469] 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.
[0470] 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 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 U.S. Pat. Nos. 8,653,832, 8,828,336, US20140197028 and US20140202863.
[0471] 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.
[0472] Examples of cleavage methods involving enzymatic cleavage are shown in FIG. **29**. The schematic shows a scaffold polynucleotide attached to a surface (via black 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.
[0473] 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

[0474] 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.

[0475] 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.

[0476] 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.

[0477] 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.

[0478] In one embodiment a reaction area may be a single area of a surface comprising a single scaffold polynucleotide molecule wherein the single scaffold polynucleotide molecule can be addressed with reagents.

[0479] 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 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.

[0480] In one embodiment a synthesis system for carrying out any of the synthesis 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.

[0481] 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 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.

[0482] 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.

[0483] 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.

Exemplary Methods

[0484] Exemplary non-limiting methods of synthesising a polynucleotide or an oligonucleotide molecule according to the invention are described herein, including in the appended claims.

[0485] In the following two exemplary methods of synthesising a polynucleotide or an oligonucleotide molecule according to the invention and variants thereof, references to synthesis method versions 1 and 2 are to be interpreted according to the reaction schematics set out respectively in FIGS. **1** to **5**, and not according to the reaction schematics set out in any of FIGS. **6** to **10** or descriptions of the same in the Examples section. The reaction schematics set out in any of FIGS. **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.

[0486] 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. The reference signs in the text below correspond with those in FIGS. **1** to **5** Such references signs are not intended to be limited to the specific 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.

[0487] Two non-limiting exemplary methods of the invention, referred to herein as synthesis method versions of the invention 1 and 2, are described below (see e.g. FIGS. **1** and **2** respectively). In step (1) of each of these exemplary methods a scaffold polynucleotide (see structure depicted in step 1 of each of FIGS. **1** and **2**) is provided (**101**, **201**) comprising a synthesis strand (see strand labelled “b” in the structure depicted in step 1 of each of FIGS. **1** and **2**) hybridized to a complementary support strand (see strand labelled “a” in the structure depicted in step 1 of each of FIGS. **1** and **2**).

[0488] 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 the strand labelled “b” in the structure depicted in step 1 of each of FIGS. **1** and **2**) and a helper strand portion (see dashed portion of the strand labelled “b” in the structure depicted in step 1 of each of FIGS. **1** and **2**). Both the primer strand portion and the helper strand portion of the synthesis strand are provided hybridised to a complementary support strand.

[0489] The terminal end of the scaffold polynucleotide comprising the primer strand portion comprises a blunt end, i.e. with no overhanging nucleotides in either strand.

[0490] In step (2) of the methods a ligation step is performed (**102**, **202**) wherein a polynucleotide ligation molecule is ligated to the double-stranded scaffold polynucleotide. The polynucleotide ligation molecule comprises a first nucleotide of the predefined nucleotide sequence. The

polynucleotide ligation molecule comprises a support strand and a helper strand hybridized to the support strand. The poly nucleotide ligation molecule comprises a blunt-ended complementary ligation end, i.e. with no overbanging nucleotides in either strand. The blunt-ended complementary ligation end is complementary with the blunt end of the double-stranded scaffold polynucleotide. The support strand of the polynucleotide ligation molecule comprises a first nucleotide of the predefined nucleotide sequence at the complementary ligation end. The first nucleotide of the predefined nucleotide sequence is the terminal nucleotide of the support strand of the polynucleotide ligation molecule at the complementary ligation end. The first nucleotide of the predefined nucleotide sequence is a ligatable nucleotide and is ligated to the terminal nucleotide of the support strand of the scaffold polynucleotide. Upon ligation the first nucleotide of the predefined nucleotide sequence is incorporated into the double-stranded scaffold polynucleotide by attachment to the support strand of the double-stranded scaffold polynucleotide at the blunt end of the double-stranded scaffold polynucleotide.

[0491] In each of the two method versions and variants thereof the support strand of the polynucleotide ligation molecule also comprises a universal nucleotide (labelled “Un” in the structures depicted in each of FIGS. **1** and **2**) at the complementary ligation end which will facilitate cleavage in the cleavage step. The role of the universal nucleotide will be apparent from the detailed description of each method below.

[0492] The terminal nucleotide of the helper strand of the polynucleotide ligation molecule at the complementary ligation end is provided such that the helper strand cannot be ligated to the primer strand portion of the synthesis strand, i.e. it is provided as a non-ligatable nucleotide. This is typically achieved by providing the terminal nucleotide of the helper strand without a phosphate group, i.e. it is provided as a nucleoside. Alternatively a 5′-protected nucleoside, a nucleoside with a non-ligatable group at the 5′ position, such as 5′-deoxynucleoside or a 5′-aminonucleoside, or any other suitable non-ligatable nucleotide or nucleoside may be used.

[0493] Thus upon ligation of the support strand of the polynucleotide ligation molecule to the support strand of the double-stranded scaffold polynucleotide, a single-strand break or “nick” is provided in the synthesis strand between the primer strand portion of the synthesis strand and the helper strand.

[0494] Upon ligation of the polynucleotide ligation molecule to the double-stranded scaffold polynucleotide a double-stranded scaffold polynucleotide is formed comprising the newly incorporated first nucleotide, a universal nucleotide for use in facilitating cleavage in the cleavage step and a “nick”.

[0495] As the first nucleotide of the predefined sequence of that cycle is ligated to the terminal nucleotide of the support strand of the double-stranded scaffold polynucleotide, the terminal nucleotide of the support strand of the double-stranded scaffold polynucleotide must be provided, prior to the ligation step, with an attached phosphate group or other ligatable group so as to allow the terminal nucleotide of the support strand of the double-stranded scaffold polynucleotide to act as a substrate for the ligase enzyme.

[0496] As described in more detail herein, in certain of the exemplary methods relating to versions 1 and 2 and variants thereof described herein, the helper strand may be removed prior to the step of incorporating a second nucleotide of the predefined sequence in that cycle of synthesis, e.g. by denaturation and release from the support strand to which it was previously hybridised.

[0497] In the context of the first cycle of synthesis the term “a first nucleotide of the predefined sequence” is not necessarily to be understood as meaning the very first nucleotide of the predefined sequence. The methods described herein relate to the synthesis of a double-stranded polynucleotide having a predefined sequence and a portion of the predefined sequence may be provided pre-synthesised in the scaffold polynucleotide before initiation of the first cycle of synthesis. In this context the term “a” first nucleotide of the predefined sequence can mean “any” nucleotide of the predefined sequence.

[0498] The terminal end of the primer strand portion of the synthesis strand provides a primer site which is a site for attachment of a second nucleotide of the predefined sequence which is attached to/incorporated into the synthesis strand by an enzyme which possesses the capability of extending an oligonucleotide or a polynucleotide molecule with a single nucleotide. Such an enzyme is typically a nucleotide transferase enzyme or a polymerase enzyme. Any suitable enzyme as defined further herein and/or known to a skilled person may be used. 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, transferase or any other enzyme which catalyses 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.
[0499] The terminal nucleotide of the helper strand portion of the synthesis strand, which 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.
[0500] In the step of incorporating the second nucleotide (step 3; **103**, **203**), the second nucleotide is provided with a reversible terminator group (depicted as the small triangle of the incorporated nucleotide in step 3 of each of FIGS. **1** and **2**) which prevents further extension by the enzyme. Thus in step (3) only a single nucleotide is incorporated.
[0501] Nucleotides comprising any suitable reversible terminator group could be used. Preferred nucleotides with reversible terminator groups are 3′-O-allyl-dNTPs and/or 3′-O-azidomethyl-dNTPs or other groups as described further herein.
[0502] As will be apparent from the description of the various methods defined herein, the term “a second nucleotide of the predefined sequence” is not to be understood as meaning the next nucleotide following on from the first nucleotide in a linear sequence in one strand comprising the predefined sequence, but merely “a” further nucleotide of the predefined sequence in the context of the synthesised double-stranded polynucleotide as a whole. In the case of the specific and non-limiting method versions 1 and 2 of the invention defined herein and certain variants thereof, each “first nucleotide” of one cycle will be sequentially ligated to the “first nucleotide” of the previous cycle in the same nucleic acid strand, thereby extending the first strand sequentially by one nucleotide per cycle. In each cycle, a “second nucleotide” will pair with a “first nucleotide” and each “second nucleotide” of one cycle will be sequentially incorporated next to the “second nucleotide” of the previous cycle in the same nucleic acid strand, thereby extending the second strand sequentially by one nucleotide per cycle. Thus, when synthesis cycles are completed the synthesised double-stranded polynucleotide molecule will comprise a predefined sequence of one strand defined by the ligated first nucleotides of each cycle, and a predefined sequence of the opposite strand defined by the incorporated second nucleotides of each cycle. The sequences of both strands are necessarily predefined, and are determined by the identity of first and second nucleotides chosen by the user in each cycle of synthesis. In method versions 1 and 2 described herein, given that first and second nucleotides of each cycle form a nucleotide pair, if the second nucleotide of each cycle is chosen by the user to be naturally complementary to the first nucleotide of each cycle, then the final synthesised strands will be perfectly complementary. If second nucleotides of certain cycles are chosen by the user to be non-complementary to the respective first nucleotides of those cycles, then the final synthesised strands will not be perfectly complementary. Nevertheless, in either situation the final synthesised strands both comprise sequence which in the context of the synthesised double-stranded polynucleotide as a whole is predefined.
[0503] Following the step of incorporating the second nucleotide (step 3), the scaffold polynucleotide is then cleaved (step 4, **104**, **204**). Cleavage results in release of the polynucleotide

ligation molecule from the scaffold polynucleotide and retention of the first nucleotide of that cycle attached to the support strand of the cleaved scaffold polynucleotide and paired with the second nucleotide of that cycle which is attached to the synthesis strand of the cleaved scaffold polynucleotide. Cleavage results in release of the helper strand, if present and hybridised to the support strand immediately prior to cleavage, and release of the support strand comprising the universal nucleotide Cleavage thus 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, and wherein the cleaved double-stranded scaffold polynucleotide comprises the first nucleotide of that cycle as the terminal nucleotide of the cleaved end of the support strand paired with the second nucleotide of that cycle as the terminal nucleotide of the primer strand portion of the synthesis strand.

[0504] In step (5) of the methods a deprotection step is performed to remove the reversible terminator group from the incorporated second nucleotide of the predefined nucleotide sequence (**105**, **205**). The deprotection step may alternatively be performed before the cleavage step (step 4), in which case the deprotection step is defined as step (4) (step 4 of FIGS. **1** and **2**; **104**, **204**) and the cleavage step is defined as step(S) (step 5 of FIGS. **1** and **2**, **105**, **205**).

[0505] Iterative cycles of synthesis comprising the same steps as described above are performed in order to generate the synthetic polynucleotide.

[0506] Specific methods are described in more detail below.

Synthesis Method Version 1

[0507] With reference to FIG. **1**, in a first specific non-limiting exemplary version of the synthesis methods of the invention a double-stranded scaffold polynucleotide is provided (step 1 of FIG. **1**, **101**). The double-stranded scaffold polynucleotide comprises a support strand and a synthesis strand hybridised thereto. The synthesis strand comprises a primer strand portion. The double-stranded scaffold polynucleotide is provided with at least one blunt end, wherein the at least one blunt end comprises the terminal end of the primer strand portion and the terminal end of the support strand hybridized thereto. The terminal nucleotide of the support strand is capable of acting as a substrate for a ligase enzyme, and preferably comprises a phosphate group, or other ligatable group.

[0508] In step (2) of the methods a polynucleotide ligation molecule (see structure depicted at the top right of the upper part of FIG. **1**) is ligated to the scaffold polynucleotide. Ligation incorporates a first nucleotide of the predefined sequence into the support strand of the scaffold polynucleotide (step 2 of FIG. **1**; **102**).

[0509] In step (3) of the methods a second nucleotide of 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 the capability of extending an oligonucleotide or a polynucleotide molecule with a single nucleotide Such an enzyme is typically a nucleotide transferase enzyme or a polymerase enzyme (step 3 of FIG. **1**; **103**). The second 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.

[0510] In each cycle of synthesis the scaffold polynucleotide is then cleaved at a cleavage site defined by a sequence comprising the universal nucleotide in the support strand (step 4, **104**). In method version 1 cleavage comprises cleaving the support strand immediately after the universal nucleotide in the direction proximal to the primer strand portion/distal to the helper strand, i.e. the support strand is cleaved between the position occupied by the universal nucleotide and the next nucleotide position in the support strand in the direction proximal to the primer strand portion/distal to the helper strand. Cleavage of the scaffold polynucleotide (step 4) results in release of the polynucleotide ligation molecule from the scaffold polynucleotide and retention of the first nucleotide of that cycle attached to the first strand of the cleaved scaffold polynucleotide and

paired with the second nucleotide of that cycle which is attached to the primer strand portion of the synthesis strand. Cleavage of the scaffold polynucleotide (step 4) results in loss of the helper strand from the scaffold polynucleotide, if present and hybridised to the support strand immediately prior to cleavage, and loss of the universal nucleotide from the scaffold polynucleotide 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 either strand, and with the first and second nucleotides as a nucleotide pair.

[0511] In step (5) of the methods a deprotection step is performed to remove the terminator group from the newly-incorporated nucleotide. The deprotection step may alternatively be performed before the cleavage step in which case the deprotection step is defined as step (4) and the cleavage step is defined as step (5), as shown in FIGS. **1** (**104** and **105** respectively).

Step 1—Provision of a Scaffold Polynucleotide

[0512] In exemplary version 1 of the synthesis methods of the invention a double-stranded scaffold polynucleotide is provided in step (1) (**101**). The double-stranded scaffold polynucleotide is provided comprising a synthesis strand and a support strand hybridised thereto, wherein the synthesis strand comprises a primer strand portion. The terminal nucleotide of the support strand is paired with the terminal nucleotide of the primer strand portion thus forming a blunt end. The terminal nucleotide of the support strand is capable of acting as a substrate for a ligase enzyme, and comprises a ligatable group, preferably a phosphate group.

Step 2—Ligation of a Polynucleotide Ligation Molecule to the Scaffold Polynucleotide and Incorporation of a First Nucleotide of the Predefined Sequence

[0513] In step (2) of the method a double-stranded polynucleotide ligation molecule is ligated (**102**) to the scaffold polynucleotide by the action of a ligase enzyme in a blunt-ended ligation reaction.

[0514] The polynucleotide ligation molecule comprises a support strand and a helper strand hybridised thereto. The polynucleotide ligation molecule further comprises a complementary ligation end comprising in the support strand a universal nucleotide and a first nucleotide of the predefined sequence.

[0515] The complementary ligation end of the polynucleotide ligation molecule is structured such that the terminal nucleotide of the support strand is the first nucleotide of the predefined sequence to be incorporated into the scaffold polynucleotide in any given cycle of synthesis. The terminal nucleotide of the support strand is paired with the terminal nucleotide of the helper strand. The terminal nucleotide of the support strand, i.e. the first nucleotide of the predefined sequence of that cycle, occupies nucleotide position n in the support strand. By position n it is meant the nucleotide position which will be opposite the second nucleotide of the predefined sequence of that cycle upon incorporation of the second nucleotide, the first and second nucleotides thereby forming a nucleotide pair. In FIG. **1** the first nucleotide of the predefined sequence is depicted as adenosine, the second nucleotide of the predefined sequence in step (3) is depicted as thymine and the terminal nucleotide of the helper strand is depicted as thymine. Adenosine and thymine are depicted purely for illustration. The first and second nucleotides and the terminal nucleotide of the helper strand can be any suitable nucleotides as chosen by the user. Nucleotides depicted as X may also be any suitable nucleotides as chosen by the user.

[0516] Typically, the terminal nucleotide of the support strand at the complementary ligation end will define the 3′ terminus of the support strand of the complementary ligation end of the polynucleotide ligation molecule.

[0517] The terminal nucleotide of the helper strand of the polynucleotide ligation molecule is configured such that it cannot be ligated to another polynucleotide in a polynucleotide strand (position labelled “T” purely for illustration in the structure depicted at the top right of the upper part of FIG. **1**). This nucleotide is referred to as a non-ligatable terminal nucleotide. Typically this

terminal nucleotide will lack a phosphate group, i.e. it will be a nucleoside. Typically, this terminal nucleotide of the helper strand will define the 5′ terminus of the helper strand.

[0518] In the complementary ligation end of the polynucleotide ligation molecule the universal nucleotide in the support strand is positioned such that it is the penultimate nucleotide of the support strand, is paired with the penultimate nucleotide of the helper strand, and occupies nucleotide position n+1 in the support strand. By position n+1 it is meant the next nucleotide position in the support strand relative to position n in the direction distal to the complementary ligation end.

[0519] The complementary ligation end is configured so that it will compatibly join with the blunt end of the scaffold polynucleotide when subjected to suitable ligation conditions. Upon ligation of the support strands of the polynucleotide ligation molecule and the scaffold polynucleotide, the first nucleotide becomes incorporated into the scaffold polynucleotide. Because the terminal nucleotide of the helper strand of the polynucleotide ligation molecule is a non-ligatable nucleotide, the ligase enzyme will be prevented from ligating the helper strand and the primer strand portion of the synthesis strand, thus creating a single-strand break or “nick” between the helper strand and the primer strand portion of the synthesis strand.

[0520] Ligation of the polynucleotide ligation molecule to the scaffold polynucleotide extends the length of the support strand of the double-stranded scaffold polynucleotide of step (1) and wherein the first nucleotide of the predefined nucleotide sequence is incorporated into the support strand of the scaffold polynucleotide.

[0521] Ligation of the support strands may be performed by any suitable means. Ligation may typically and preferably 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 or other enzymes described herein. The use of such enzymes will result in the maintenance of the single-strand 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 or presence of a non-ligatable blocking group.

[0522] Following ligation of the polynucleotide ligation molecule to the scaffold polynucleotide the first nucleotide of the predefined nucleotide sequence is referred to as occupying nucleotide position n, the universal nucleotide is referred to as occupying nucleotide position n+1 and the nucleotide which prior to ligation was the terminal nucleotide of the support strand of the scaffold polynucleotide proximal to the primer strand portion is referred to as occupying nucleotide position n−1. By nucleotide position n−1 it is meant the next nucleotide position in the support strand relative to position n in the direction proximal to the primer strand portion/distal to the helper strand.

Step 3—Incorporation of a Second Nucleotide of the Predefined Sequence

[0523] In step (3) of the method, following ligation of the polynucleotide ligation molecule to the scaffold polynucleotide a second nucleotide of the predefined sequence is then incorporated into the synthesis strand by extension of the primer strand portion.

[0524] Extension of the primer strand portion may be achieved by the action of any suitable enzyme which possesses the capability of extending an oligonucleotide or a polynucleotide molecule with a single nucleotide. Such an enzyme is typically a nucleotide transferase enzyme or a polymerase enzyme. Any suitable enzyme as defined further herein or known to the skilled person may be used.

[0525] If immediately prior to extension of the primer strand portion a helper strand is present in the scaffold polynucleotide, and particularly if a polymerase enzyme is used, the enzyme may act to “invade” the helper strand and displace the terminal nucleotide of the helper strand. The incorporated second nucleotide will then occupy the position previously occupied by the displaced terminal nucleotide of the helper strand (see the structure depicted in the middle of the lower part of FIG. **1**). In certain embodiments the helper strand may be removed prior to

extension/incorporation step (3) in which case the enzyme may gain access to and extend the terminal nucleotide of the primer strand portion of the synthesis strand without the need to displace the helper strand or a portion thereof.

[0526] The second nucleotide of the predefined sequence which is incorporated during step (3) comprises a reversible terminator group which prevents further extension by the enzyme, or comprises any other analogous functionality which prevents further extension by the enzyme. Any suitable reversible terminator group or functionality as defined further herein or known to the skilled person may be used.

[0527] Upon its incorporation into the primer strand portion of the scaffold polynucleotide the second nucleotide of the predefined sequence of that cycle becomes paired with the first nucleotide of the predefined sequence of that cycle to form a nucleotide pair of that cycle. A nucleotide pair may be any suitable nucleotide pair as defined further herein.

Step 4—Cleavage

[0528] In step (4) of the method the ligated scaffold polynucleotide is cleaved (**104**) 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-strand break in the scaffold polynucleotide. Cleavage of the scaffold polynucleotide (step 4) results in loss of the helper strand, if present and hybridised to the support strand immediately prior to cleavage, and loss of the support strand comprising the universal nucleotide.

[0529] Cleavage of the scaffold polynucleotide thereby releases the polynucleotide ligation molecule from the scaffold polynucleotide but leads to the retention of the first nucleotide of that cycle attached to the first strand of the cleaved scaffold polynucleotide and paired with the second nucleotide of that cycle. Cleavage of the scaffold polynucleotide leaves in place a cleaved blunt-ended double-stranded scaffold polynucleotide comprising the first nucleotide of the predefined sequence at the terminal end of the support strand and the second nucleotide of the predefined sequence at the terminal end of the primer strand portion of the synthesis strand, wherein the first and second nucleotides form a nucleotide pair.

[0530] The synthesis strand is already provided with a single-strand break or “nick” in this exemplary method, thus only cleavage of the support strand is necessary to provide a double-strand break in the scaffold polynucleotide. Furthermore, as noted previously 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, and 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. When the support strand of the scaffold polynucleotide is cleaved between nucleotide positions n+1 and n the polynucleotide ligation molecule is released from the scaffold polynucleotide (see structure depicted at the top left of the upper part of FIG. **1**) except that the first nucleotide of that cycle is retained in the scaffold polynucleotide attached to the support strand of the cleaved scaffold polynucleotide.

[0531] 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. This ensures that the support strand of the cleaved scaffold polynucleotide can be ligated to the support strand of the polynucleotide ligation molecule in the ligation step of the next cycle of synthesis. Cleavage is performed so that the terminal nucleotide of the support strand of the cleaved scaffold polynucleotide retains ligatable group, preferably a terminal phosphate group and such that a phosphorylation step need not therefore be performed.

[0532] Thus in method version 1 the universal nucleotide occupies position n+1 in the support strand at steps (2), (3) and (4) and the support strand is cleaved between nucleotide positions n+1 and n at step (4).

[0533] Preferably, the support strand is cleaved by cleavage of the phosphodiester bond between nucleotide positions n+1 and n (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).
[0534] The support strand may be cleaved by cleavage of one ester bond of the phosphodiester bond between nucleotide positions n+1 and n.
[0535] 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.
[0536] Any suitable mechanism may be employed to effect cleavage of the support strand between nucleotide positions n+1 and n when the universal nucleotide occupies position n+1.
[0537] Cleavage of the support strand between nucleotide positions n+1 and n as described above may be performed by the action of an enzyme.
[0538] Cleavage of the support strand between nucleotide positions n+1 and n as described above may be performed as a two-step cleavage process.
[0539] 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+1, and the second cleavage step may comprise cleaving the support strand at the abasic site, between positions n+1 and n.
[0540] One mechanism of cleaving the support strand at a cleavage site defined by a sequence comprising a universal nucleotide in the manner outlined above 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.
[0541] 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 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 an enzyme which catalyses the release of uracil from polynucleotides, such as Uracil-DNA Glycosylase (UDG).
[0542] 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.
[0543] Thus in embodiments wherein the universal nucleotide is at position n+1 of the support strand at steps (1) and (2) and the support strand is cleaved between positions n+1 and n, 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 be performed with an enzyme having abasic site lyase activity such as Endonuclease VIII or Endonuclease III.
[0544] Cleavage of the support strand between nucleotide positions n+1 and n 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. Other enzymes which may be used in any such process include enzymes which cleave 8-oxoguanosine, such as formamidopyrimidine DNA glycosylase (Fpg) and 8-oxoguanine DNA glycosylase (hOGG1)

Step 5—Deprotection

[0545] 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; **105**). This can be performed at various stages of the first cycle. It may be performed as step (5) of the method, after cleavage step (4). However, the deprotection step could be performed after incorporation of the second nucleotide at step (3) and before cleavage step (4), in which case the deprotection step is defined as step (4) and the cleavage step is defined as step(S), as shown in FIG. **1** (**104** and **105** respectively). Regardless of which stage the deprotection step is performed, enzyme and residual unincorporated second nucleotides should first be removed following step (3) in order to prevent multiple incorporation of second nucleotides in the same cycle of synthesis.

[0546] Removal of the reversible terminator group from the first nucleotide can be performed by any suitable means known to the skilled person. For example, removal can be performed by the use of a chemical, such as tris (carboxyethyl) phosphine (TCEP).

Further Cycles

[0547] Following completion of the first cycle of synthesis, second and further cycles of synthesis may be performed using the same method steps.

[0548] The cleavage product of steps (4) and (5) of the previous cycle is provided (in step 6) as the double-stranded scaffold polynucleotide for the next cycle of synthesis.

[0549] In step (6) of the next and each further cycle of synthesis a further double-stranded polynucleotide ligation molecule is ligated to the cleavage product of steps (4) and(S) of the previous cycle. The polynucleotide ligation molecule may be structured in the same way as described above for step (2) of the previous cycle, except that the polynucleotide ligation molecule comprises a first nucleotide of the further cycle of synthesis. The polynucleotide ligation molecule may be ligated to the cleavage product of steps (4) and (5) of the previous cycle in the same way as described above for step (2).

[0550] In step (7) of the next and each further cycle of synthesis the terminal end of the primer strand portion of the synthesis strand of the double-stranded scaffold polynucleotide is further extended by the incorporation of a second nucleotide of the further cycle of synthesis by the action of the nucleotide transferase enzyme, polymerase enzyme or other enzyme. The second nucleotide of the further cycle of synthesis may be incorporated in the same way as described above for step (3).

[0551] In step (8) of the next and each further cycle of synthesis the ligated scaffold polynucleotide is cleaved at the cleavage site. Cleavage results in a double-strand break in the scaffold polynucleotide. Cleavage of the scaffold polynucleotide (step 8) results in loss of the helper strand, if present and hybridised to the support strand immediately prior to cleavage, and loss of the support strand comprising the universal nucleotide, Cleavage of the scaffold polynucleotide thereby releases the further polynucleotide ligation molecule from the scaffold polynucleotide but leads to the retention of the first nucleotide of that further cycle attached to the support strand of the cleaved scaffold polynucleotide and paired with the second nucleotide of that further cycle. Cleavage of the scaffold polynucleotide leaves in place a cleaved blunt-ended double-stranded scaffold polynucleotide comprising the first nucleotide of the further cycle at the terminal end of the support strand of the scaffold polynucleotide and the second nucleotide of the further cycle at the terminal end of the primer strand portion of the synthesis strand of the scaffold polynucleotide. Cleavage at step (8) may be performed in the same way as described above for step (4).

[0552] In step (9) the reversible terminator group is removed from the second nucleotide of the

further cycle (deprotection step; 109). As described above for first cycle, this can be performed at various stages. It may be performed as step (9) of the method, after cleavage step (8). Alternatively, the deprotection step could be performed at any step after incorporation step (7) and before cleavage step (8), in which case the deprotection step is defined as step (8) and the cleavage step is defined as step (9), as shown in FIG. **1** (**109** and **110** respectively). Deprotection by removal of the reversible terminator group in the next cycle and further cycles may be performed as described above with respect to the first synthesis cycle.

[0553] 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

[0554] With reference to FIG. **2**, in a second specific non-limiting exemplary version of the synthesis methods of the invention a double-stranded scaffold polynucleotide is provided (step) of FIG. **2**; **201**). The double-stranded scaffold polynucleotide comprises a support strand and a synthesis strand hybridised thereto. The synthesis strand comprises a primer strand portion. The double-stranded scaffold polynucleotide is provided with at least one blunt end, wherein the at least one blunt end comprises the terminal end of the primer strand portion and the terminal end of the support strand hybridized thereto. The terminal nucleotide of the support strand is capable of acting as a substrate for a ligase enzyme, and preferably comprises a phosphate group or other ligatable group.

[0555] In step (2) of the methods a polynucleotide ligation molecule (see structure depicted at the top right of the upper part of FIG. **2**) is ligated to the scaffold polynucleotide Ligation incorporates a first nucleotide of the predefined sequence into the support strand of the scaffold polynucleotide (step 2 of FIG. **2**; **202**).

[0556] In step (3) of the methods a second nucleotide of 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 the capability of extending an oligonucleotide or a polynucleotide molecule with a single nucleotide. Such an enzyme is typically a nucleotide transferase enzyme or a polymerase enzyme (step 3 of FIG. **2**; **203**). The second 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.

[0557] In each cycle of synthesis the scaffold polynucleotide is then cleaved at a cleavage site defined by a sequence comprising the universal nucleotide in the support strand (step 4. **204**). In method version 2 cleavage comprises cleaving the support strand immediately after the nucleotide which is in the nucleotide position next to the universal nucleotide in the direction proximal to the primer strand portion/distal to the helper strand. Cleavage of the scaffold polynucleotide (step 4) results in release of the polynucleotide ligation molecule from the scaffold polynucleotide and retention of the first nucleotide of that cycle attached to the first strand of the cleaved scaffold polynucleotide and paired with the second nucleotide of that cycle which is attached to the primer strand portion of the synthesis strand. Cleavage of the scaffold polynucleotide (step 4) results in loss of the helper strand from the scaffold polynucleotide, if present and hybridised to the support strand immediately prior to cleavage, and loss of the universal nucleotide from the scaffold polynucleotide. 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 either strand, and with the first and second nucleotides as a nucletoide pair.

[0558] In step (5) of the methods a deprotection step is performed to remove the terminator group from the newly-incorporated nucleotide. The deprotection step may alternatively be performed before the cleavage step in which case the deprotection step is defined as step (4) and the cleavage

step is defined as step (5), as shown in FIGS. **2** (**204** and **205** respectively).

Step 1—Provision of a Scaffold Polynucleotide

[0559] In exemplary version 2 of the synthesis methods of the invention a double-stranded scaffold polynucleotide is provided in step (1) (**201**). The double-stranded scaffold polynucleotide is provided comprising a synthesis strand and a support strand hybridised thereto, wherein the synthesis strand comprises a primer strand portion. The terminal nucleotide of the support strand is paired with the terminal nucleotide of the support strand thus forming a blunt end. The terminal nucleotide of the support strand is capable of acting as a substrate for a ligase enzyme, and comprises a ligatable group, preferably a phosphate group.

Step 2—Ligation of a Polynucleotide Ligation Molecule to the Scaffold Polynucleotide and Incorporation of a First Nucleotide of the Predefined Sequence

[0560] In step (2) of the method a double-stranded polynucleotide ligation molecule is ligated (**202**) to the scaffold polynucleotide by the action of a ligase enzyme in a blunt-ended ligation reaction.

[0561] The polynucleotide ligation molecule comprises a support strand and a helper strand hybridised thereto. The polynucleotide ligation molecule further comprises a complementary ligation end comprising in the support strand a universal nucleotide and a first nucleotide of the predefined sequence.

[0562] The complementary ligation end of the polynucleotide ligation molecule is structured such that the terminal nucleotide of the support strand is the first nucleotide of the predefined sequence to be incorporated into the scaffold polynucleotide in any given cycle of synthesis. The terminal nucleotide of the support strand is paired with the terminal nucleotide of the helper strand. The terminal nucleotide of the support strand, i.e. the first nucleotide of the predefined sequence of that cycle, occupies nucleotide position n in the support strand. By position n it is meant the nucleotide position which will be opposite the second nucleotide of the predefined sequence of that cycle upon incorporation of the second nucleotide, the first and second nucleotides thereby forming a nucleotide pair. In FIG. **2** the first nucleotide of the predefined sequence is depicted as adenosine, the second nucleotide of the predefined sequence in step (3) is depicted as thymine and the terminal nucleotide of the helper strand is depicted as thymine. Adenosine and thymine are depicted purely for illustration. The first and second nucleotides and the terminal nucleotide of the helper strand can be any suitable nucleotides. Nucleotides depicted as X may also be any suitable nucleotides as chosen by the user.

[0563] Typically, the terminal nucleotide of the support strand at the complementary ligation end will define the 3′ terminus of the support strand of the complementary ligation end of the polynucleotide ligation molecule.

[0564] The terminal nucleotide of the helper strand of the polynucleotide ligation molecule is configured such that it cannot be ligated to another polynucleotide in a polynucleotide strand (position labelled “T” purely for illustration in the structure depicted at the top right of the upper part of FIG. **2**). This nucleotide is referred to as a non-ligatable terminal nucleotide. Typically this terminal nucleotide will lack a phosphate group, i.e. it will be a nucleoside. Typically, this terminal nucleotide of the helper strand will define the 5′ terminus of the helper strand.

[0565] In the complementary ligation end of the polynucleotide ligation molecule the universal nucleotide in the support strand is positioned such that it occupies nucleotide position n+2 in the support strand and is paired with a partner nucleotide in the helper strand. By position n+2 it is meant the second nucleotide position in the support strand relative to position n in the direction distal to the complementary ligation end.

[0566] The complementary ligation end is configured so that it will compatibly join with the blunt end of the scaffold polynucleotide when subjected to suitable ligation conditions. Upon ligation of the support strands of the polynucleotide ligation molecule and the scaffold polynucleotide, the first nucleotide becomes incorporated into the scaffold polynucleotide. Because the terminal nucleotide of the helper strand of the polynucleotide ligation molecule is a non-ligatable nucleotide, the ligase

enzyme will be prevented from ligating the helper strand and the primer strand portion of the synthesis strand, thus creating a single-strand break or “nick” between the helper strand and the primer strand portion of the synthesis strand.

[0567] Ligation of the polynucleotide ligation molecule to the scaffold polynucleotide extends the length of the support strand of the double-stranded scaffold polynucleotide of step (1) and wherein the first nucleotide of the predefined nucleotide sequence is incorporated into the support strand of the scaffold polynucleotide.

[0568] Ligation of the support strands may be performed by any suitable means Ligation may typically and preferably 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 or other enzymes described further herein. The use of such enzymes will result in the maintenance of the single-strand 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 or presence of a non-ligatable blocking group.

[0569] Following ligation of the polynucleotide ligation molecule to the scaffold polynucleotide the first nucleotide of the predefined nucleotide sequence is referred to as occupying nucleotide position n, the universal nucleotide is referred to as occupying nucleotide position n+2 and the nucleotide which prior to ligation was the terminal nucleotide of the support strand of the scaffold polynucleotide proximal to the primer strand portion is referred to as occupying nucleotide position n−1. By nucleotide position n−1 it is meant the next nucleotide position in the support strand relative to position n in the direction proximal to the primer strand portion/distal to the helper strand.

Step 3—Incorporation of a Second Nucleotide of the Predefined Sequence

[0570] In step (3) of the method, following ligation of the polynucleotide ligation molecule to the scaffold polynucleotide a second nucleotide of the predefined sequence is then incorporated into the synthesis strand by extension of the primer strand portion.

[0571] Extension of the primer strand portion may be achieved by the action of any suitable enzyme which possesses the capability of extending an oligonucleotide or a polynucleotide molecule with a single nucleotide. Such an enzyme is typically a nucleotide transferase enzyme or a polymerase enzyme. Any suitable enzyme as defined further herein or known to the skilled person may be used.

[0572] If immediately prior to extension of the primer strand portion a helper strand is present in the scaffold polynucleotide, and particularly if a polymerase enzyme is used, the enzyme may act to “invade” the helper strand and displace the terminal nucleotide of the helper strand. The incorporated second nucleotide will then occupy the position previously occupied by the displaced terminal nucleotide of the helper strand (see the structure depicted in the middle of the lower part of FIG. **2**). In certain embodiments the helper strand may be removed prior to extension/incorporation step (3) in which case the enzyme may gain access to and extend the terminal nucleotide of the primer strand portion of the synthesis strand without the need to displace the helper strand or a portion thereof.

[0573] The second nucleotide of the predefined sequence which is incorporated during step (3) comprises a reversible terminator group which prevents further extension by the enzyme, or comprises any other analogous functionality which prevents further extension by the enzyme. Any suitable reversible terminator group or functionality as defined further herein or known to the skilled person may be used.

[0574] Upon its incorporation into the primer strand portion of the scaffold polynucleotide the second nucleotide of the predefined sequence of that cycle becomes paired with the first nucleotide of the predefined sequence of that cycle to form a nucleotide pair of that cycle. A nucleotide pair may be any suitable nucleotide pair as defined further herein.

Step 4—Cleavage

[0575] In step (4) of the method the ligated scaffold polynucleotide is cleaved (**204**) 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-strand break in the scaffold polynucleotide. Cleavage of the scaffold polynucleotide (step 4) results in loss of the helper strand, if present and hybridised to the support strand immediately prior to cleavage, and loss of the support strand comprising the universal nucleotide.

[0576] Cleavage of the scaffold polynucleotide thereby releases the polynucleotide ligation molecule from the scaffold polynucleotide but leads to the retention of the first nucleotide of that cycle attached to the first strand of the cleaved scaffold polynucleotide and paired with the second nucleotide of that cycle. Cleavage of the scaffold polynucleotide leaves in place a cleaved blunt-ended double-stranded scaffold polynucleotide comprising the first nucleotide of the predefined sequence at the terminal end of the support strand and the second nucleotide of the predefined sequence at the terminal end of the primer strand portion of the synthesis strand, wherein the first and second nucleotides form a nucleotide pair.

[0577] The synthesis strand is already provided with a single-strand break or “nick” in this exemplary method, thos only cleavage of the support strand is necessary to provide a double-strand break in the scaffold polynucleotide. Furthermore, as noted previously in this exemplary method version cleavage generates a blunt-ended cleaved double-stranded scaffold polynucleotide with no overhang in either the synthesis strand of the support strand, and 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. When the support strand of the scaffold polynucleotide is cleaved between nucleotide positions n+1 and n the polynucleotide ligation molecule is released from the scaffold polynucleotide (see structure depicted at the top left of the upper part of FIG. **1**) except that the first nucleotide of that cycle is retained in the scaffold polynucleotide attached to the support strand of the cleaved scaffold polynucleotide.

[0578] 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. This ensures that the support strand of the cleaved scaffold polynucleotide can be ligated to the support strand of the polynucleotide ligation molecule in the ligation step of the next cycle of synthesis. Cleavage is performed so that the terminal nucleotide of the support strand of the cleaved scaffold polynucleotide retains a ligatable group, preferably a terminal phosphate group and such that a phosphorylation step need not therefore be performed.

[0579] Thus in method version 1 the universal nucleotide occupies position n+2 in the support strand at steps (2), (3) and (4) and the support strand is cleaved between nucleotide positions n+1 and n at step (4).

[0580] Preferably, the support strand is cleaved by cleavage of the phosphodiester bond between nucleotide positions n+1 and n (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).

[0581] The support strand may be cleaved by cleavage of one ester bond of the phosphodiester bond between nucleotide positions n+1 and n.

[0582] 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.

[0583] Any suitable mechanism may be employed to effect cleavage of the support strand between nucleotide positions n+1 and n when the universal nucleotide occupies position n+2.

[0584] Cleavage of the support strand between nucleotide positions n+1 and n as described above may be performed by the action of an enzyme.

[0585] Cleavage of the support strand between nucleotide positions n+1 and n when the universal nucleotide occupies position n+2 in the support strand, as described above, may be performed by the action of an enzyme such as Endonuclease V.
[0586] One mechanism of cleaving the support strand between nucleotide positions n+1 and n at a cleavage site defined by a sequence comprising a universal nucleotide which is occupying position n+2 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.
[0587] 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 when the universal nucleotide occupies position n+2 in the support strand.
Step 5—Deprotection
[0588] 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; 205). This can be performed at various stages of the first cycle. It may be performed as step (5) of the method, after cleavage step (4). However, the deprotection step could be performed after incorporation of the second nucleotide at step (3) and before cleavage step (4), in which case the deprotection step is defined as step (4) and the cleavage step is defined as step (5), as shown in FIGS. **2** (**204** and **205** respectively). Regardless of which stage the deprotection step is performed, enzyme and residual unincorporated second nucleotides should first be removed following step (3) in order to prevent multiple incorporation of second nucleotides in the same cycle of synthesis.
[0589] Removal of the reversible terminator group from the first nucleotide can be performed by any suitable means known to the skilled person. For example, removal can be performed by the use of a chemical, such as tris (carboxyethyl) phosphine (TCEP).
Further Cycles
[0590] Following completion of the first cycle of synthesis, second and further cycles of synthesis may be performed using the same method steps.
[0591] The cleavage product of steps (4) and (5) of the previous cycle is provided (in step 6) as the double-stranded scaffold polynucleotide for the next cycle of synthesis.
[0592] In step (6) of the next and each further cycle of synthesis a further double-stranded polynucleotide ligation molecule is ligated to the cleavage product of steps (4) and (5) of the previous cycle. The polynucleotide ligation molecule may be structured in the same way as described above for step (2) of the previous cycle, except that the polynucleotide ligation molecule comprises a first nucleotide of the further cycle of synthesis. The polynucleotide ligation molecule may be ligated to the cleavage product of steps (4) and (5) of the previous cycle in the same way as described above for step (2).
[0593] In step (7) of the next and each further cycle of synthesis the terminal end of the primer strand portion of the synthesis strand of the double-stranded scaffold polynucleotide is further extended by the incorporation of a second nucleotide of the further cycle of synthesis by the action of the nucleotide transferase enzyme, polymerase enzyme or other enzyme. The second nucleotide of the further cycle of synthesis may be incorporated in the same way as described above for step (3).
[0594] In step (8) of the next and each further cycle of synthesis the ligated scaffold polynucleotide is cleaved at the cleavage site. Cleavage results in a double-strand break in the scaffold polynucleotide. Cleavage of the scaffold polynucleotide (step 8) results in loss of the helper strand, if present and hybridised to the support strand immediately prior to cleavage, and loss of the support strand comprising the universal nucleotide. Cleavage of the scaffold polynucleotide thereby releases the further polynucleotide ligation molecule from the scaffold polynucleotide but leads to

the retention of the first nucleotide of that further cycle attached to the support strand of the cleaved scaffold polynucleotide and paired with the second nucleotide of that further cycle. Cleavage of the scaffold polynucleotide leaves in place a cleaved blunt-ended double-stranded scaffold polynucleotide comprising the first nucleotide of the further cycle at the terminal end of the support strand of the scaffold polynucleotide and the second nucleotide of the further cycle at the terminal end of the primer strand portion of the synthesis strand of the scaffold polynucleotide. Cleavage at step (8) may be performed in the same way as described above for step (4).

[0595] In step (9) the reversible terminator group is removed from the second nucleotide of the further cycle (deprotection step; 209). As described above for first cycle, this can be performed at various stages. It may be performed as step (9) of the method, after cleavage step (8). Alternatively, the deprotection step could be performed at any step after incorporation step (7) and before cleavage step (8), in which case the deprotection step is defined as step (8) and the cleavage step is defined as step (9), as shown in FIGS. **2** (**209** and **210** respectively). Deprotection by removal of the reversible terminator group in the next cycle and further cycles may be performed as described above with respect to the first synthesis cycle.

[0596] Synthesis cycles are repeated as described above for as many times as necessary to synthesise the double-stranded polynucleotide having the predefined nucleotide sequence.

Variants of Synthesis Method Version 2

[0597] In addition to the above-described methods, variants of synthesis method version 2 are provided wherein the method is performed in the same way as synthesis method version 2 described above except for the following variations.

[0598] In the ligation step of the first cycle (step 2) the complementary ligation end of the polynucleotide ligation molecule is structured such that the universal nucleotide instead occupies nucleotide position n+2+x in the support strand and is paired with a partner nucleotide in the helper strand which is 2+x positions removed from the terminal nucleotide of the helper strand at the complementary ligation end; wherein nucleotide position n+2 is the second nucleotide position in the support strand relative to nucleotide position n in the direction distal to the complementary ligation end.

[0599] In the cleavage step of the first cycle (step 4) the universal nucleotide instead occupies nucleotide position n+2+x in the support strand of the scaffold polynucleotide, wherein nucleotide position n+2 is the second nucleotide position in the support strand relative to nucleotide 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+1 and n.

[0600] In the ligation step of the second cycle (step 6) and in ligation steps of all further cycles the complementary ligation end of the polynucleotide ligation molecule is structured such that the universal nucleotide occupies nucleotide position n+2+x in the support strand and is paired with a partner nucleotide which is 2+x positions removed from the terminal nucleotide of the helper strand at the complementary ligation end; wherein nucleotide position n+2 is the second nucleotide position in the support strand relative to nucleotide position n in the direction distal to the complementary ligation end.

[0601] Finally, in the cleavage step of the second cycle (step 8) and in cleavage steps of all further cycles the universal nucleotide instead occupies nucleotide position n+2+x in the support strand of the scaffold polynucleotide, wherein nucleotide position n+2 is the second nucleotide position in the support strand relative to nucleotide 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+1 and n.

[0602] In all of these variant methods, x is a whole number from 1 to 10 or more, and wherein x is the same whole number in steps (2), (4), (6) and (8).

[0603] As with synthesis method version 2, with variant methods based on version 2 it will be noted that in any given cycle the nucleotide position occupied by the first nucleotide of that cycle

in the support strand following ligation and during the incorporation and cleavage steps is defined as nucleotide position n, and upon completion of the given cycle of synthesis the position occupied by that first nucleotide of that cycle in the support strand of the cleaved scaffold polynucleotide is defined as nucleotide position n−1 in the next cycle of synthesis.

[0604] Thus in these particular variants of synthesis method version 2 the position of the cleavage site relative to nucleotide position n is held constant, and the position of the universal nucleotide relative to nucleotide position n is increased by moving the position of the universal nucleotide in the direction proximal to the helper strand/distal to the primer strand portion by a number of nucleotide positions determined by the number selected for x.

[0605] A diagrammatic representation of these variant methods is provided in FIG. **3**, wherein the deprotection step is shown as step (4) and the cleavage step is shown as step (5). As discussed above, the order in which these steps may be performed can be switched.

Variants of Synthesis Method Versions 1 and 2 Involving Incorporation of More than Two Nucleotides Per Cycle

[0606] The invention further provides further additional variant methods wherein more than two nucleotides are incorporated per cycle, including further additional variant methods based on specific versions 1 and 2 and the variants thereof described above. Any of these further variant methods may be performed as described above, except that the following modifications may be made.

[0607] In step (2) the polynucleotide ligation molecule is provided with a complementary ligation end comprising a first nucleotide of the predefined sequence of the first cycle and further comprising one or more further nucleotides of the predefined sequence of the first cycle. The polynucleotide ligation molecule is then ligated to the scaffold polynucleotide. The first nucleotide of the predefined sequence of the first cycle is the terminal nucleotide of the support strand of the complementary ligation end. The complementary ligation end is preferably structured such that the first and further nucleotides of the predefined sequence of the first cycle comprise a linear sequence of nucleotides wherein each nucleotide in the sequence occupies the next nucleotide position in the support strand in the direction distal to the complementary ligation end.

[0608] In step (3) the terminal end of the primer strand portion of the synthesis strand of the double-stranded scaffold polynucleotide is extended by the incorporation of a second nucleotide of the predefined sequence of the first cycle by the action of the nucleotide transferase or polymerase enzyme, and wherein the terminal end of the primer strand portion is further extended by the incorporation of one or more further nucleotides of the predefined sequence of the first cycle by the action of the nucleotide transferase or polymerase enzyme, wherein each one of the second and further nucleotides of the first cycle comprises a reversible terminator group which prevents further extension by the enzyme, and wherein following each further extension the reversible terminator group is removed from a nucleotide in a deprotection step (step 4) before the incorporation of the next nucleotide.

[0609] The complementary ligation end of the polynucleotide ligation molecule is structured such that in step (4) prior to cleavage the universal nucleotide occupies a position in the support strand which is after the nucleotide positions of the first and further nucleotides in the direction distal to the complementary ligation end.

[0610] In step (4) following cleavage the first, second and further nucleotides of the predefined sequence of the first cycle are retained in the cleaved scaffold polynucleotide.

[0611] In step (6) the polynucleotide ligation molecule is provided with a complementary ligation end comprising a first nucleotide of the predefined sequence of the further cycle and further comprising one or more further nucleotides of the predefined sequence of the further cycle. As in step (2) the polynucleotide ligation molecule is then ligated to the scaffold polynucleotide. As in step (2) the first nucleotide of the predefined sequence of the further cycle is the terminal nucleotide of the support strand of the complementary ligation end. The complementary ligation

end is preferably structured such that the first and further nucleotides of the predefined sequence of the further cycle comprise a linear sequence of nucleotides wherein each nucleotide in the sequence occupies the next nucleotide position in the support strand in the direction distal to the complementary ligation end.

[0612] In step (6) the terminal end of the primer strand portion of the synthesis strand of the double-stranded scaffold polynucleotide is extended by the incorporation of a second nucleotide of the predefined sequence of the further cycle by the action of the nucleotide transferase or polymerase enzyme, and wherein the terminal end of the primer strand portion is further extended by the incorporation of one or more further nucleotides of the predefined sequence of the further cycle by the action of the nucleotide transferase or polymerase enzyme, wherein each one of the second and further nucleotides of the further cycle comprises a reversible terminator group which prevents further extension by the enzyme, and wherein following each further extension the reversible terminator group is removed from a nucleotide before the incorporation of the next nucleotide.

[0613] In step (8) following cleavage the first, second and further nucleotides of the predefined sequence of the further cycle are retained in the cleaved scaffold polynucleotide.

[0614] In these variant methods cleavage is always performed as steps (5) and (9) following the final deprotection step (steps 4 and 8), except that the reversible terminator group of the very last further nucleotide to be incorporated in any given cycle may alternatively be removed from the very last further nucleotide after the step of cleaving the ligated scaffold polynucleotide at the cleavage site.

[0615] In these variant methods in any given cycle of synthesis, prior to cleavage, in the support strand the position occupied by the first nucleotide of the predefined sequence may be referred to as position n, the position occupied by the first further nucleotide of the predefined sequence in the support strand may be referred to as position n+1, and the position occupied by the universal nucleotide may be referred to as position n+2+x, wherein x is a whole number from zero to 10 or more, wherein x is zero if the support strand comprises only one further nucleotide and wherein x is one if the support strand comprises only two further nucleotides, and so on and so forth.

[0616] A diagrammatic representation of these variant methods is provided in FIG. **4**, wherein the deprotection step is shown as step (4) and the cleavage step is shown as step (5). As discussed above, the order in which these steps may be performed can be switched with respect to the very last nucleotide to be incorporated.

[0617] In any of these variant methods the complementary ligation end of the polynucleotide ligation molecule is structured such that in steps (4) and (8) prior to cleavage the universal nucleotide occupies a position in the support strand which is the next nucleotide position in the support strand after the nucleotide positions of the first and further nucleotides in the direction distal to the complementary ligation end, and the support strand is cleaved between the position occupied by the last further nucleotide and the position occupied by the universal nucleotide.

[0618] In any of these variant methods the complementary ligation end of the polynucleotide ligation molecule is alternatively structured such that in steps (4) and (8) prior to cleavage the universal nucleotide occupies a position in the support strand which is the next+1 nucleotide position in the support strand after the nucleotide positions of the first and further nucleotides in the direction distal to the complementary ligation end, and the support strand is alternatively cleaved between the position occupied by the last further nucleotide and the position occupied by the next nucleotide in the support strand.

[0619] It will be appreciated that in order to accommodate the incorporation of further nucleotides during the first and further cycles of synthesis, in addition to each first and second nucleotides, routine adaptations may need to be made to the configuration of the complementary ligation end of the polynucleotide ligation molecule in terms of the placement of the universal nucleotide relative to nucleotide position n. In all methods of the invention described and defined herein nucleotide

position n is invariably the nucleotide position in the support strand which is or will be opposite the second nucleotide of the predefined sequence of any given cycle prior to or upon its incorporation. Thus the skilled person is readily able to make routine modifications to the positioning of the universal nucleotide and the selection of the cleavage site relative to nucleotide position n in order to adapt any of the method versions and variants thereof to accommodate the incorporation of further nucleotides of the first and further cycles of synthesis.

EXAMPLES

[0620] 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.

[0621] 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.

[0622] 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 scaffold polynucleotide, cleavage of the scaffold polynucleotide at a cleavage site defined by a universal nucleotide and ligation of a polynucleotide ligation molecule 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 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 Therminator X DNA polymerase using an incorporation step according to methods of the invention, e.g. synthesis method versions of the invention 1 and 2 and variants thereof (FIGS. **1** to **5**).

[0623] In the following Examples, and in corresponding FIGS. **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 FIGS. **6**, **7** and **8** and not according to the reaction schematics set out in any of FIGS. **1** to **5** or descriptions of the same herein. Example 13 and FIG. **51** are to be interpreted according to synthesis methods the invention. In particular according to synthesis methods of the invention 1 and 2 and variants thereof and associated reaction schematics set out in FIGS. **1** to **5**, and more particularly incorporation step 3 of such methods.

Example 1. Synthesis in the Absence of a Helper Strand

[0624] 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.

Step 1: Incorporation

[0625] The first step describes controlled addition of a 3′-O-protected single nucleotide to an oligonucleotide by enzymatic incorporation by DNA polymerase (FIG. **12***a*).

Materials and Methods

Materials

[0626] 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, Juliesta E. Sylvester, Modified Template-Independent Enzymes for Polydeoxynucleotide Synthesis, Molecular Assemblies US2016/0108382A1. [0627] 2. Oligonucleotides were designed in house and obtained from Sigma-Aldrich (FIG. **12***h*). The stock solutions were prepared at a concentration of 100 μM. [0628] 3. Therminator 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.

[0629] Two types of reversible terminators were tested:
##STR00036##
Methods
[0630] 1. 2 μl of 10× Thermopol® buffer (20 mM Tris-HCl, 10 mM (NH.sub.4).sub.2SO.sub.4, 10 mM KCl, 2 mM MgSO.sub.4, 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. [0631] 2. 0.5 μl of 10 μM primer (synthesised strand) (5 pmol, 1 equiv) (SEQ ID NO: 1, FIG. **12***h*) and 0.75 μl of 10 μM template (support strand) (6 pmol, 1.5 equiv) (SEQ ID NO: 2, FIG. **12***h*) were added to the reaction mixture. [0632] 3. 3′-O-modified-dTTP (2 μl of 100 μM) and MnCl.sub.2 (1 μl of 40 mM) were added. [0633] 4. 1.5 μl of Therminator IX DNA polymerase (15 U, New England BioLabs) was then added. However, any DNA polymerase that could incorporate modified dNTPs could be used. [0634] 5. The reaction was incubated for 20 minutes at 65° C. [0635] 6. The reaction was stopped by addition of TBE-Urea sample buffer (Novex). [0636] 7. The reaction was separated on polyacrylamide gel (15%) with TBE buffer and visualized by ChemiDoc MP imaging system (BioRad).
Gel Electrophoresis and DNA Visualization:
[0637] 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). [0638] 2. 5 μl of the sample was then loaded into the wells of a 15% TBE-Urea gel 1.0 mm×10 well (Invitrogen) which contained preheated 1×TBE buffer Thermo Scientific (89 mM Tris, 89 mM boric acid and 2 mM EDTA). [0639] 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. [0640] 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
[0641] Customised engineered Therminator IX DNA polymerase from New England BioLabs is an efficient DNA polymerase able to incorporate 3′-O-modified-dNTPs opposite a universal nucleotide e.g. inosine (FIG. **12***b-c*).
[0642] Efficient incorporation opposite inosine occurred at a temperature of 65° C. (FIG. **12***d-e*).
[0643] Incorporation of 3′-O-modified-d′TTPs opposite inosine requires the presence of Mn.sup.2+ ions (FIG. **12***f-g*). Successful conversion is marked in bold in FIGS. **12** *c, e, g* and *h.*
Conclusion
[0644] Incorporation of 3-O-modified-dTTPs opposite inosine can be achieved with particularly high efficiency using customized engineered Therminator IX DNA polymerase from New England BioLabs, in the presence of Mn.sup.2+ ions and at a temperature at 65° C.
Step 2: Cleavage
[0645] The second step describes a two-step cleavage of polynucleotides with either hAAG/Endo VIII or bAAG/chemical base (FIG. **13***a*).
Materials and Methods
Materials
[0646] 1. Oligonucleotides utilized in Example 1 were designed in-house and synthesised by Sigma Aldrich (see table in FIG. **13** (*e*) for sequences). [0647] 2. The oligonucleotides were diluted to a stock concentration of 100 uM using sterile distilled water (ELGA VEOLIA).
Methods
[0648] A cleavage reaction on oligonucleotides was carried out using the procedure below: [0649] 1. A pipette (Gilson) was used to transfer 41 μl sterile distilled water (ELGA. VEOLIA) into a 1.5 ml Eppendorf tube. [0650] 2. 5 μl of 10× ThermoPol® reaction buffer NEB (20 mM Tris-HCl, 10 mM (NH.sub.4).sub.2SO.sub.4, 10 mM KCl, 2 mM MgSO.sub.4, 0.1% Triton® X-100, pH 8.8) were then added into the same Eppendorf tube. [0651] 3. 1 μl each of oligonucleotides (FIG. **13***e*), 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 5 pmols were added into the same tube. [0652] 4. 1 μl of Human Alkyladenine DNA Glycosylase (HAAG) NEB (10 units/μl) was added into the same tube. [0653] 5. 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. [0654] 6. Typically after incubation time had elapsed, the reaction was terminated by enzymatic heat inactivation (i.e. 65° C. for 20 minutes).

[0655] Purification under ambient conditions. The sample mixture was purified using the protocol outlined below: [0656] 1. 500 μl of buffer PNI QIAGEN (5M guanidinium chloride) was added to the sample and mixed by gentle resuspension with a pipette. [0657] 2. The mixture was transferred into a QIAquick spin column (QIAGEN) and centrifuged for 1 min at 6000 rpm. [0658] 3. After centrifugation, flow-through was discarded and 750 μl of buffer PE QIAGEN (10 mM Tris-HCl pH 7.5 and 80% ethanol) was added into the spin column and centrifuged for 1 min at 6000 rpm. [0659] 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. [0660] 5. The spin column was then placed in a sterile 1.5 ml Eppendorf tube. [0661] 6. For DNA elution, 50 μl of Buffer EB QIAGEN (10 mM Tris.CL, pH 8.5) was added to the centre of the column membrane and left to stand for 1 min at room temperature. [0662] 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.

[0663] Measurement of purified DNA concentration was determined using the protocol below. [0664] 1. NanoDrop one (Thermo Scientific) was equilibrated by adding 2 μl of sterile distilled water (ELGA VEOLIA) onto the pedestal. [0665] 2. After equilibration, the water was gently wiped off using a lint-free lens cleaning tissue (Whatman). [0666] 3. NanoDrop one was blanked by adding 2 μl of Buffer EB QIAGEN (10 mM. Tris.CL, pH 8.5). Then step 2 was repeated after blanking. [0667] 4. DNA concentration was measured by adding 2 μl of the sample onto the pedestal and selecting the measure icon on the touch screen.

[0668] Cleavage of the generated abasic site was carried out using the procedure below: [0669] 1. 2 μl (10-100 ng/μl) DNA was added into a sterile 1.5 ml Eppendorf tube. [0670] 2. 40 μl (0.2M) NaOH or 1.5 μl Endo VIII NEB (10 units/μl) and 5 μl 10× 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. [0671] 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 1 hr. [0672] 4. After incubation time had elapsed, the reaction mixture was purified using steps 1-7 of purification protocol as outlined above.

Gel Electrophoresis and DNA Visualization:

[0673] 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). [0674] 2. The DNA mixtures were then loaded into the wells of a 15% TBE-Urea gel 1.0 mm×10 well (Invitrogen) which contained preheated 1×TBE buffer Thermo Scientific (89 mM Tris, 89 mM boric acid and 2 mM. EDTA). [0675] 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. [0676] 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

[0677] The cleavage reaction without a helper strand showed a low percentage yield of cleaved to uncleaved DNA ratio of ˜7%:93% (FIG. **13***b-d*).

[0678] 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 Endo VIII cleavage.

Step 3: Ligation

[0679] The third step describes ligation of polynucleotides with DNA ligase in the absence of a helper strand. A diagrammatic illustration is shown in FIG. **14**.

Materials and Methods

Materials

[0680] 1. Oligonucleotides utilized in Example 1 were designed in-house and synthesised by Sigma Aldrich (see table in FIG. **14***c* for sequences). [0681] 2. The oligonucleotides were diluted to a stock concentration of 100 uM using sterile distilled water (ELGA VEOLIA).

Methods

[0682] Ligation reaction on oligonucleotides was carried out using the procedure below: [0683] 1. A pipette (Gilson) was used to transfer 16 μl sterile distilled water (ELGA VEOLIA) into a 1.5 ml Eppendorf tube. [0684] 2. 10 μl of 2× Quick Ligation Reaction buffer NEB (132 mM Tris-HCl, 20 mM MgCl.sub.2. 2 mM dithiothreitol, 2 mM ATP, 15% Polyethylene glycol (PEG6000) and pH 7.6 at 25° C.) was then added into the same Eppendorf tube. [0685] 3. 1 μl each of oligonucleotides (FIG. **14***c*); 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. [0686] 4. 1 μl of Quick T4 DNA Ligase NEB (400 units/μl) was added into the same tube. [0687] 5. The 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. [0688] 6. Typically after incubation time had elapsed, reaction was terminated with the addition of TBE-Urea sample Buffer (Novex). [0689] 7. The reaction mixture was purified using the protocol outlined in purification steps 1-7 as described above.

[0690] Measurement of purified DNA concentration was determined using the protocol below: [0691] 1. NanoDrop one (Thermo Scientific) was equilibrated by adding 2 μl of sterile distilled water (ELGA VEOLIA) onto the pedestal. [0692] 2. After equilibration, the water was gently wiped off using a lint-free lens cleaning tissue (Whatman) [0693] 3. NanoDrop one was blanked by adding 2 μl of Buffer EB QIAGEN (10 mM Tris.CL, pH 8.5), then step 2 was repeated after blanking. [0694] 4. DNA concentration was measured by adding 2 μl of the sample onto the pedestal and selecting the measure icon on the touch screen. [0695] 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.

Results and Conclusion

[0696] 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 (FIG. **14***b*).

Example 2. Version 1 Chemistry with a Helper Strand

[0697] 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.

Step 1: Incorporation

[0698] The first step describes controlled addition of 3′-O-protected single nucleotide to oligonucleotide by enzymatic incorporation using DNA polymerase (FIG. **15***a*).

Materials and Methods

Materials

[0699] 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, Juliesta E. Sylvester, Modified Template-Independent

Enzymes for Polydeoxynucleotide Synthesis, Molecular Assemblies US2016/0108382A1. [0700] 2. Oligonucleotides were designed in house and obtained from Sigma-Aldrich. The stock solutions were prepared at a concentration of 100 μM. Oligonucleotides are shown in FIG. **15***b*. [0701] 3. Therminator IX DNA polymerase was used that has been engineered by New England BioLabs with enhanced ability to incorporate 3-O-modified dNTPs.
[0702] Two types of reversible terminators were tested:
##STR00037##
Methods
[0703] 1. 2 μl of 10× Thermopol® buffer (20 mM. Tris-HCl, 10 mM (NH.sub.4).sub.2SO.sub.4, 10 mM KCl, 2 mM MgSO.sub.4, 0.1% Triton® X-100, pH 8.8, New England BioLabs) was mixed with 10.25 μl of sterile deionized water (ELGA VEOLIA) in 1.5 ml Eppendorf tube. [0704] 2. 0.5 μl of 10 μM primer (5 pmol, 1 equiv) (SEQ ID NO. 10, Table in FIG. **15** (*b*), 0.75 μl of 10 μM template (6 pmol, 1.5 equiv) (SEQ ID NO: 11, Table in FIG. **15** (*b*)), 2 μl of 10 μM of helper strand (SEQ ID NO: 12, Table in FIG. **15** (*b*)) were added to the reaction mixture. [0705] 3. 3′-O-modified-dTTP (2 μl of 100 μM) and MnCl.sub.2 (1 μl of 40 mM) were added. [0706] 4. 1.5 μl of Therminator IX DNA polymerase (15 U, New England BioLabs) was then added. [0707] 5. The reaction was incubated for 20 minutes at 65° C. [0708] 6. The reaction was stopped by addition of TBE-Urea sample buffer (Novex). [0709] 7. The reaction was separated on polyacrylamide gel (15%) TBE buffer and visualized by ChemiDoc MP imaging system (BioRad).
Gel Electrophoresis and DNA Visualization:
[0710] 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 beat ThermoMixer (Eppendorf). [0711] 2. 5 μl of the sample were then loaded into the wells of a 15% TBE-Urea gel 1.0 mm×10 well (Invitrogen) which contained preheated 1×TBE buffer Thermo Scientific (89 mM Tris, 89 mM Boric acid and 2 mM EDTA). [0712] 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. [0713] 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.
[0714] The incorporation step can be studied according to the protocol described above.
Step 2: Cleavage
[0715] The second step describes a two-step cleavage of polynucleotides with either hAAG/Endo VIII or hAAG/chemical base (×2) (FIG. **16***a*).
Materials and Methods
Materials
[0716] 1. Oligonucleotides utilized in Example 2 were designed in-house and synthesised by Sigma Aldrich (see FIG. **16***f* for sequences). [0717] 2. The oligonucleotides were diluted to a stock concentration of 100 uM using sterile distilled water (ELGA VEOLIA).
Methods
[0718] Cleavage reaction on oligonucleotides was carried out using the procedure below: [0719] 1. A pipette (Gilson) was used to transfer 41 μl sterile distilled water (ELGA. VEOLIA) into a 1.5 ml Eppendorf tube. [0720] 2. 5 μl of 10× ThermoPol® Reaction buffer NEB (20 mM Tris-HCl, 10 mM (NH.sub.4).sub.2SO.sub.4, 10 mM KCl, 2 mM MgSO.sub.4, 0.1% Triton® X-100, pH 8.8) was then added into the same Eppendorf tube. [0721] 3. 1 μl each of oligonucleotides (FIG. **16***f*); 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. [0722] 4. 1 μl of Human Alkyladenine DNA Glycosylase (HAAG) NEB (10 units/μl) was added into the same tube. [0723] 5. In the reaction using alternative base, 1 μl of Human Alkyladenine DNA Glycosylase (hAAG) NEB (100 units/μl) was added. [0724] 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. [0725] 7. Typically after incubation time had

elapsed, the reaction was terminated by enzymatic heat inactivation (i.e. 65° C. for 20 minutes).
[0726] Purification under ambient conditions. The sample mixture was purified using the protocol outlined below: [0727] 1. 500 μl of buffer PNI QIAGEN (5M guanidinium chloride) was added to the sample and mixed by gentle resuspension with a pipette. [0728] 2. The mixture was transferred into a QIAquick spin column (QIAGEN) and centrifuged for 1 min at 6000 rpm [0729] 3. After centrifugation, flow-through was discarded and 750 μl of buffer PE QIAGEN (10 mM Tris-HCl pH 7.5 and 80% ethanol) was added into the spin column and centrifuged for 1 min at 6000 rpm.
[0730] 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. [0731] 5. The spin column was then placed in a sterile 1.5 ml Eppendorf tube. [0732] 6. For DNA elution, 50 μl of Buffer EB QIAGEN (10 mM Tris CL, pH 8.5) was added to the centre of the column membrane and left to stand for Imin at room temperature. [0733] 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.
[0734] Measurement of purified DNA concentration was determined using the protocol below:
[0735] 1. NanoDrop one (Thermo Scientific) was equilibrated by adding 2 μl of sterile distilled water (ELGA VEOLIA) onto the pedestal. [0736] 2. After equilibration, the water was gently wiped off using a lint-free lens cleaning tissue (Whatman). [0737] 3. NanoDrop one was blanked by adding 2 μl of Buffer EB QIAGEN (10 mM Tris.CL, pH 8.5). Then step 2 was repeated after blanking. [0738] 4. DNA concentration was measured by adding 2 μl of the sample onto the pedestal and selecting the measure icon on the touch screen.
[0739] Cleavage of generated abasic site was carried out using the procedure below: [0740] 1. 2 μl (10-100 ng/μl) DNA was added into a sterile 1.5 ml Eppendorf tube. [0741] 2. 40 μl (0.2M) NaOH or 1.5 μl Endo VIII NEB (10 units/μl) and 5 μl 10× 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. [0742] 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 1 hr. [0743] 4. After incubation time had elapsed, the reaction mixture was purified using steps 1-7 of purification protocol as stated above.
[0744] Cleavage of generated abasic site using alternative basic chemical was carried out using the procedure below: [0745] 1. 1 μl (10-100 ng/μl) DNA was added into a sterile 1.5 ml Eppendorf tube. 2 μl of N,N′ dimethylethylenediamine Sigma (100 mM) 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.
[0746] 2. 20 μl of sterile distilled water (ELGA VEOLIA) was added into the tube and gently mixed by resuspension and centrifugation at 13000 rpm for 5 sec. [0747] 3. The resulting mixture was incubated at 37° C. for 20 minutes. [0748] 4. After incubation time had elapsed, the reaction mixture was purified using steps 1-7 of the purification protocol stated above.
Gel Electrophoresis and DNA Visualization:
[0749] 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). [0750] 2. The DNA mixtures were then loaded into the wells of a 15% TBE-Urea gel 1.0 mm×10 well (Invitrogen) which contained preheated 1×TBE buffer Thermo Scientific (89 mM Tris, 89 mM boric acid and 2 mM EDTA). [0751] 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. [0752] 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
[0753] 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 of helper strand to 50% in presence of helper strand (FIG. **16***b*). 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 (FIG. **16***c*). Mild N,N′-dimethylethylenediamine at neutral pH has high efficiency to cleave abasic sites as 0.2M NaOH, and therefore it is preferable compared with Endonuclease VIII and NaOH (FIGS. **16***d-e*).

Conclusion

[0754] 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.

[0755] 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.

[0756] 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.

[0757] 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 of 10× bAAG enzyme, had a significant increase on cleaved DNA (see FIG. **16***e*).

Step 3: Ligation

[0758] The third step describes ligation of polynucleotides with DNA ligase in the presence of a helper strand. A diagrammatic illustration is shown in FIG. **17***a*.

Materials and Methods

Materials

[0759] 1. Oligonucleotides were designed in-house and synthesised by Sigma Aldrich (see FIG. **17***d* for sequences). [0760] 2. The oligonucleotides were diluted to a stock concentration of 100 uM using sterile distilled water (ELGA VEOLIA).

Methods

[0761] Ligation reaction on oligonucleotides was carried out using the procedure below: [0762] 1. A pipette (Gilson) was used to transfer 16 μl sterile distilled water (ELGA VEOLIA) into a 1.5 ml Eppendorf tube. [0763] 2. 10 μl of 2× Quick Ligation Reaction buffer NEB (132 mM Tris-HCl, 20 mM MgCl.sub.2, 2 mM dithiothreitol, 2 mM ATP, 15% Polyethylene glycol (PEG6000) and pH 7.6 at 25° C.) was then added into the same Eppendorf tube. [0764] 3. Id each of oligonucleotides (FIG. **17***d*); 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. [0765] 4. 1 μl of Quick T4 DNA Ligase NEB (400 units/μl) was added into the same tube. [0766] 5. 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. [0767] 6. Typically after incubation time had elapsed, reaction was terminated with the addition of TBE-Urea sample Buffer (Novex). [0768] 7. The reaction mixture was purified using the protocol outlined in purification steps 1-7 as described above.

[0769] Measurement of purified DNA concentration was determined using the protocol below: [0770] 1. NanoDrop one (Thermo Scientific) was equilibrated by adding 2 μl of sterile distilled water (ELGA VEOLIA) onto the pedestal. [0771] 2. After equilibration, the water was gently wiped off using a lint-free lens cleaning tissue (Whatman). [0772] 3. NanoDrop one was blanked by adding 2 μl of Buffer EB QIAGEN (10 mM Tris.CL, pH 8.5). Then step 2 was repeated after blanking. [0773] 4. DNA concentration was measured by adding 2 μl of the sample onto the pedestal and selecting the measure icon on the touch screen. [0774] 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.

Results and Conclusion

[0775] In this specific example, and based on the specific reagents used, reduced ligation activity is

observed in the absence of a helper strand (FIG. **17***b*), whereas ligation proceeds with high efficiency in presence of a helper strand (FIG. **17***c*) and the product is formed in high yield.

Example 3. Version 2 Chemistry with a Helper Strand

[0776] 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.

Step 1: Incorporation

Materials and Methods

Materials

[0777] The first step describes controlled addition of 3′-O-protected single nucleotide to oligonucleotide by enzymatic incorporation by DNA polymerase (FIG. **18***a*) [0778] 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, Juliesta E. Sylvester, Modified Template-Independent Enzymes for Polydeoxynucleotide Synthesis, Molecular Assemblies US2016/0108382A1. [0779] 2. Oligonucleotides were designed in house and obtained from Sigma-Aldrich (FIG. **18***j*). The stock solutions are prepared in concentration of 100 μM. [0780] 3. Therminator IX DNA polymerase was used that has been engineered by New England BioLabs with enhanced ability to incorporate 3-O-modified dNTPs.

[0781] 3′-O-azidomethyl reversible terminators of all dNTPs were tested independently for incorporation:

##STR00038##

Methods

[0782] 1. 2 μl of 10× Thermopol® buffer (20 mM Tris-HCl, 10 mM (NH.sub.4).sub.2SO.sub.4, 10 mM KCl, 2 mM MgSO.sub.4. 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. [0783] 2. 0.5 μl of 10 μM primer (S pmol, 1 equiv) (SEQ ID NO: 22, FIG. **18***j*) and 0.75 μl of 10 μM template-A/G/T/C (6 pmol, 1.5 equiv) (SEQ ID NOS: 23 to 26, FIG. **18***j*) and 1 μl of 10 μM helper strand-T/C/A/G (10 pmol, 2 equiv) (SEQ ID NOS: 27 to 30, FIG. **18***j*) were added to the reaction mixture. [0784] 3. 3′-O-modified-dTTP/dCTP/dATP/dGTP (2 μl of 100 μM) and MnCl.sub.2 (1 μl of 40 mM) were added. [0785] 4. 1.5 μl of Therminator IX DNA polymerase (15 U, New England BioLabs) was then added. [0786] 5. The reaction was incubated for 20 minutes at 65° C. [0787] 6. The reaction was stopped by addition of TBE-Urea sample buffer (Novex). [0788] 7. The reaction was separated on polyacrylamide gel (15%) TBE buffer and visualized by ChemiDoc MP imaging system (BioRad).

Gel Electrophoresis and DNA Visualization:

[0789] 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). [0790] 2. 5 μl of the sample were then loaded into the wells of a 15% TBE-Urea gel 1.0 mm×10 well (Invitrogen) which contained preheated 1×TBE buffer Thermo Scientific (89 mM Tris, 89 mM boric acid and 2 mM EDTA). [0791] 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. [0792] 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

[0793] 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.
[0794] Therminator IX DNA polymerase at 2 mM Mn.sup.2+ 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).
Step 2: Cleavage
[0795] The second step describes a one-step cleavage of polynucleotides with Endonuclease V (FIG. **19***a*).
Materials and Methods
Materials
[0796] 1. Oligonucleotides utilized in Example 3 were designed in-house and synthesised by Sigma Aldrich (see table in FIG. **19***d* for sequences). [0797] 2. The oligonucleotides were diluted to a stock concentration of 100 uM using sterile distilled water (ELGA VEOLIA).
Methods
[0798] Cleavage reaction on oligonucleotides was carried out using the procedure below: [0799] 1. A pipette (Gilson) was used to transfer 41 μl sterile distilled water (ELGA VEOLIA) into a 1.5 ml Eppendorf tube. [0800] 2. 5 μl of 10× 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. [0801] 3. 1 μl each of oligonucleotides (FIG. **19***d*); 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 5 pmols, were added into the same tube. [0802] 4. 1 μl of Human Endonuclease V (Endo V) NEB (10 units/μl) was added into the same tube. [0803] 5. 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. [0804] 6. Typically after incubation time had elapsed, reaction was terminated by enzymatic heat inactivation (i.e. 65° C. for 20 minutes).
[0805] The sample mixture was purified using the protocol outlined below: [0806] 1. 500 μl of buffer PNI QIAGEN (5M guanidinium chloride) was added to the sample and mixed by gentle resuspension with a pipette. [0807] 2. The mixture was transferred into a QIAquick spin column (QIAGEN) and centrifuged for 1 min at 6000 rpm. [0808] 3. After centrifugation, flow-through was discarded and 750 μl of buffer PE QIAGEN (10 mM Tris-HCl pH 7.5 and 80% ethanol) was added into the spin column and centrifuged for 1 min at 6000 rpm. [0809] 4. The Bow-through was discarded and the spin column was centrifuged for an additional 1 min at 13000 rpm to remove residual PE buffer. [0810] 5. The spin column was then placed in a sterile 1.5 ml Eppendorf tube. [0811] 6. For DNA elution, 50 μl of Buffer EB QIAGEN (10 mM Tris.CL, pH 8.5) was added to the centre of the column membrane and left to stand for Imin at room temperature. [0812] 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.
[0813] Measurement of purified DNA concentration was determined using the protocol below: [0814] 1. NanoDrop one (Thermo Scientific) was equilibrated by adding 2 μl of sterile distilled water (ELGA VEOLIA) onto the pedestal. [0815] 2. After equilibration, the water was gently wiped off using a lint-free lens cleaning tissue (Whatman). [0816] 3. NanoDrop one was blanked by adding 2 μl of Buffer EB QIAGEN (10 mM Tris.CL, pH 8.5). Then step 2 was repeated after blanking. [0817] 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:
[0818] 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). [0819] 2. The DNA mixtures were then loaded into the wells of a 15% TBE-Urea gel 1.0 mm×10 well (Invitrogen) which contained preheated 1×TBE buffer Thermo Scientific (89 mM Tris, 89 mM

boric acid and 2 mM EDTA). [0820] 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. [0821] 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 Conclusions

[0822] 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 (FIG. **19***c*).

Step 3: Ligation

[0823] The third step describes ligation of polynucleotides with DNA ligase in the presence of a helper strand. A diagrammatic illustration is shown in FIG. **20***a*.

Materials and Methods

Materials

[0824] 1. Oligonucleotides utilized in Example 3 were designed in-house and synthesised by Sigma Aldrich (see table in FIG. **20***b* for sequences). [0825] 2. The oligonucleotides were diluted to a stock concentration of 100 μM using sterile distilled water (ELGA VEOLIA).

Methods

[0826] Ligation reaction on oligonucleotides was carried out using the procedure below [0827] 1. A pipette (Gilson) was used to transfer 16 μl sterile distilled water (ELGA VEOLIA) into a 1.5 mil Eppendorf tube. [0828] 2. 10 μl of 2× Quick Ligation Reaction buffer NEB (132 mM Tris-HCl, 20 mM MgCl.sub.2, 2 mM dithiothreitol, 2 mM ATP, 15% Polyethylene glycol (PEG6000) and pH 7.6 at 25° C.) was then added into the same Eppendorf tube. [0829] 3. 1 μl each of oligonucleotides (FIG. **20***b*); 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 into the same tube. [0830] 4. 1 μl of Quick T4 DNA Ligase NEB (400 units/μl) was added into the same tube. [0831] 5. 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. [0832] 6. Typically after the incubation time had elapsed, the reaction was terminated with the addition of TBE-Urea sample Buffer (Novex). [0833] 7. The reaction mixture was purified using the protocol outlined in purification steps 1-7 as described above.

[0834] Measurement of purified DNA concentration was determined using the protocol below: [0835] 1. NanoDrop one (Thermo Scientific) was equilibrated by adding 2 μl of sterile distilled water (ELGA VEOLIA) onto the pedestal. [0836] 2. After equilibration, the water was gently wiped off using a lint-free lens cleaning tissue (Whatman). [0837] 3. NanoDrop one was blanked by adding 2 μl of Buffer EB QIAGEN (10 mM Tris.CL, pH 8.5). Then step 2 was repeated after blanking. [0838] 4. DNA concentration was measured by adding 2 μl of the sample onto the pedestal and selecting the measure icon on the touch screen. [0839] 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.

Gel Electrophoresis and DNA Visualization:

[0840] 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). [0841] 2. The DNA mixtures were then loaded into the wells of a 15% TBE-Urea gel 1.0 mm×10 well (Invitrogen) which contained preheated 1×TBE buffer Thermo Scientific (89 mM Tris, 89 mM boric acid and 2 mM EDTA). [0842] 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 [0843] 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.

Step 4: Deprotection

[0844] Deprotection step (FIG. **21***a*) was studied on DNA model bearing 3′-O-azidomethyl group that is introduced to DNA by incorporation of 3′-O-azidomethyl-dNTPs by Therminator 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.

Materials and Methods

Materials

[0845] 1. Oligonucleotides utilized in Example 3 were designed in-house and synthesised by Sigma Aldrich (see FIG. **21***i* for sequences). [0846] 2. The oligonucleotides were diluted to a stock concentration of 100 uM using sterile distilled water (ELGA VEOLIA). [0847] 3. Enzymes were purchased from New England BioLabs.

Methods

[0848] 1. 2 μl of 10× Thermopol@ buffer (20 mM Tris-HCl, 10 mM (NH.sub.4).sub.2SO.sub.4, 10 mM KCl, 2 mM MgSO.sub.4, 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. [0849] 2. 1 μl of 10 μM primer (10 pmol, 1 equiv) (SEQ ID NO: 39, FIG. **21***i*) and 1.5 μl of either 10 μM template-A/G/T/C (15 pmol, 1.5 equiv) (SEQ ID NOS: 40 to 43, FIG. **21***i*) were added to the reaction mixture. [0850] 3. 3′-O-modified-dTTP/dCTP/dATP/dGTP (2 μl of 100 μM) and MnCl.sub.2 (1 μl of 40 mM) were added. [0851] 4. 1.5 μl of Therminator IX DNA polymerase (15 U, New England BioLabs) was then added. [0852] 5. The reaction was incubated for 5 minutes at 37° C. [0853] 6. 4 μL of the sample was taken out and mixed with 0.5 μl of 5 mM dNTP mix and allowed to react for 10 minutes for control reaction. [0854] 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. [0855] 8. The reaction mixture was purified using QIAGEN Nucleotide removal kit eluting by 20 μL of 1× Thermopol® buffer. [0856] 9. 1 μL of 5 mM dNTP mix and 1 μL of Deep Vent (exo-) DNA polymerase were added to the purified reaction mixture and allowed to react 10 minutes. [0857] 10. The reaction was stopped by addition of TBE-Urea sample buffer (Novex). [0858] 11. The reaction was separated on polyacrylamide gel (15%) TBE buffer and visualized by ChemiDoc MP imaging system (BioRad).

Results and Conclusion

[0859] 50 mM TCEP was not sufficient to cleave 3′-O-azidomethyl group with high efficiency on 0.2 μM DNA model (FIG. **21***h*). In contrast, 300 mM TCEP successfully cleaved 3′-O-azidomethyl group with 95% efficiency on 0.2 μM DNA model (FIG. **21***h*).

Example 4. Version 2 Chemistry with Double Hairpin Model

[0860] 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.

Step 1: Incorporation

[0861] The first step describes controlled addition of 3′-O-protected single nucleotide to oligonucleotide by enzymatic incorporation by DNA polymerase (FIG. **22***a*).

Materials and Methods

Materials

[0862] 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. Juliesta E. Sylvester, Modified Template-Independent Enzymes for Polydeoxynucleotide Synthesis, Molecular Assemblies US2016/0108382A1. [0863] 2. Oligonucleotides were designed in house and obtained from Sigma-Aldrich (FIG. **22***c*). The

stock solutions were prepared in concentration of 100 μM. [0864] 3. Therminator IX DNA polymerase was used that has been engineered by New England BioLabs with enhanced ability to incorporate 3-O-modified dNTPs.
[0865] 3′-O-azidomethyl-dTTP was tested for incorporation:
##STR00039##
Method
[0866] 1. 2 μl of 10× Thermopol® buffer (20 mM Tris-HCl, 10 mM (NH.sub.4).sub.2SO.sub.4, 10 mM KCl, 2 mM MgSO.sub.4, 0.1% Triton® X-100, pH 8.8, New England BioLabs) was mixed with 10.25 μl of sterile deionized water (ELGA VEOLIA) in 1.5 ml Eppendorf tube. [0867] 2. 0.5 μl of 10 μM hairpin oligonucleotide (5 pmol, 1 equiv) (SEQ ID NO: 44, FIG. **22***c*) was added to the reaction mixture. [0868] 3. 3′-O-modified-dTTP (2 μl of 100 μM) and MnCl.sub.2 (1 μl of 40 mM) were added. [0869] 4. 1.5 μl of Therminator IX DNA polymerase (15 U, New England BioLabs) was then added.
[0870] The reaction was incubated for 20 minutes at 65° C. [0871] 6. The reaction was stopped by addition of TBE-Urea sample buffer (Novex). [0872] 7. The reaction was separated on polyacrylamide gel (15%) TBE buffer and visualized by ChemiDoc MP imaging system (BioRad).
Gel Electrophoresis and DNA Visualization:
[0873] 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).
[0874] 2. 5 μl of the sample were then loaded into the wells of a 15% TBE-Urea gel 1.0 mm×10 well (Invitrogen) which contained preheated 1×TBE buffer Thermo Scientific (89 mM Tris, 89 mM boric acid and 2 mM EDTA). [0875] 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. [0876] 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
[0877] DNA polymerases incorporate 3′-O-modified-dTTPs opposite its naturally complementary base in a hairpin construct.
Step 2: Cleavage
[0878] The second step describes a one-step cleavage of a hairpin model in this particular case with Endonuclease V (FIG. **23***a*).
Materials and Methods
Materials
[0879] 1. Oligonucleotides utilized in Example 4 were designed in-house and synthesised by Sigma Aldrich (see FIG. **23***c* for sequences). [0880] 2. The oligonucleotides were diluted to a stock concentration of 100 uM using sterile distilled water (ELGA VEOLIA).
Methods
[0881] Cleavage reaction on hairpin oligonucleotides was carried out using the procedure below:
[0882] 1. A pipette (Gilson) was used to transfer 43 μl sterile distilled water (ELGA VEOLIA) into a 1.5 ml Eppendorf tube. [0883] 2. 5 μl of 10× 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. [0884] 3. 1 μl of hairpin oligonucleotide (SEQ ID NO: 45, FIG. **23***c*) having an amount of 5 pmols was added into the same tube. [0885] 4. 1 μl of Human Endonuclease V (Endo V) NEB (30 units/μl) was added into the same tube. [0886] 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. [0887] 6. Typically after incubation time had elapsed, the reaction was terminated by enzymatic heat inactivation (i.e. 65° C. for 20 minutes).
[0888] The sample mixture was purified using the protocol outlined below: [0889] 1. 500 μl of buffer PNI QIAGEN (5M guanidinium chloride) was added to the sample and mixed by gentle resuspension with a pipette. [0890] 2. The mixture was transferred into a QIAquick spin column

(QIAGEN) and centrifuged for Imin at 6000 rpm. [0891] 3. After centrifugation, flow-through was discarded and 750 μl of buffer PE QIAGEN (10 mM Tris-HCl pH 7.5 and 80% ethanol) was added into the spin column and centrifuged for Imin at 6000 rpm. [0892] 4. The flow-through was discarded and the spin column was centrifuged for an additional Imin at 13000 rpm to remove residual PE buffer. [0893] 5. The spin column was then placed in a sterile 1.5 ml Eppendorf tube. [0894] 6. For DNA elution, 50 al of Buffer EB QIAGEN (10 mM Tris.CL, pH 8.5) was added to the centre of the column membrane and left to stand for Imin at room temperature. [0895] 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.

[0896] Measurement of purified DNA concentration was determined using the protocol below. [0897] 1. NanoDrop One (Thermo Scientific) was equilibrated by adding 2 μl of sterile distilled water (ELGA VEOLIA) onto the pedestal. [0898] 2. After equilibration, the water was gently wiped off using a lint-free lens cleaning tissue (Whatman) [0899] 3. NanoDrop One was blanked by adding 2 μl of Buffer EB QIAGEN (10 mM Tris CL, pH. 8.5). Then step 2 was repeated after blanking [0900] 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:

[0901] 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 ThermoMixer (Eppendorf). [0902] 2. The DNA mixtures were then loaded into the wells of a 15% TBE-Urea gel 1.0 mm×10 well (Invitrogen) which contained preheated 1×TBE buffer Thermo Scientific (89 mM Tris, 89 mM boric acid and 2 mM EDTA). [0903] 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. [0904] 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.

[0905] Cleavage results from Example 4 showed that a significantly high yield of digested hairpin DNA was obtained with Endonuclease V at 37° C. (FIG. **23***b*).

Step 3: Ligation

[0906] The third step describes ligation of a hairpin model with DNA ligase. Diagrammatic illustration is shown in FIG. **24***a*.

Materials and Methods

Materials

[0907] 1. Oligonucleotides utilized in Example 4 were designed in-house and synthesised by Sigma Aldrich (see FIG. **24***c* for sequences). [0908] 2. The oligonucleotides were diluted to a stock concentration of 100 uM using sterile distilled water (ELGA. VEOLIA).

Method

[0909] Ligation reaction on oligonucleotides was carried out using the procedure below: [0910] 1. A pipette (Gilson) was used to transfer 1 μl (5 pmols) of TAMRA or any fluorescently tagged phosphate hairpin oligo (SEQ ID NO: 46) into a 1.5 ml Eppendorf tube. [0911] 2. 15 μl (100 pmols) 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. [0912] 3. 40 μl of Blunt/TA DNA Ligase NEB (180 units/μl) was added into the same tube. [0913] 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. [0914] 5. Typically after incubation time bad elapsed, the reaction was terminated with the addition of TBE-Urea sample buffer (Novex). [0915] 6. The reaction mixture was purified using the protocol outlined in purification steps 1-7 above.

[0916] Measurement of purified DNA concentration was determined using the protocol below: [0917] 1. NanoDrop One (Thermo Scientific) was equilibrated by adding 2 μl of sterile distilled water (ELGA VEOLIA) onto the pedestal. [0918] 2. After equilibration, the water was gently

wiped off using a lint-free lens cleaning tissue (Whatman) [0919] 3. NanoDrop One was blanked by adding 2 μl of Buffer EB QIAGEN (10 mM Tris.CL, pH 8.5). Then step 2 was repeated after blanking. [0920] 4. DNA concentration was measured by adding 2 μl of the sample onto the pedestal and selecting the measure icon on the touch screen. [0921] 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 introduced

Gel Electrophoresis and DNA Visualization.

[0922] 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 ThermoMixer (Eppendorf). [0923] 2. The DNA mixtures were then loaded into the wells of a 15% TBE-Urea gel 1.0 mm×10 well (Invitrogen) which contained preheated 1×TBE buffer Thermo Scientific (89 mM Tris, 89 mM boric acid and 2 mM EDTA). [0924] 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. [0925] 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

[0926] 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% (FIG. **24***b*).

Example 5. Version 2 Chemistry-Complete Cycle on Double Hairpin Model

[0927] 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.

[0928] 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 (FIG. **25***a*).

Materials and Methods

Materials

[0929] 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, Juliesta E. Sylvester, Modified Template-Independent Enzymes for Polydeoxynucleotide Synthesis, Molecular Assemblies US2016/0108382A1 [0930] 2. Oligonucleotides were designed in house and obtained from Sigma-Aldrich (FIG. **25***c*). The stock solutions are prepared in concentration of 100 μM. [0931] 3. Therminator IX DNA polymerase was used that has been engineered by New England BioLabs with enhanced ability to incorporate 3-O-modified dNTPs.

[0932] 3′-O-azidomethyl-dTTP was tested for incorporation.

##STR00040##

Method

[0933] 1. 2 μl of 10× Thermopol® buffer (20 mM Tris-HCl, 10 mM (NH.sub.4).sub.2SO.sub.4, 10 mM KCl, 2 mM MgSO.sub.4, 0.1% Triton® X-100, pH 8.8, New England BioLabs) was mixed with 12.5 μl of sterile deionized water (ELGA VEOLIA) in 1.5 ml Eppendorf tube. [0934] 2. 2 μl

of 10 μM double hairpin model oligonucleotide (20 pmol, 1 equiv) (SEQ ID NO: 48, FIG. **25***c*) were added to the reaction mixture. [0935] 3. 3′-O-modified-dTTP (2 μl of 100 μM) and MnCl.sub.2 (1 μl of 40 mM) were added. [0936] 4. 1.5 μl of Therminator IX DNA polymerase (15 U, New England BioLabs) was then added. [0937] 5. The reaction was incubated for 10 minutes at 37° C. [0938] 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. [0939] 7. The reaction mixture was purified using the protocol outlined in purification steps 1-7. [0940] 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. [0941] 9. 1 μl of Human Endonuclease V (Endo V) NEB (30 units/μl) was added into the same tube. [0942] 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. [0943] 11. After incubation time had elapsed, reaction was terminated by enzymatic heat inactivation (i.e. 65° C. for 20 minutes). [0944] 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). [0945] 13. Reaction mixture was purified using the protocol outlined in purification steps 1-7 above. [0946] 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. [0947] 15. 10 μl of 100 μM strand for ligation (1 nmol) (SEQ ID NO: 49, FIG. **25***c*) were added to the reaction mixture. [0948] 16. 40 μl of Blunt/TA DNA Ligase NEB (180 units/μl) was added into the purified DNA sample. [0949] 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. [0950] 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. [0951] 19. The reaction mixture was purified using QIAGEN nucleotide removal kit eluting by 20 μL of 1× Thermopol® buffer.

Gel Electrophoresis and DNA Visualization:

[0952] 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). [0953] 2. 5 μl of the sample were then loaded into the wells of a 15% TBE-Urea gel 1.0 mm×10 well (Invitrogen) which contained preheated 1×TBE buffer Thermo Scientific (89 mM Tris, 89 mM boric acid and 2 mM EDTA). [0954] 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. [0955] 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.

[0956] Measurement of purified DNA concentration was determined using the protocol below: [0957] 1. NanoDrop One (Thermo Scientific) was equilibrated by adding 2 μl of sterile distilled water (ELGA VEOLIA) onto the pedestal. [0958] 2. After equilibration, the water was gently wiped off using a lint-free lens cleaning tissue (Whatman). [0959] 3. NanoDrop One was blanked by adding 2 μl of Buffer EB QIAGEN (10 mM Tris.CL, pH 8.5). Then step 2 was repeated after blanking. [0960] 4. DNA concentration was measured by adding 2 μl of the sample onto the pedestal and selecting the measure icon on the touch screen. [0961] 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.

[0962] The sample mixture was purified after each step using the protocol outlined below: [0963] 1. 500 μl of buffer PNI QIAGEN (5M guanidinium chloride) was added to the sample and mixed by gentle resuspension with a pipette. [0964] 2. The mixture was transferred into a QIAquick spin column (QIAGEN) and centrifuged for 1 min at 6000 rpm. [0965] 3. After centrifugation, flow-through was discarded and 750 μl of buffer PE QIAGEN (10 mM Tris-HCl pH 7.5 and 80% ethanol) was added into the spin column and centrifuged for 1 min at 6000 rpm. [0966] 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. [0967] 5. The spin column was then placed in a sterile 1.5 ml Eppendorf tube. [0968] 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 min at room temperature. [0969] 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.

Results

[0970] DNA polymerase incorporates 3′-O-modified-dTTP's opposite its naturally complementary base in a double hairpin construct (FIG. **25***b*).

Example 6. Version 2 Chemistry-Complete Cycle on Single Hairpin Model Using Helper Strand

[0971] This Example describes the synthesis of polynucleotides using 4 steps on single-hairpin 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.

[0972] 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 (FIG. **26***a*).

Materials and Methods

Materials

[0973] 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, Juliesta E. Sylvester, Modified Template-Independent Enzymes for Polydeoxynucleotide Synthesis, Molecular Assemblies US2016/0108382A1 [0974] 2. Oligonucleotides were designed in house and obtained from Sigma Aldrich (FIG. **26***b*). The stock solutions are prepared in concentration of 100 μM [0975] 3. Therminator IX DNA polymerase was used that has been engineered by New England BioLabs with enhanced ability to incorporate 3-O-modified dNTPs.

[0976] 3′-O-azidomethyl-dTTP was tested for incorporation.

##STR00041##

Method

[0977] 1. 2 μl of 10× Thermopol® buffer (20 mM Tris-HCl, 10 mM (NH.sub.4).sub.2SO.sub.4, 10 mM KCl, 2 mM MgSO.sub.4, 0.1% Triton® X-100, pH 8.8, New England BioLabs) was mixed with 12.5 μl of sterile deionized water (ELGA VEOLIA) in 1.5 ml Eppendorf tube. [0978] 2. 2 μl of 10 μM Single hairpin model oligonucleotide (20 pmol, 1 equiv) (SEQ ID NO: 50, FIG. **26***b*) and Helper strand (30 pmol, 1.5 equiv) (SEQ ID NO: 51, FIG. **26***b*) were added to the reaction mixture. [0979] 3. 3′-O-modified-dTTP (2 μl of 100 μM) and MnCl.sub.2 (1 μl of 40 mM) were added [0980] 4. 1.5 μl of Therminator IX DNA polymerase (15 U, New England BioLabs) was then added. [0981] 5. The reaction was incubated for 10 minutes at 37° C. [0982] 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. [0983] 7. The reaction mixture was purified using the protocol outlined in purification steps 1-7 above. [0984] 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 a clean Eppendorf tube [0985] 9. 1 μl of Human Endonuclease V (Endo V) NEB (30 units/μl) was added into the same tube. [0986] 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. [0987] 11. After incubation time had elapsed, the reaction was terminated by enzymatic heat inactivation (i.e. 65° C. for 20 minutes). [0988] 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). [0989] 13. The reaction mixture was purified using the protocol outlined in purification steps 1-7 above. [0990] 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. [0991] 15. 10 μl of 100 μM strand for ligation (1 nmol) (SEQ ID NO: 52, FIG. **26***b*) and 10 μl of 100 μM helper strand for ligation (1 nmol) (SEQ ID NO: 53, FIG. **26***b*) were added to the reaction mixture. [0992] 16. 40 μl of Blunt/TA DNA Ligase NEB (180 units/μl) was added into the same tube. [0993] 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. [0994] 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. [0995] 19. The reaction mixture was purified using QIAGEN Nucleotide removal kit eluting by 20 μL of 1×NEB Thermopol® buffer. [0996] 20. Typically after incubation time had elapsed, reaction was terminated with the addition of TBE-Urea sample Buffer (Novex).

Gel Electrophoresis and DNA Visualization:

[0997] 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). [0998] 2. 5 μl of the sample were then loaded into the wells of a 15% TBE-Urea gel 1.0 mm×10 well (Invitrogen) which contained preheated 1×TBE buffer Thermo Scientific (89 mM Tris, 89 mM boric acid and 2 mM EDTA). [0999] 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. [1000] 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.

[1001] Measurement of purified DNA concentration was determined using the protocol below: [1002] 1. NanoDrop One (Thermo Scientific) was equilibrated by adding 2 μl of sterile distilled water (ELGA VEOLIA) onto the pedestal. [1003] 2. After equilibration, the water was gently wiped off using a lint-free lens cleaning tissue (Whatman). [1004] 3. NanoDrop One was blanked by adding 2 μl of Buffer EB QIAGEN (10 mM Tris.CL, pH 8.5). Then step 2 was repeated after blanking. [1005] 4. DNA concentration was measured by adding 2 μl of the sample unto the pedestal and selecting the measure icon on the touch screen. [1006] 5. Purified DNA was run on a polyacrylamide gel and visualized in accordance with the procedure noted above in steps 5-8. No change in conditions or reagents was introduced.

[1007] The sample mixture was purified after each step using the protocol outlined below: [1008] 1. 500 μl of buffer PNI QIAGEN (5M guanidinium chloride) was added to the sample and mixed by gentle resuspension with a pipette. [1009] 2. The mixture was transferred into a QIAquick spin column (QIAGEN) and centrifuged for Imin at 6000 rpm. [1010] 3. After centrifugation, flow-through was discarded and 750 μl of buffer PE QIAGEN (10 mM Tris-HCl pH 7.5 and 80% ethanol) was added into the spin column and centrifuged for Imin at 6000 rpm. [1011] 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. [1012] 5. The spin column was then placed in a sterile 1.5 ml Eppendorf tube. [1013] 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. [1014] 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.

Example 7. Version 3 Chemistry-Complete Cycle on Double Hairpin Model

[1015] This Example describes the synthesis of polynucleotides using 4 steps on a double-hairpin 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.

[1016] 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 (FIG. **27***a*).

Materials and Methods

Materials

[1017] 1. 3′-O-modified dNTPs were synthesised in-housed 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, Juliesta E. Sylvester, Modified Template-Independent Enzymes for Polydeoxynucleotide Synthesis, Molecular Assemblies US2016/0108382A1. [1018] 2. Oligonucleotides were designed in house and obtained from Sigma-Aldrich (FIG. **27***b*). The stock solutions are prepared in concentration of 100 μM. [1019] 3. Therminator IX DNA polymerase that has been engineered by New England BioLabs has enhanced ability to incorporate 3-O-modified dNTPs.

[1020] 3′-O-azidomethyl-dTTP was tested for incorporation.

##STR00042##

Method

[1021] 1. 2 μl of 10× Thermopol® buffer (20 mM Tris-HCl, 10 mM (NH.sub.4).sub.2SO.sub.4, 10 mM KCl, 2 mM MgSO.sub.4, 0.1% Triton® X-100, pH 8.8, New England BioLabs) was mixed with 12.5 μl of sterile deionized water (ELGA. VEOLIA) in 1.5 ml Eppendorf tube. [1022] 2. 2 μl of 10 μM double hairpin model oligonucleotide (20 pmol, 1 equiv) (SEQ ID NO: 54, FIG. **27***b*) were added to the reaction mixture. [1023] 3. 3′-O-modified-dTTP (2 μl of 100 μM) and MnCl.sub.2 (1 μl of 40 mM) were added. [1024] 4. 1.5 μl of Therminator IX DNA polymerase (15 U, New England BioLabs) was then added. [1025] 5. The reaction was incubated for 10 minutes at 37° C. [1026] 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. [1027] 7. The reaction mixture was purified using the protocol outlined in purification steps 1-7. [1028] 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. [1029] 9. 1 μl of Human Endonuclease V (Endo V) NEB (30 units/μl) was added into the same tube. [1030] 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 [1031] 11. After the incubation time had elapsed, the reaction was terminated by enzymatic beat inactivation (i.e. 65° C. for 20 minutes) [1032] 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). [1033] 13. Reaction mixture was purified using the protocol outlined in purification steps 1-7 above. [1034] 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. [1035] 15. 10 μl of 100 μM strand for ligation (1 nmol) (SEQ ID NO: 55, FIG. **27***b*), were added to the reaction mixture. [1036] 16. 40 μl of Blunt/TA DNA Ligase NEB (180 units/μl) was added into the same tube. [1037] 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. [1038] 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. [1039] 19. The reaction mixture was purified using QIAGEN Nucleotide removal kit eluting by 20 μL of 1×NEB Thermopol® buffer. [1040] 20. Typically after incubation time had elapsed, reaction was terminated with the addition of TBE-Urea sample Buffer (Novex).

Gel Electrophoresis and DNA Visualization:

[1041] 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). [1042] 2. 5 μl of the sample were then loaded into the wells of a 15% TBE-Urea gel 1.0 mm×10 well (Invitrogen) which contained preheated 1×TBE buffer Thermo Scientific (89 mM Tris, 89 mM

boric acid and 2 mM EDTA). [1043] 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. [1044] 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.
[1045] Measurement of purified DNA concentration was determined using the protocol below: [1046] 1. NanoDrop One (Thermo Scientific) was equilibrated by adding 2 μl of sterile distilled water (ELGA VEOLIA) onto the pedestal. [1047] 2. After equilibration, the water was gently wiped off using a lint-free lens cleaning tissue (Whatman). [1048] 3. NanoDrop One was blanked by adding 2 μl of Buffer EB QIAGEN (10 mM Tris.CL, pH 8.5). Step 2 was then repeated after blanking. [1049] 4. DNA concentration was measured by adding 2 μl of the sample unto the pedestal and selecting the measure icon on the touch screen. [1050] 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.
[1051] The sample mixture was purified after each step using the protocol outlined below: [1052] 1. 500 μl of buffer PNI QIAGEN (5M guanidinium chloride) was added to the sample and mixed by gentle resuspension with a pipette. [1053] 2. The mixture was transferred into a QIAquick spin column (QIAGEN) and centrifuged for 1 min at 6000 rpm. [1054] 3. After centrifugation, Now-through was discarded and 750 μl of buffer PE QIAGEN (10 mM Tris-HCl pH 7.5 and 80% ethanol) was added into the spin column and centrifuged for 1 min at 6000 rpm. [1055] 4. The flow-through was discarded and the spin column was centrifuged for an additional Imin at 13000 rpm to remove residual PE buffer. [1056] 5. The spin column was then placed in a sterile 1.5 ml Eppendorf tube. [1057] 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 Imin at room temperature. [1058] 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.
Example 8. Version 2 Chemistry-Complete Two-Cycle Experiment on Double-Hairpin Model
[1059] 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 place opposite a complementary base.
[1060] 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 shown in FIG. **28***a*. FIG. **28***b* shows a reaction schematic for the second cycle.
Materials and Methods
Materials
[1061] 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, Juliesta E. Sylvester, Modified Template-Independent Enzymes for Polydeoxynucleotide Synthesis, Molecular Assemblies US2016/0108382A1. [1062] 2. Oligonucleotides were designed in house and obtained from Sigma-Aldrich (FIG. **28***d*). The stock solutions are prepared in concentration of 100 μM. [1063] 3. Therminator IX DNA polymerase that has been engineered by New England BioLabs has enhanced ability to incorporate 3′-O-modified dNTPs.
[1064] 3′-O-azidomethyl-dTTP and 3′-O-azidomethyl-dCTP were used for incorporation: ##STR00043##
Method
1.SUP.St .Cycle:
[1065] 1. 2 μl of 10× Thermopol® buffer (20 mM Tris-HCl, 10 mM (NH.sub.4).sub.2SO.sub.4, 10

mM KCl, 2 mM MgSO.sub.4, 0.1% Triton® X-100, pH 8.8, New England BioLabs) was mixed with 12.5 μl of sterile deionized water (ELGA VEOLIA) in 1.5 ml Eppendorf tube. [1066] 2. 2 μl of 10 μM double hairpin model oligonucleotide (20 pmol, 1 equiv) (SEQ ID NO: 56, FIG. **28***d*) were added to the reaction mixture. [1067] 3. 3′-O-modified-dTTP (2 μl of 100 μM) and MnCl.sub.2 (1 μl of 40 mM) were added. [1068] 4. 1.5 μl of Therminator IX DNA polymerase (15 U, New England BioLabs) was then added. [1069] 5. The reaction was incubated for 10 minutes at 37° C. [1070] 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 min. The reaction was analysed by gel electrophoresis. [1071] 7. 40 μL of the 500 mM TCEP in 1 M TRIS buffer pH=7.4 was added to the reaction mixture and allowed to react for 10 minutes at 37° C. [1072] 8. The reaction mixture was purified using the protocol outlined in purification steps 1-7. [1073] 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. [1074] 10. 1 μl of Human Endonuclease V (Endo V) NEB (30 units/μl) was added into the same tube. [1075] 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. [1076] 12. After incubation time had elapsed, the reaction was terminated by enzymatic heat inactivation (i.e. 65° C. for 20 mins). [1077] 13. 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). [1078] 14. Reaction mixture was purified by QIAGEN Nucleotide Removal kit using the protocol outlined in purification steps 1-7. [1079] 15. The DNA sample was eluted by 20 al 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. [1080] 16. 10 μl of 100 μM strand for ligation (1 nmol) (SEQ ID NO: 57, FIG. **28***d*), were added to the reaction mixture. [1081] 17. 40 μl of Blunt/TA DNA Ligase NEB (180 units/μl) was added into the same tube. [1082] 18. 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 mins. [1083] 19. Reaction mixture was purified by Streptavidin Magnetic Beads kit using the protocol outlined in purification steps 1-5. [1084] 20. Unligated oligonucleotide was digested by Lambda Exonuclease. [1085] 21. Reaction mixture was purified by QIAGEN Nucleotide Removal kit using the protocol outlined in purification steps 1-7. [1086] 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.

2.SUP.nd .Cycle:

[1087] 23. 3′-O-modified-dCTP (2 μl of 100 μM) and MnCl.sub.2 (1 μl of 40 mM) were added. [1088] 24. 1.5 μl of Therminator IX DNA polymerase (15 U, New England BioLabs) was then added. [1089] 25. The reaction was incubated for 10 minutes at 37° C. [1090] 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. [1091] 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. [1092] 28. The reaction mixture was purified using the protocol outlined in purification steps 1-7. [1093] 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. [1094] 30. 1 μl of Human Endonuclease V (Endo V) NEB (30 units/μl) was added into the same tube. [1095] 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. [1096] 32. After incubation time had elapsed, the reaction was terminated by enzymatic heat inactivation (i.e. 65° C. for 20 mins). [1097] 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). [1098] 34. The reaction mixture was purified using the protocol outlined in

purification steps 1.7. [1099] 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. [1100] 36. 10 μl of 100 μM strand for ligation (1 nmol) (SEQ ID NO: 58, FIG. **28***d*), were added to the reaction mixture. [1101] 37. 40 μl of Blunt/TA DNA Ligase NEB (180 units/μl) was added into the same tube. [1102] 38. 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 10 mins. [1103] 39. After incubation time had elapsed, the reaction was terminated with the addition of TBE-Urea sample Buffer (Novex).

Gel Electrophoresis and DNA Visualization:

[1104] 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 mins using a heat ThermoMixer (Eppendorf). [1105] 2. 5 μl of the sample were then loaded into the wells of a 15% TBE-Urea gel 1.0 mm×10 well (Invitrogen) which contained preheated 1×TBE buffer Thermo Scientific (89 mM Tris, 89 mM boric acid and 2 mM EDTA). [1106] 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. [1107] 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.

[1108] Measurement of purified DNA concentration was determined using the protocol below: [1109] 1. NanoDrop One (Thermo Scientific) was equilibrated by adding 2 μl of sterile distilled water (ELGA VEOLIA) onto the pedestal. [1110] 2. After equilibration, the water was gently wiped off using a lint-free lens cleaning tissue (Whatman). [1111] 3. NanoDrop One was blanked by adding 2 μl of Buffer EB QIAGEN (10 mM Tris.CL, pH 8.5). Then step 2 was repeated after blanking. [1112] 4. DNA concentration was measured by adding 2 μl of the sample unto the pedestal and selecting the measure icon on the touch screen.

[1113] The sample mixture was purified by QIAGEN Nucleotide Removal kit using the protocol outlined below: [1114] 1. 500 μl of buffer PNI QIAGEN (5M guanidinium chloride) was added to the sample and mixed by gentle resuspension with a pipette. [1115] 2. The mixture was transferred into a QIAquick spin column (QIAGEN) and centrifuged for 1 min at 6000 rpm. [1116] 3. After centrifugation, flow-through was discarded and 750 μl of buffer PE QIAGEN (10 mM Tris-HCl pH 7.5 and 80% ethanol) was added into the spin column and centrifuged for 1 min at 6000 rpm. [1117] 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. [1118] 5. The spin column was then placed in a sterile 1.5 ml Eppendorf tube. [1119] 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 min at room temperature. [1120] 7. The tube was then centrifuged at 13000 rpm for 1 min.

[1121] After the ligation step, the sample mixture was purified using Streptavidin Magnetic Beads via the protocol outlined below: [1122] 1. 100 μl of Streptavidin Magnetic Beads (New England BioLabs) were washed 3 times by 200 μl of binding buffer (20 mM TRIS, 500 mM NaCl, pH=7.4). [1123] 2. Reaction mixture after ligation step is mixed with 10 volumes of binding buffer (20 mM TRIS, 500 mM NaCl, pH=7.4) and incubated with Streptavidin Magnetic Beads for 15 minutes at 20° C. [1124] 3. Streptavidin Magnetic Beads were washed 3 times by 200 μl of binding buffer (20 mM TRIS, 500 mM NaCl, pH=7.4). [1125] 4. Streptavidin Magnetic Beads were washed 3 times by deionized water. [1126] 5. The oligonucleotides were eluted by 40 μl of deionized water by heating to 95° C. for 3 minutes.

[1127] The results shown in FIG. **28***c* 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

[1128] 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.
[1129] Exemplary schematic overviews of the method are shown in FIGS. **33**, **34** and **35**.
[1130] 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. FIG. **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. FIG. **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. FIG. **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
[1131] 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, Juliesta E. Sylvester, Modified Template-Independent Enzymes for Polydeoxynucleotide Synthesis, Molecular Assemblies US2016/0108382A1. [1132] 2. Oligonucleotides were designed in house and obtained from Integrated DNA Technologies, Sigma-Aldrich (FIG. **36**). The stock solutions are prepared in concentration of 100 μM. [1133] 3. Therminator 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.
[1134] 3′-O-azidomethyl-dTTP was used for incorporation:
##STR00044##
Method
1.SUP.St .Cycle:
[1135] 1. 20 μl of 10× Thermopol® buffer (20 mM Tris-HCl, 10 mM (NH.sub.4).sub.2SO.sub.4, 10 mM KCl, 2 mM MgSO.sub.4, 0.1% Triton® X-100, pH 8.8, New England BioLabs) and MnCl.sub.2 solution (10 μl of 40 mM) were mixed with 139 μl of sterile deionized water (ELGA VEOLIA) in 1.5 ml Eppendorf tube. [1136] 2. 20 μl of 100 μM single hairpin model oligonucleotide (2 nmol, 1 equiv) (SEQ ID NO: 59, FIG. **36**) was added to the reaction mixture. [1137] 3. The aliquot (4 μl) was taken out of the reaction mixture and 0.5 μl of natural dNTP mix (4 mM) 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. [1138] 4. 3′-O-modified-dTTP (10 μl of 2 mM) was added. [1139] 5. 5 μl of Therminator 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. [1140] 6. The reaction was incubated for 30 minutes at 37° C. [1141] 7. The reaction mixture was purified using QIAGEN Nucleotide Removal kit outlined in purification steps 66-72. [1142] 8. The DNA sample was eluted by 200 μl of TE buffer into a clean Eppendorf tube. [1143] 9. The aliquot (4 μl) was taken out of the reaction mixture and 0.5 μl of natural dNTP mix (4 mM) 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. [1144] 10. 400 μL of the 500 mM TCEP was added to the reaction mixture and allowed to react for 10 minutes at 37° C. [1145] 11. The reaction mixture was purified using QIAGEN Nucleotide Removal kit outlined in purification steps 66-72. [1146] 12. The DNA sample was eluted by 150 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. [1147] 13. The aliquot (4 μl) was taken out of the reaction mixture and 0.5 μl of natural dNTP mix (4 mM) 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. [1148] 14. 5 μl of Human Endonuclease V (Endo V) NEB (30 units/μl) was added to the eluate and incubated at 37° C. for 30 minutes. Any suitable alternative endonuclease could be used. [1149] 15. After incubation time had elapsed, the reaction was terminated by enzymatic heat inactivation at 65° C. for 20 mins. [1150] 16. An aliquot (5 μl) was taken out of the reaction mixture and analysed on a polyacrylamide gel. [1151] 17. The reaction mixture was purified by QIAGEN Nucleotide Removal kit using the protocol outlined in purification steps 66-72. [1152] 18. The DNA sample was eluted by 100 μl of T3 DNA ligase buffer (2× concentrate) into a clean Eppendorf tube. [1153] 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, FIG. **36**), and 40 μl of water were added to the reaction mixture. [1154] 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. [1155] The reaction mixture was purified using the protocol for Streptavidin Magnetic Beads kit including the denaturation step outlined in purification steps 73˜78. [1156] 21. The reaction mixture was purified using the protocol for QIAGEN Nucleotide Removal kit outlined in purification steps 66-72. [1157] 22. The DNA sample was eluted by 100 μl of TE buffer into a clean Eppendorf tube.
2.SUP.Nd .Cycle:
[1158] 23. 15 μl of 10× Thermopol® buffer (20 mM Tris-HCl, 10 mM (NH.sub.4).sub.2SO.sub.4, 10 mM KCl, 2 mM MgSO.sub.4, 0.1% Triton® X-100, pH 8.8, New England BioLabs), MnCl.sub.2 solution (7.5 μl of 40 mM) and 19 μl of deionized water was added. [1159] 24. An aliquot (4 μl) was taken out of the reaction mixture and 0.5 μl of natural dNTP mix (4 mM) 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. [1160] 25. 3′-O-modified-dTTP (7.5 μl of 2 mM) was added. [1161] 26. 5 μl of Therminator X DNA polymerase (50 U, New England BioLabs) was then added. Any DNA polymerase that could incorporate modified dNTPs could be used. [1162] 27. The reaction was incubated for 30 minutes at 37° C. [1163] 28. The reaction mixture was purified using QIAGEN Nucleotide Removal kit outlined in purification steps 66-72 [1164] 29. The DNA sample was eluted by 100 μl of TE buffer into a clean Eppendorf tube [1165] 30. An aliquot (4 μl) was taken out of the reaction mixture and 0.5 μl of natural dNTP mix (4 mM) 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. [1166] 31. 200 μL of the 500 mM TCEP was added to the reaction mixture and allowed to react for 10 minutes at 37° C. [1167] 32. The reaction mixture was purified using QIAGEN Nucleotide Removal kit outlined in purification steps 66-72 [1168] 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 a clean Eppendorf tube. [1169] 34. The aliquot (4 μl) was taken out of the reaction mixture and 0.5 μl of natural dNTP mix (4 mM) 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. [1170] 35. 5 μl of Human Endonuclease V (Endo V) NEB (30 units/μl) was added to the eluate, and incubated at 37° C. for 30 minutes. Any suitable alternative endonuclease could be used. [1171] 36. After incubation time had elapsed, the reaction was terminated by enzymatic heat inactivation at 65° C. for 20 mins. [1172] 37. The aliquot (5 μl) was taken out of the reaction mixture and analysed on a polyacrylamide gel. [1173] 38. The reaction mixture was purified by QIAGEN Nucleotide Removal kit using the protocol outlined in purification steps 66-72. [1174] 39. The DNA sample was eluted by 60 μl of T3 DNA ligase buffer (2× concentrate) into a clean Eppendorf tube. [1175] 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, FIG. **36**), and 10 μl of deionized water were added to the reaction mixture. [1176] 41. 10 μl of T3 DNA Ligase NEB (3000 units/al) was added into the same tube and incubated at room temperature for 30 mins. Any suitable DNA ligase could be used. [1177] 42. The reaction mixture

was purified using the protocol for Streptavidin Magnetic Beads kit including denaturation step outlined in purification steps 73-78. [1178] 43. The reaction mixture was purified using the protocol for QIAGEN Nucleotide Removal kit outlined in purification steps 66-72. [1179] 44. The DNA sample was eluted by 46 μl of TE buffer into a clean Eppendorf tube.
3.sup.rd cycle: [1180] 45. 6 μl of 10× Thermopol® buffer (20 mM Tris-HC), 10 mM (NH.sub.4).sub.2SO.sub.4, 10 mM KCl, 2 mM MgSO.sub.4, 0.1% Triton® X-100, pH 8.8, New England BioLabs), MnCl.sub.2 solution (3 μl of 40 mM) was added. [1181] 46. An aliquot (4 μl) was taken out of the reaction mixture and 0.5 μl of natural dNTP mix (4 mM) 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. [1182] 47. 3′-O-modified-dTTP (6 μl of 200 μM) was added. [1183] 48. 3 μl of Therminator 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. [1184] 49. The reaction was incubated for 30 minutes at 37° C. [1185] 50. The reaction mixture was purified using QIAGEN Nucleotide Removal kit outlined in purification steps 66-72. [1186] 51. The DNA sample was eluted by 50 μl of TE buffer into a clean Eppendorf tube. [1187] 52. The aliquot (4 μl) was taken out of the reaction mixture and 0.5 μl of natural dNTP mix (4 mM) 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. [1188] 53. 100 μL of the 500 mM TCEP was added to the reaction mixture and allowed to react for 10 minutes at 37° C. [1189] 54. The reaction mixture was purified using QIAGEN Nucleotide Removal kit outlined in purification steps 66-72. [1190] 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. [1191] 56. An aliquot (4 μl) was taken out of the reaction mixture and 0.5 μl of natural dNTP mix (4 mM) 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. [1192] 57. 5 μl of Human Endonuclease V (Endo V) NEB (30 units/μl) was added to the eluate and incubated at 37° C. for 30 minutes. Any suitable endonuclease could alternatively be used. [1193] 58. After incubation time had elapsed, the reaction was terminated by enzymatic heat inactivation at 65° C. for 20 mins. [1194] 59. The aliquot (5 μl) was taken out of the reaction mixture and analysed on a polyacrylamide gel. [1195] 60. The reaction mixture was purified by QIAGEN Nucleotide Removal kit using the protocol outlined in purification steps 66-72. [1196] 61. The DNA sample was eluted by 30 μl of T3 DNA ligase buffer (2× concentrate) into a clean Eppendorf tube [1197] 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, FIG. **36**) and 5 μl of water were added to the reaction mixture. [1198] 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. [1199] 64. The reaction mixture was analysed by gel electrophoresis.
[1200] Purification of the reaction mixture by QIAGEN Nucleotide Removal kit after incorporation, deblock and cleavage steps using the protocol outlined below: [1201] 65. 10 volumes of buffer PNI QIAGEN (5M guanidinium chloride) was added to the sample and mixed by gentle resuspension with a pipette. [1202] 66. The mixture was transferred into a QIAquick spin column (QIAGEN) and centrifuged for 1 min at 6000 rpm. [1203] 67. After centrifugation, flow-through was discarded and 750 μl of buffer PE QIAGEN (10 mM Tris-HCl pH 7.5 and 80% ethanol) was added into the spin column and centrifuged for 1 min at 6000 rpm. [1204] 68. The Bow-through was discarded and the spin column was centrifuged for an additional 1 min at 13000 rpm to remove residual PE buffer. [1205] 69. The spin column was then placed in a sterile 1.5 ml Eppendorf tube. [1206] 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. [1207] 71. The tube was then centrifuged at 13000 rpm for 1 min.

[1208] Purification of the reaction after the ligation step using Streptavidin Magnetic Beads involving denaturation step was performed via the protocol outlined below: [1209] 72. 100 μl of Streptavidin Magnetic Beads (New England BioLabs) were washed 3 times by 200 μl of binding buffer (20 mM TRIS, 500 mM NaCl, pH=7.4). [1210] 73. Reaction mixture after ligation step is mixed with 10 volumes of binding buffer (20 mM TRIS, 500 mM NaCl, pH=7.4) and allowed to incubate with Streptavidin Magnetic Beads for 15 minutes at 20° C. [1211] 74. Streptavidin Magnetic Beads were washed 3 times by 200 μl of binding buffer (20 mM TRIS, 500 mM NaCl, pH=7.4). [1212] 75. To remove the helper strand, Streptavidin Magnetic Beads were heated to 80° C. in 200 μl of binding buffer (20 mM TRIS, 500 mM NaCl, pH=7.4), placed to magnet and supernatant was quickly discarded. [1213] 76. Streptavidin Magnetic Beads were washed 3 times with deionized water. [1214] 77. The oligonucleotides were eluted by 50-100 μl of deionized water by heating to 95° C. for 3 minutes.

Results and Conclusion

[1215] FIG. **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

[1216] 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

[1217] 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.

[1218] The polyacrylamide surfaces were subsequently exposed to thiolated polyethylene glycol (1 kDa) fluorescein (FITC-PEG-SH), and carboxylated polyethylene glycol (1 kDa) fluorescein (FITC-PEG-COOH) as a negative control in sodium phosphate buffer (10 mM, pH 8) for 1 hr and subsequently washed sequentially with sodium phosphate buffer (10 mM, 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

[1219] FIG. **38** shows fluorescence signals and FIG. **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.

[1220] 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.

[1221] 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.

Example 11. Surface Immobilisation of Hairpin DNA Oligomers and Subsequent Incorporation of Fluorescently Labelled Deoxynucleoside Triphosphates

[1222] This example describes: [1223] (1) a method of presenting bromoacetyl groups on a thin polyacrylamide surface; [1224] (2) the subsequent immobilisation of hairpin DNA via covalent coupling of thiophosphate functionalised hairpin DNA with or without a linker; and [1225] (3) the incorporation of 2′-deoxynucleotide triphosphate (dNTP) into hairpin DNA.

[1226] The method is compatible with virtually any type of material surface (e.g. metals, polymers etc).

(1): Fabrication of a Bromoacetyl Functionalised Thin Polyacrylamide Surface

Materials and Methods

[1227] 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.

[1228] 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 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

[1229] 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 μM 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 (10 mM Tris, 10 mM 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.

[1230] FIG. **40***a* shows the sequences of hairpin DNA without a linker immobilised on different samples. FIG. **40***b* shows the sequences of hairpin DNA with a linker immobilised on different

samples.

Results

[1231] Results are shown in FIGS. **41** and **42**. FIG. **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.

[1232] FIG. **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 BRAPA and oligomer control surfaces (as described in (2)), due to successful covalent immobilisation of DNA onto bromoacetyl functionalised polyacrylamide surfaces.

Conclusion

[1233] 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.

(3): Incorporation of Triphosphates into Hairpin DNA Oligomer with a Linker

Materials and Methods

[1234] Rubber gaskets with a 9 mm diameter circular opening were placed on the BRAPA 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 MgSO.sub.4, 0.05% tween20 and 2 mM MnCl.sub.2) for 10 mins. The surfaces were subsequently exposed to incorporation buffer containing DNA polymerase (0.5 U/μl Therminator X DNA polymerase) and triphosphates (20 μM Alexa 488 labelled dUTP) and incubated for 1 hr (referred to as ‘polymerase surface’ in this example hereafter). Additional set of surfaces were also exposed to incorporation buffer without Therminator 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 1 M 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 and Alexa 488 channels to monitor both the presence of hairpin DNA (Alexa 647) and incorporation of dUTP (Alexa 488).

Results

[1235] FIG. **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 488 were only observed from the polymerase surfaces after incorporation reaction showing the successful incorporation of dUTPs only with the presence of polymerase.

[1236] FIG. **44** shows measured fluorescence signals in the Alexa 647 (hairpin DNA) and Alexa 488 (dUTP) channels obtained from ‘polymerase surfaces’ and ‘negative surfaces’ before 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 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

[1237] 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.

Example 12. Cleavage and Ligation of Hairpin DNA Oligomers Immobilised to Derivatized Surfaces Via a Linker and Thiophosphate Covalent Linkage

[1238] 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.

(1): Cleavage of Immobilised Hairpin DNA Oligomers with a Linker

Materials and Methods

[1239] 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 FIG. **45***a*. FIG. **45***b* shows the sequences of hairpin DNA immobilised on different samples.

[1240] 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 1× 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/μl 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 1×T3 DNA Ligase buffer (66 mM Tris-HCl, 10 mM MgCl2, 1 mM ATP, 7.5% PEG6000, 1 mM DTT, pH 7.6). 1×13 DNA Ligase buffer containing 1M sodium chloride and 0.05% (v/v) Tween20, washed again with 1×T3 DNA Ligase buffer and scanned on ChemiDoc Imager in the Alexa 647 channel.

Results

[1241] FIG. **46** shows fluorescence signals from hairpin DNA oligomers before and after cleavage reactions.

[1242] FIG. **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).

[1243] 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

[1244] After the cleavage reaction as described in (1), samples A and B (as described in FIG. **45***a*) were exposed to 1×T3 DNA Ligase buffer containing MnCl.sub.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 FIG. **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 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. FIG. **48** shows the sequences for the inosine-containing strand and the complimentary ‘helper’ strand for ligation

reactions.

Results

[1245] FIG. **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 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.

[1246] FIG. **50** shows that a significant increase in the Alexa 647 fluorescence signal was 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 FIG. **47**. The fluorescence signals from the sample B remained the same after the ligation reaction due to the absence of T3 DNA ligase.

Conclusion

[1247] 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

[1248] This example describes the incorporation of 3′-O-azidomethyl-dNTPs to the 3′ end of blunt-ended double-stranded DNA.

[1249] 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 FIGS. **1** to **5**.

Materials and Methods

Materials

[1250] 1. In-house synthesized 3′-O-azidomethyl-dNTPs. [1251] 2. Therminator X DNA polymerase that has been engineered by New England Biolabs to possess enhanced ability to incorporate 3-O-modified dNTPs. [1252] 3. Blunt-ended double-stranded DNA oligonucleotide.

[1253] Four types of reversible terminators were tested:

##STR00045##

Method

[1254] 1. 5 μl of 10× Thermopol® buffer (20 mM Tris-HCl, 10 mM (NH.sub.4).sub.2SO.sub.4, 10 mM KCL, 2 mM MgSO.sub.4, 0.1% Triton® X-100, pH 8.8, New England Biolabs) was mixed with 33.5 μl of sterile deionized water (ELGA VEOLIA) in a 1.5 ml Eppendorf tube. [1255] 2. 2 μl of 20 μM primer (40 pmol, 1 equiv) (SEQ ID: NO: 68, FIG. **51***a*) and 3 μl of 20 μM template (60 pmol, 1.5 equiv) (SEQ ID: NO: 69, FIG. **51***a*) were added to the reaction mixture. [1256] 3. 3′-O-modified-dTTP (2 μl of 100 μM) and MnCl.sub.2 (2.5 μl of 40 mM) were added. [1257] 4. 2 μl of Therminator X DNA polymerase (20 U, New England BioLabs) was then added. [1258] 5. The reaction was incubated for 30 minutes at 37° C. [1259] 6. The reaction was stopped by addition of TBE-Urea sample buffer (Novex). [1260] 7. The reaction was separated on polyacrylamide gel (15%) TBE buffer and visualized by ChemiDoc MP imaging system (BioRad).

Results

[1261] FIG. **51***b* depicts a gel showing results of incorporation of 3′-O-modified-dNTPs by Therminator X DNA polymerase in the presence of Mn.sup.2+ ions at 37° C. The data show that Therminator 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.

Example 14. Exemplary Cleavage of a Scaffold Polynucleotide and its Ligation to a Polynucleotide Ligation Molecule

[1262] This example describes the cleavage of a scaffold polynucleotide at a cleavage site defined by a universal nucleotide and the ligation of a polynucleotide ligation molecule, using DNA ligase, to the cleaved scaffold polynucleotide. This example involves ligation of molecules with blunt ends, consistent with synthesis method of the invention version 1 as depicted in FIG. **1**.
[1263] FIG. **52** provides a scheme depicting a DNA synthesis reaction cycle. The scheme is intended to be consistent with synthesis method of the invention version 1 as depicted in FIG. **1**. Thus the scheme in FIG. **52** shows the provision of a scaffold polynucleotide (hairpin structure in the middle panel on the far right of the scheme) having a blunt end, the left strand corresponds to the support strand and the right strand corresponds to the synthesis strand. The terminal nucleotide of the support strand is paired with the terminal nucleotide of the synthesis strand. The terminal nucleotide of the support strand comprises a phosphate group at the 5′ end. In the next step of the cycle a polynucleotide ligation molecule (far right structure in the lowermost panel of the scheme) is provided. The polynucleotide ligation molecule has a support strand (the left strand) and a helper strand (the right strand). The polynucleotide ligation molecule has a complementary ligation end having a blunt end and which comprises a universal nucleotide which is 2-deoxyuridine (U). The terminal end of the helper strand at the complementary ligation end comprises a non-ligatible nucleotide and is paired with the terminal nucleotide of the support strand. The terminal nucleotide of the support strand at the complementary ligation end comprises a nucleotide of the predefined sequence, depicted as “A”, purely for illustration. Upon ligation of the polynucleotide ligation molecule to the cleaved scaffold polynucleotide, the terminal nucleotide of the support strand at the complementary ligation end of the polynucleotide ligation molecule is ligated to the terminal nucleotide of the support strand of the scaffold polynucleotide, and a single strand break (“nick”) is created between the helper strand of the polynucleotide ligation molecule and the synthesis strand of the scaffold polynucleotide. The terminal nucleotide of the support strand of the polynucleotide ligation molecule comprises a nucleotide of the predefined sequence and occupies position n. The universal nucleotide consequently occupies position n+1. In the next step a further nucleotide, depicted as “T”, purely for illustration, is incorporated in this case into the synthesis strand of the scaffold polynucleotide by the action of a polymerase enzyme or a nucleotide transferase enzyme. The further nucleotide comprises a reversible terminator group or blocking group. The further nucleotide pairs with the partner nucleotide in the support strand, which in this case is the “T” nucleotide which was incorporated during the ligation step, thus forming a nucleotide pair. The scheme then depicts a deprotection or deblocking step wherein the reversible terminator group or blocking group is removed. In the reaction scheme shown in FIG. **52** the helper strand is shown removed, as an optional step, prior to the incorporation step. Following incorporation, the polynucleotide ligation molecule is cleaved by cleaving the support strand between positions n and n+1. Following cleavage the first and second nucleotides of the predefined sequence to be incorporated, “A” and “T” respectively, are retained in the scaffold polynucleotide as paired nucleotides in a blunt end arrangement.
[1264] As detailed below, this Example 14 describes the steps of cleaving the scaffold polynucleotide and ligating a polynucleotide ligation molecule to the cleaved scaffold polynucleotide, as shown in the dashed box in FIG. **52**.
Materials and Methods for the Cleavage Step
Materials
[1265] 1. Oligonucleotides utilized in this example were designed in house and synthesised by Integrated DNA technologies. These are described in FIG. **53**. [1266] 2. The oligonucleotides were diluted to a stock concentration of 100 uM using sterile distilled water (ELGA VEOLIA).
Method:
[1267] 1. 39 µl sterile distilled water (ELGA VEOLIA) were added into a 1.5 ml Eppendorf tube.
[1268] 2. 5 µl of 10× Cut Smart buffer (50 mM Potassium Acetate, 20 mM Tris-acetate, 10 mM Magnesium Acetate, 100 µg/ml bovine, pH 7.9) were then added into the same Eppendorf tube.

[1269] 3. 1 μl TAMRA (any fluorescent tag could be used) hairpin polynucleotide containing 2′-deoxyuridine (SEQ ID: No 70) (20 μmol/l) was added into the same tube. [1270] 4. 5 μl of USER enzyme (mixture of Uracil DNA Glycosylase (UDG) and Endonuclease VIII (NEB) (1 unit/μl) was then added into the same tube. [1271] 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 30 minutes. [1272] 6. After incubation time had elapsed, the reaction was terminated by enzymatic heat inactivation (i.e. 95° C. for 10 mins). [1273] 7. After the incubation time had elapsed, the reaction mixture was purified using a QIAGEN nucleotide removal kit.

Materials and Methods for the Ligation Step

Materials

[1274] 1. Oligonucleotides utilized in this example were designed in house and synthesised by Integrated DNA technologies. These are described in FIG. **53**. [1275] 2. The oligonucleotides were dilated to a stock concentration of 100 uM using sterile distilled water (ELGA VEOLIA).

Method

[1276] Ligation reactions with oligonucleotides were carried out using the procedure below: [1277] 1. 13 μl of TAMRA (any fluorescent tag could be used) cleaved blunt ended polynucleotide from the cleavage reaction described above were added into a 1.5 ml Eppendorf tube. [1278] 2. 30 μl of 2×T3 DNA Ligase Reaction buffer NEB (132 mM Tris-HCl, 20 mM MgCl.sub.2, 2 mM Dithiothreitol, 2 mM ATP, 15% Polyethylene glycol (PEG6000) and pH 7.6 at 25° C.) and 2 μl of 40 mM MnCl.sub.2 were then added into the same Eppendorf tube. [1279] 3. 5 μl of the 2-deoxyuridine (U) strand (200 μmol/l) (SEQ ID: No 71) and 5 μl of helper strand (200 μmol/l) (SEQ ID: No 72) were added into the same tube. [1280] 4. 5 μl of T3 DNA Ligase NEB (3000 units/μl) were added into the same tube. [1281] 5. The reaction mixture was then incubated at room temperature for 30 minutes. [1282] 6. After the incubation time had elapsed, the reaction was terminated with the addition of TBE-Urea sample Buffer (Novex).

Results

[1283] The results are shown in FIG. **54**.

[1284] In the above Examples, all oligonucleotides presented in SEQ ID NOS 1-72 have a hydroxyl group at the 3° terminus. All oligonucleotides presented in SEQ ID NOS 1-72 lack a phosphate group at the 5′ terminus except for SEQ ID NO 7. SEQ ID NO 18, SEQ ID NO 35 and SEQ ID NO 70.

[1285] 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.

[1286] 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 polynucleotide ligation molecule” includes two or more such polynucleotides, reference to “a scaffold polynucleotide” includes two or more such scaffold polynucleotides, and the like.

[1287] 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 repeating cycles of synthesis, wherein in each cycle: (A) a first strand of a double-stranded polynucleotide is extended by incorporation of a first nucleotide of the predefined sequence and a universal nucleotide by the action of a ligase enzyme in a blunt-ended ligation reaction, wherein the universal nucleotide defines a cleavage site; (B) a

second strand of the double-stranded polynucleotide which is hybridized to the first strand is then extended by incorporation of a second nucleotide of the predefined sequence by a nucleotide transferase or polymerase enzyme; and (C) the double-stranded polynucleotide is then cleaved at the cleavage site; and wherein the first and second nucleotides of the predefined sequence of each cycle are retained in the double-stranded polynucleotide following cleavage.

2. The method according to claim 1, wherein (a) in each cycle the first nucleotide is a partner nucleotide for the second nucleotide, and wherein upon incorporation into the double-stranded polynucleotide the first and second nucleotides form a nucleotide pair, (b) the first nucleotide and the universal nucleotide are components of a polynucleotide ligation molecule, and wherein the polynucleotide ligation molecule is ligated to the double-stranded polynucleotide during step (A) by the action of the ligase enzyme in the blunt-ended ligation reaction, and wherein upon ligation of the polynucleotide ligation molecule to the double-stranded polynucleotide the first strand of the double-stranded polynucleotide is extended and the cleavage site is created, and/or (c) a given cycle of synthesis the second nucleotide of that cycle which is added to the second strand of the double-stranded polynucleotide comprises a reversible terminator group which prevents further extension by the enzyme, and wherein the reversible terminator group is removed from the incorporated second nucleotide of that cycle prior to the addition in the next cycle of synthesis of the second nucleotide of the next cycle.

3.-**7**. (canceled)

8. The method according to claim 2, the method comprising performing a first cycle of synthesis 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 wherein the support strand is the first strand of the double-stranded polynucleotide and the synthesis strand is the strand is the second strand of the double-stranded polynucleotide; (2) ligating a double-stranded polynucleotide ligation molecule to the scaffold polynucleotide by the action of the ligase enzyme in a blunt-ended ligation reaction, the polynucleotide ligation molecule 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 first nucleotide of the predefined sequence; and (ii) in the helper strand a non-ligatable terminal nucleotide; wherein upon ligation the first strand of the double-stranded polynucleotide is extended with the first nucleotide and the cleavage site is created by incorporation of the universal nucleotide into the first strand; (3) extending the terminal end of the primer strand portion of the synthesis strand of the double-stranded scaffold polynucleotide by the incorporation of a second nucleotide of the predefined sequence by the action of the nucleotide transferase or polymerase enzyme, the second nucleotide comprising a reversible terminator group which prevents further extension by the enzyme, wherein the second nucleotide is a partner for first nucleotide and wherein upon incorporation the second nucleotide and the first nucleotide form a nucleotide pair; (4) cleaving the ligated scaffold polynucleotide at the cleavage site, 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 the incorporated nucleotide pair; and (5) removing the reversible terminator group from the second nucleotide; the method further comprising performing a further cycle of synthesis comprising: (6) ligating a further double-stranded polynucleotide ligation molecule to the cleaved scaffold polynucleotide by the action of the ligase enzyme in the blunt-ended ligation reaction, the polynucleotide ligation molecule 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 first nucleotide of the further cycle of synthesis; and (ii) in the helper strand a non-ligatable terminal nucleotide; wherein upon ligation the first strand of the double-stranded polynucleotide is extended with the first nucleotide of the further cycle of synthesis and the cleavage site is created by the incorporation of the universal nucleotide into the

first strand; (7) extending the terminal end of the primer strand portion of the synthesis strand of the double-stranded scaffold polynucleotide by incorporation of a second nucleotide of the further cycle of synthesis by the action of the nucleotide transferase or polymerase enzyme, the second nucleotide comprising a reversible terminator group which prevents further extension by the enzyme, wherein the second nucleotide of the further cycle of synthesis is a partner for the first nucleotide of the further cycle of synthesis, and wherein upon incorporation the second and first nucleotides of the further cycle form a further nucleotide pair; (8) cleaving the ligated scaffold polynucleotide at the cleavage site, 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 the incorporated nucleotide pair from step (4) and the incorporated further nucleotide pair; (9) removing the reversible terminator group from the second nucleotide; and (10) repeating steps 6 to 9 multiple times to provide the double-stranded polynucleotide having a predefined nucleotide sequence.

9. The method according to claim 8, wherein in any one one more, or all cycles of synthesis the reversible terminator group is alternatively removed from the second nucleotide before the step of cleaving the ligated scaffold polynucleotide at the cleavage site.

10. The method according to claim 8, wherein: (a) in the ligation step of the first cycle (step 2) and in ligation steps of all further cycles the complementary ligation end of the polynucleotide ligation molecule is structured such that: i. the first nucleotide of the predefined sequence of that cycle is the terminal nucleotide of the support strand, occupies nucleotide position n in the support strand and is paired with the terminal nucleotide of the helper strand; ii. the universal nucleotide is the penultimate nucleotide of the support strand, occupies nucleotide position n+1 in the support strand and is paired with the penultimate nucleotide of the helper strand; and iii. the terminal nucleotide of the helper strand is a non-ligatable nucleotide; wherein position n is the nucleotide position which is opposite the second nucleotide of the predefined sequence of that cycle upon its incorporation, and wherein position n+1 is the next nucleotide position in the support strand relative to position n in the direction distal to the complementary ligation end; and wherein upon ligation the terminal nucleotide of the support strand of the polynucleotide ligation molecule is ligated to the terminal nucleotide of the scaffold polynucleotide proximal to the primer strand portion of the synthesis strand and a single-strand break is created between the terminal nucleotides of the helper strand and the primer strand portion of the synthesis strand; (b) in the extension step of the first cycle (step 3) and in all further cycles the second nucleotide of that cycle is incorporated into the second strand opposite the first nucleotide in the first strand and is paired therewith; (c) in the cleavage step of the first cycle (step 4) and in all further cycles the support strand of the ligated scaffold polynucleotide is cleaved between positions n+1 and n, thereby releasing the polynucleotide ligation molecule from the scaffold polynucleotide and retaining the first nucleotide of that cycle attached to the first strand of the cleaved scaffold polynucleotide and paired with the second nucleotide of that cycle, and whereupon the position occupied by the first nucleotide of that cycle in the support strand of the cleaved scaffold polynucleotide is defined as nucleotide position n−1 in the next cycle of synthesis.

11. The method according to claim 8, wherein: (a) in the ligation step of the first cycle (step 2) and in ligation steps of all further cycles the complementary ligation end of the polynucleotide ligation molecule is structured such that: i. the first nucleotide of the predefined sequence of that cycle is the terminal nucleotide of the support strand, occupies nucleotide position n in the support strand and is paired with the terminal nucleotide of the helper strand; ii. the universal nucleotide occupies nucleotide position n+2 in the support strand and is paired with a partner nucleotide in the helper strand; and iii. the terminal nucleotide of the helper strand is a non-ligatable nucleotide; wherein position n is the nucleotide position which is opposite the second nucleotide of the predefined sequence of that cycle upon its incorporation, and wherein position n+2 is the second nucleotide position in the support strand relative to position n in the direction distal to the complementary

ligation end; and wherein upon ligation the terminal nucleotide of the support strand of the polynucleotide ligation molecule is ligated to the terminal nucleotide of the scaffold polynucleotide proximal to the primer strand portion of the synthesis strand and a single-strand break is created between the terminal nucleotides of the helper strand and the primer strand portion of the synthesis strand; (b) in the extension step of the first cycle (step 3) and in all further cycles the second nucleotide of that cycle is incorporated into the second strand opposite the first nucleotide in the first strand and is paired therewith; (c) in the cleavage step of the first cycle (step 4) and in all further cycles the support strand of the ligated scaffold polynucleotide is cleaved between positions n+1 and n, thereby releasing the polynucleotide ligation molecule from the scaffold polynucleotide and retaining the first nucleotide of that cycle attached to the first strand of the cleaved scaffold polynucleotide and paired with the second nucleotide of that cycle, and whereupon the position occupied by the first nucleotide of that cycle in the support strand of the cleaved scaffold polynucleotide is defined as nucleotide position n−1 in the next cycle of synthesis.

12. The method according to claim 8, wherein: (a) in the ligation step of the first cycle (step 2) and in ligation steps of all further cycles the complementary ligation end of the polynucleotide ligation molecule is structured such that: i. the first nucleotide of the predefined sequence of that cycle is the terminal nucleotide of the support strand, occupies nucleotide position n in the support strand and is paired with the terminal nucleotide of the helper strand; ii. the universal nucleotide occupies nucleotide position n+2+x in the support strand and is paired with a partner nucleotide in the helper strand; and iii. the terminal nucleotide of the helper strand is a non-ligatable nucleotide; wherein position n is the nucleotide position which is opposite the second nucleotide of the predefined sequence of that cycle upon its incorporation, wherein position n+2 is the second nucleotide position in the support strand relative to position n in the direction distal to the complementary ligation end, and wherein x is a number of nucleotide positions relative to position n+2 in the direction distal to the complementary ligation end wherein the number is a whole number from 1 to 10 or more; and wherein upon ligation the terminal nucleotide of the support strand of the polynucleotide ligation molecule is ligated to the terminal nucleotide of the scaffold polynucleotide proximal to the primer strand portion of the synthesis strand and a single-strand break is created between the terminal nucleotides of the helper strand and the primer strand portion of the synthesis strand; (b) in the extension step of the first cycle (step 3) and in all further cycles the second nucleotide of that cycle is incorporated into the second strand opposite the first nucleotide in the first strand and is paired therewith; (c) in the cleavage step of the first cycle (step 4) and in all further cycles the support strand of the ligated scaffold polynucleotide is cleaved between positions n+1 and n, thereby releasing the polynucleotide ligation molecule from the scaffold polynucleotide and retaining the first nucleotide of that cycle attached to the first strand of the cleaved scaffold polynucleotide and paired with the second nucleotide of that cycle, and whereupon the position occupied by the first nucleotide of that cycle in the support strand of the cleaved scaffold polynucleotide is defined as nucleotide position n−1 in the next cycle of synthesis.

13. The method according to claim 8, wherein the method is modified such that: (i) in step (2) the polynucleotide ligation molecule is provided with a complementary ligation end comprising a first nucleotide of the predefined sequence of the first cycle and further comprising one or more further nucleotides of the predefined sequence of the first cycle; (ii) in step (3) the terminal end of the primer strand portion of the synthesis strand of the double-stranded scaffold polynucleotide is extended by the incorporation of a second nucleotide of the predefined sequence of the first cycle by the action of the nucleotide transferase or polymerase enzyme, and wherein the terminal end of the primer strand portion is further extended by the incorporation of one or more further nucleotides of the predefined sequence of the first cycle by the action of the nucleotide transferase or polymerase enzyme, wherein each one of the second and further nucleotides of the first cycle comprises a reversible terminator group which prevents further extension by the enzyme, and wherein following each further extension the reversible terminator group is removed from a

nucleotide before the incorporation of the next nucleotide; (iii) in step (4) following cleavage the first, second and further nucleotides of the predefined sequence of the first cycle are retained in the cleaved scaffold polynucleotide; (iv) in step (6) the polynucleotide ligation molecule is provided with a complementary ligation end comprising a first nucleotide of the predefined sequence of the further cycle and further comprising one or more further nucleotides of the predefined sequence of the further cycle; (v) in step (6) the terminal end of the primer strand portion of the synthesis strand of the double-stranded scaffold polynucleotide is extended by the incorporation of a second nucleotide of the predefined sequence of the further cycle by the action of the nucleotide transferase or polymerase enzyme, and wherein the terminal end of the primer strand portion is further extended by the incorporation of one or more further nucleotides of the predefined sequence of the further cycle by the action of the nucleotide transferase or polymerase enzyme, wherein each one of the second and further nucleotides of the further cycle comprises a reversible terminator group which prevents further extension by the enzyme, and wherein following each further extension the reversible terminator group is removed from a nucleotide before the incorporation of the next nucleotide; (vi) in step (8) following cleavage the first, second and further nucleotides of the predefined sequence of the further cycle are retained in the cleaved scaffold polynucleotide.

14. The method according to claim 13, wherein: (a) the complementary ligation end of the polynucleotide ligation molecule is structured such that in steps (4) and (8) prior to cleavage the universal nucleotide occupies a position in the support strand which is the next nucleotide position in the support strand after the nucleotide positions of the first and further nucleotides in the direction distal to the complementary ligation end, and the support strand is cleaved between the position occupied by the last further nucleotide and the position occupied by the universal nucleotide or (b) the complementary ligation end of the polynucleotide ligation molecule is structured such that in steps (4) and (8) prior to cleavage the universal nucleotide occupies a position in the support strand which is the next+1 nucleotide position in the support strand after the nucleotide positions of the first and further nucleotides in the direction distal to the complementary ligation end, and the support strand is cleaved between the position occupied by the last further nucleotide and the position occupied by the next nucleotide in the support strand.

15. (canceled)

16. The method according to claim 14, wherein the reversible terminator group of the last further nucleotide of a further cycle to be incorporated is alternatively removed from the last nucleotide before the step of cleaving the ligated scaffold polynucleotide at the cleavage site.

17. The method according to claim 1, wherein in any one or more, or all cycles of synthesis a partner nucleotide which pairs with the first nucleotide of the predefined sequence is a nucleotide which is complementary with the first nucleotide.

18. The method according to claim 8, wherein in any one or more, or all cycles of synthesis, prior to step (3) and/or (7): (a) the scaffold polynucleotide is provided comprising a synthesis strand and a support strand hybridized thereto, wherein the synthesis strand is provided without a helper strand; and/or (b) the helper strand portion of the synthesis strand is removed from the scaffold polynucleotide.

19.-**20**. (canceled)

21. The method according to claim 1, wherein each cleavage step comprises: (a) 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; or (b) 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) formamidopirimidine DNA glycosylase (Fpg); or (iv) 8-oxoguanine DNA glycosylase (hOGG1).

22. The method according to claim 21, wherein the first step of (a) is performed with a nucleotide-excising enzyme and/or the second step of (a) is performed with an enzyme having abasic site lyase

activity, wherein the enzyme having abasic site lyase activity is: (i) AP Endonuclease 1; (ii) Endonuclease III (Nth); or (iii) Endonuclease VIII.

23.-**30**. (canceled)

31. The method according to claim 1, wherein the cleavage step comprises cleaving the support strand with an enzyme, wherein: (a) the enzyme cleaves the support strand between nucleotide positions n+1 and n; and/or (b) the enzyme is Endonuclease V.

32.-**52**. (canceled)

53. The method according to claim 8, wherein in any one or more, or all cycles of synthesis: a) in steps (1)/(6) in the scaffold polynucleotide the synthesis strand comprising the primer strand portion and the portion of the support strand hybridized thereto are connected by a hairpin loop; and b) in steps (2)/(6) in the polynucleotide ligation molecule the helper strand and the portion of the support strand hybridized thereto are connected by a hairpin loop at the end opposite the complementary ligation end.

54.-**62**. (canceled)

63. The method according to claim 8, wherein the synthesis strand comprising the primer strand portion and the portion of the support strand hybridized thereto are tethered to a common surface via one or more covalent bonds.

64. The method according to claim 63, 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, a thioamide group, or a bromoacetyl group.

65. The method according to claim 64, wherein the functional group on the common surface is a bromoacetyl group, wherein the bromoacetyl group is provided on a polyacrylamide surface derived using N-(5-bromoacetamidylpentyl) acrylamide (BRAPA).

66. The method according to claim 1, wherein: (a) synthesis cycles are performed in droplets within a microfluidic system; 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.

67.-**87**. (canceled)