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C12Q 1/68 (2018.01)

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(56) Documents Cited:
WO 2012/078312 A **WO 2011/150168 A**
WO 2010/025310 A **WO 2001/088173 A**
US 5681947 A **US 20160046974 A**
Nucleic Acids Research,Vol 28, 200, M Berger et al, "Universal bases for hybridization, replication and chain termination", 2911-2914

(58) Field of Search:
As for published application 2559117 A viz:
INT CL **C07H, C12N, C12P, C12Q**
Other: **ONLINE:EPODOC, WPI, BIOSIS, MEDLINE**
updated as appropriate

Additional Fields
INT CL **B01J**
Other: **None**

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GB 2559117 B

Figure 1

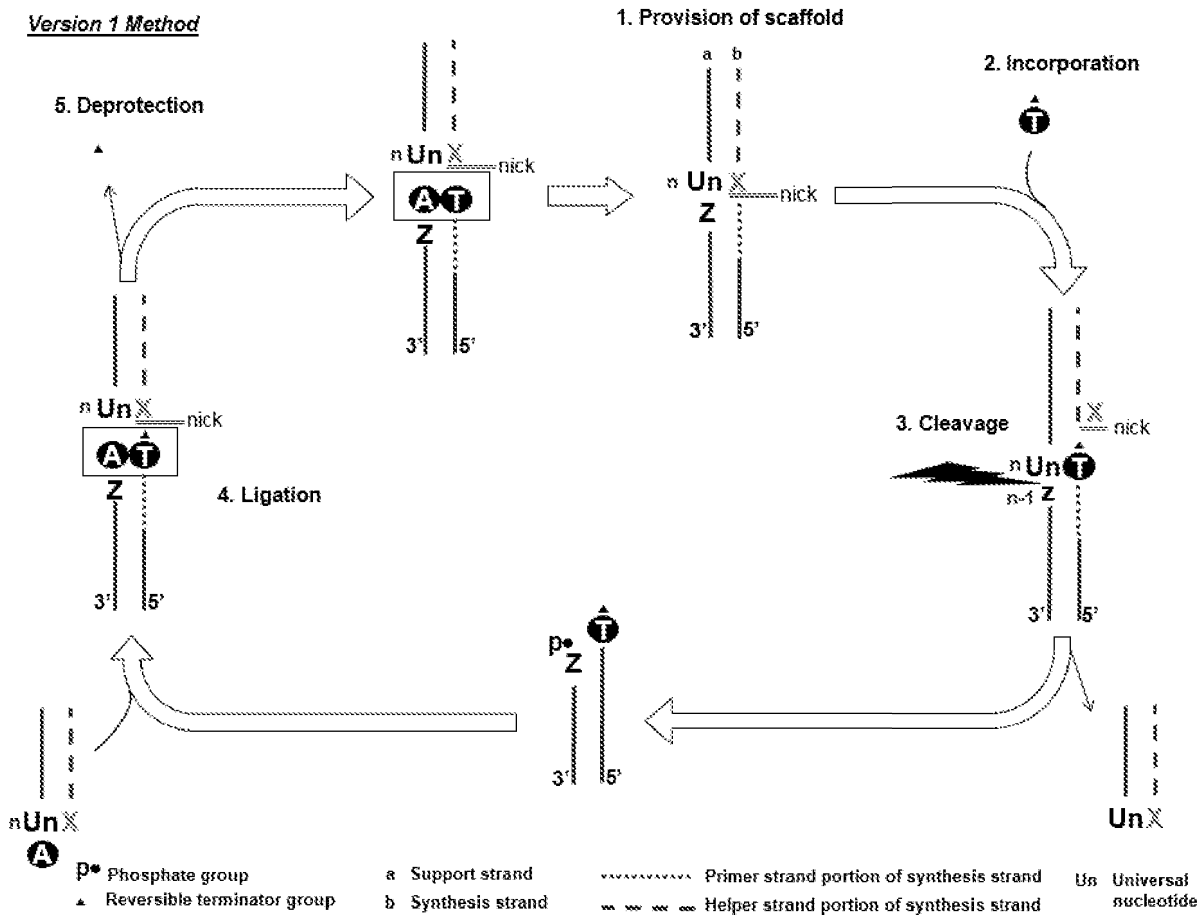

Version 1 Method
1. Provision of scaffold
2. Incorporation
3. Cleavage
4. Ligation
5. Deprotection
nick
3'
5'
Phosphate group
Reversible terminator group
Support strand
Synthesis strand
Primer strand portion of synthesis strand
Helper strand portion of synthesis strand
Universal nucleotide

Figure 2

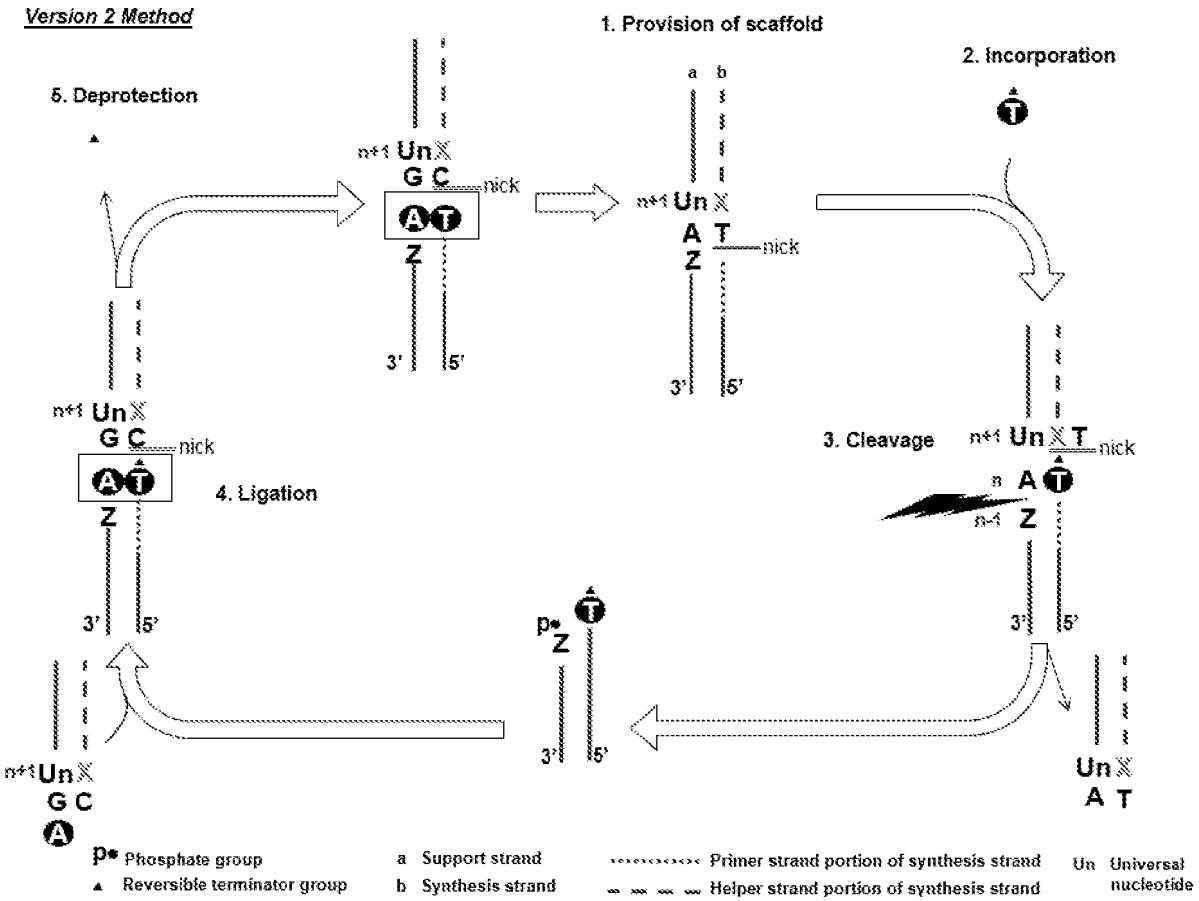

Version 2 Method
1. Provision of scaffold
2. Incorporation
3. Cleavage
4. Ligation
5. Deprotection
nick
3'
5'
Phosphate group
Reversible terminator group
a Support strand
b Synthesis strand
Primer strand portion of synthesis strand
Helper strand portions of synthesis strand
Un Universal nucleotide

Figure 3

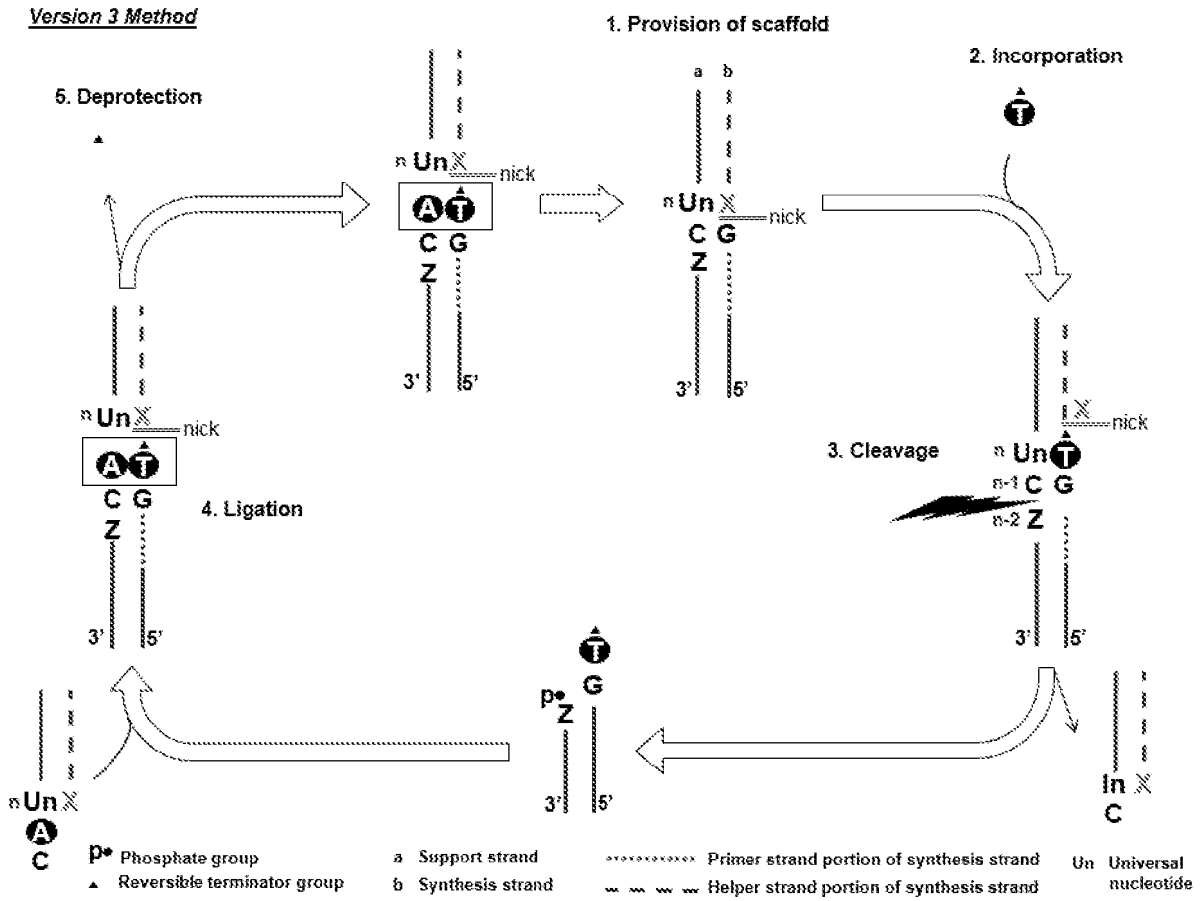
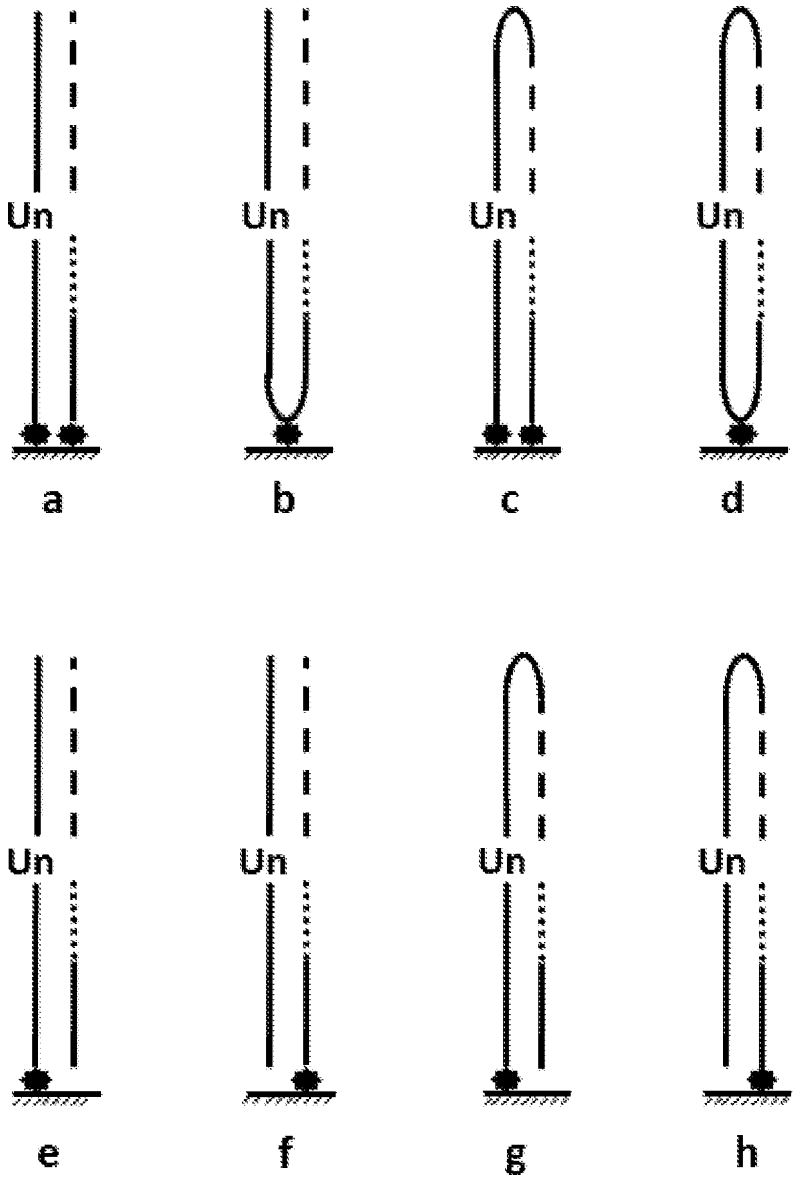
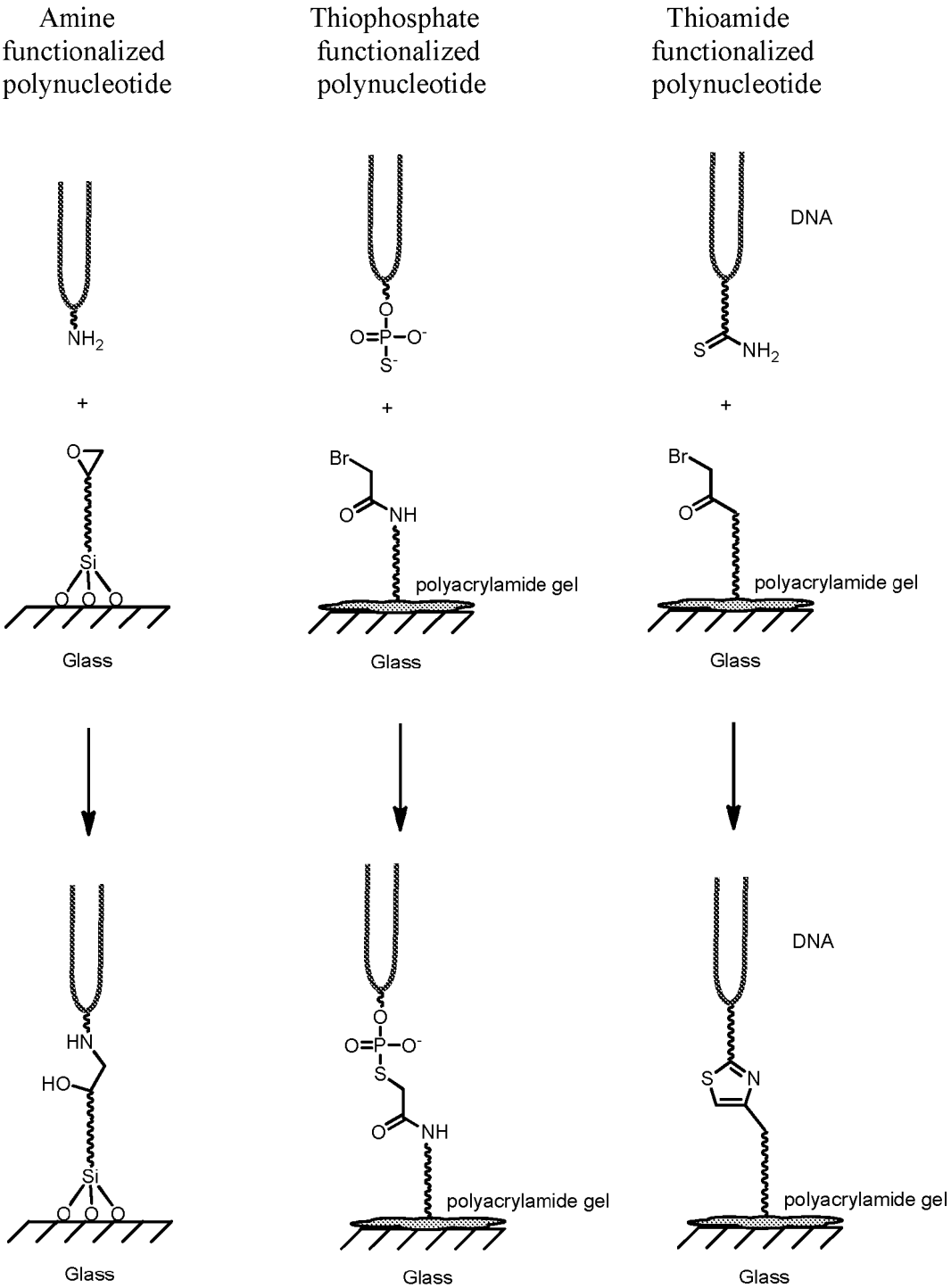

Version 3 Method
1. Provision of scaffold
2. Incorporation
3. Cleavage
4. Ligation
5. Deprotection
nick
3'
5'
Phosphate group
Reversible terminator group
Support strand
Synthesis strand
Primer strand portion of synthesis strand
Helper strand portion of synthesis strand
Universal nucleotide

Figure 4

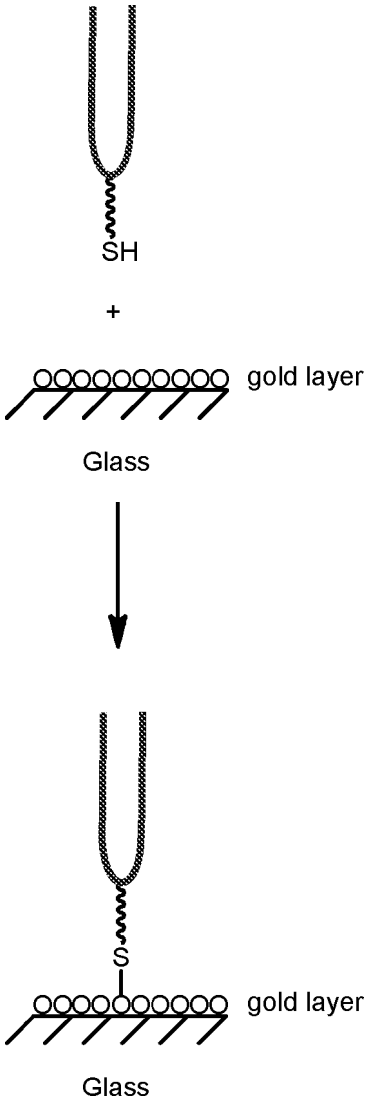


i


Amine functionalized polynucleotide
Thiophosphate functionalized polynucleotide
Thioamide functionalized polynucleotide
DNA
polyacrylamide gel
Glass

j

Example method for attaching polynucleotide to gold surface:



Example method for attaching polynucleotide by biotin-streptavidin binding:

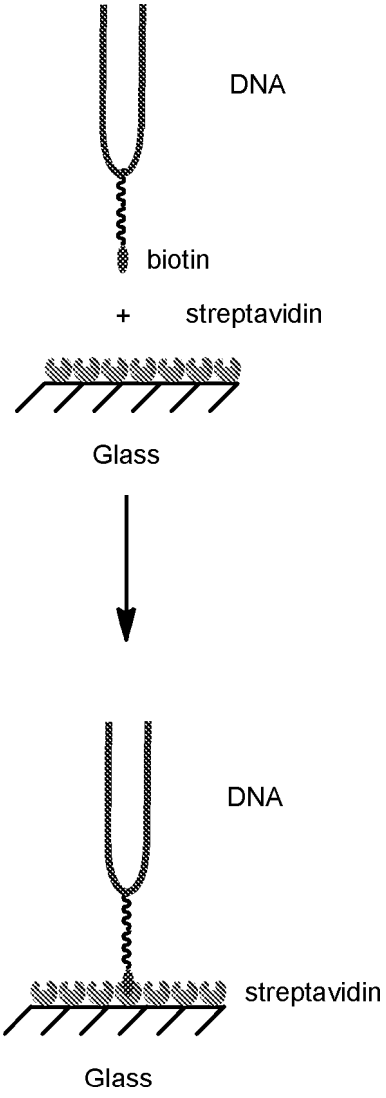
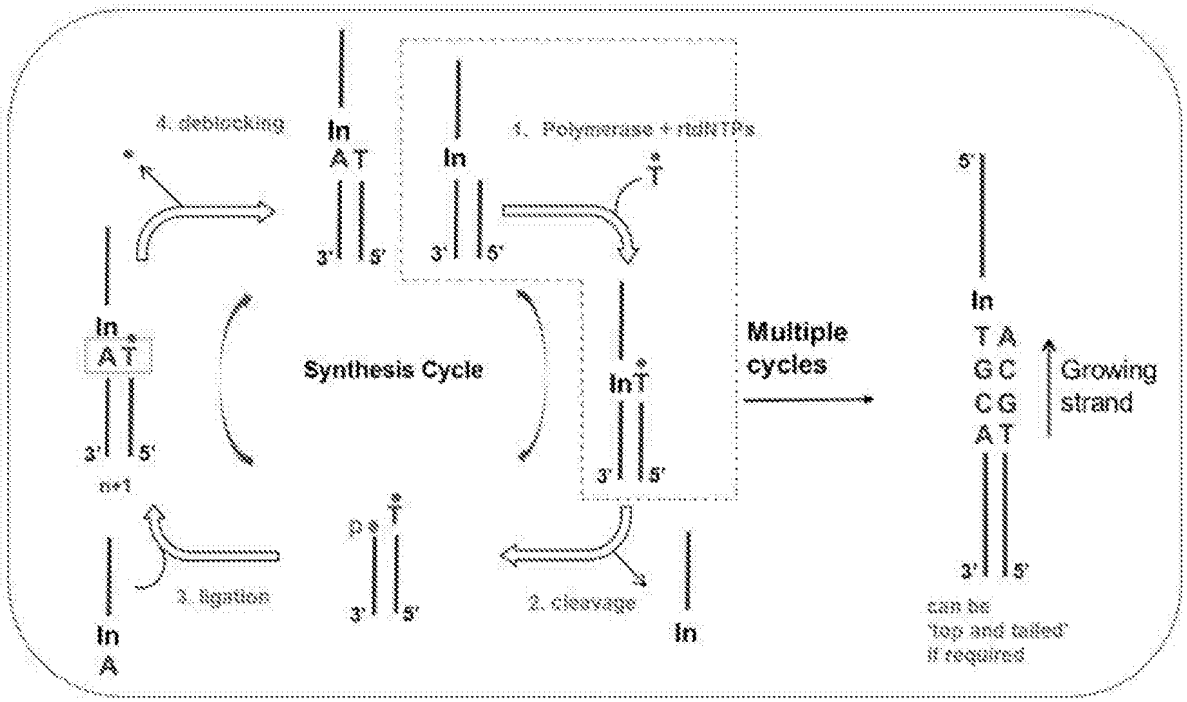


Figure 5

a



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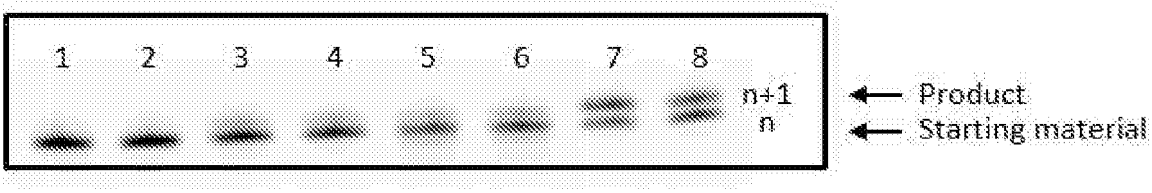
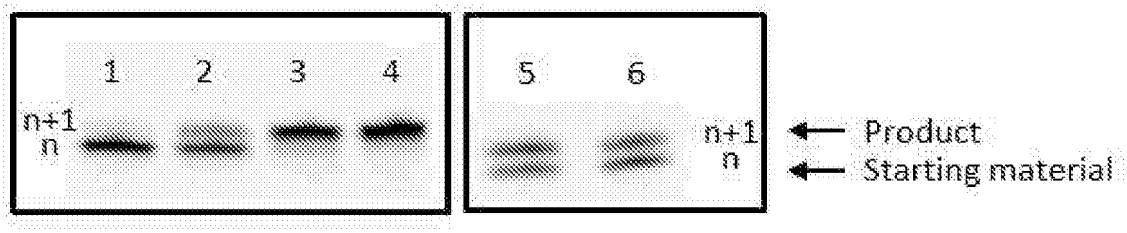


c

Lane no.	Incorporation at 50°C	DNA polymerase	conversion
1	3'-*O*-allyl-dTTPs	Bst	<1%
2	3'-*O*-azidomethyl-dTTPs	Bst	<1%
3	3'-*O*-allyl-dTTPs	Deep Vent (exo-)	5%
4	3'-*O*-azidomethyl-dTTPs	Deep Vent (exo-)	5%
5	3'-*O*-allyl-dTTPs by	Therminator I	8%
6	3'-*O*-azidomethyl-dTTPs	Therminator I	8%
7	**3'-*O*-allyl-dTTPs**	**Therminator IX**	**55%**
8	**3'-*O*-azidomethyl-dTTPs**	**Therminator IX**	**48%**

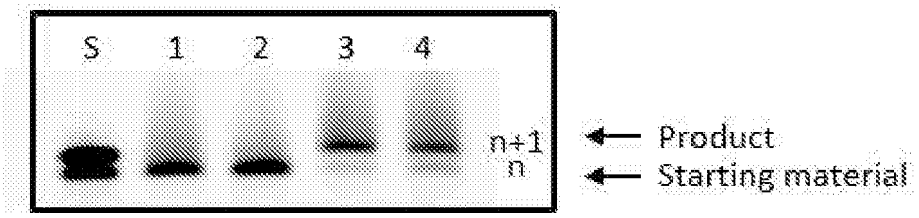
d



e

Lane No.	Incorporation	Enzyme	Temp.	Conversion
1	3'-*O*-allyl-dTTPs	Therminator IX	37 °C	<5%
2	3'-*O*-azidomethyl-dTTPs	Therminator IX	37 °C	15%
3	3'-*O*-allyl-dTTPs	Therminator IX	50 °C	55%
4	3'-*O*-azidomethyl-dTTPs	Therminator IX	50 °C	48%
5	**3'-*O*-allyl-dTTPs**	**Therminator IX**	**65** °C	**>90%**
6	**3'-*O*-azidomethyl-dTTPs**	**Therminator IX**	**65** °C	**>90%**

f



g

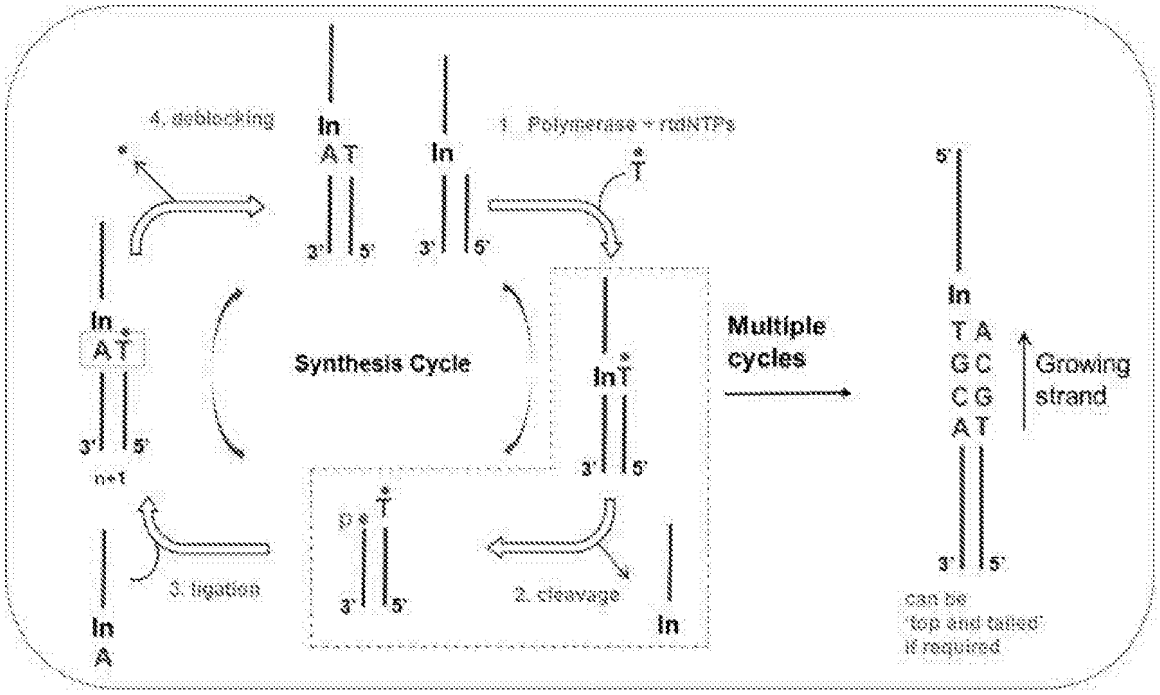
Lane No.	incorporation	Enzyme	Temp	2mM Mn^{2+}	conversion
1	3'-*O*-allyl-dTTPs	Therminator IX	65 °C	No	<1%
2	3'-*O*-azidomethyl-dTTPs	Therminator IX	65 °C	No	<1%
3	**3'-*O*-allyl-dTTPs**	**Therminator IX**	**65** °C	**Yes**	**>90%**
4	**3'-*O*-azidomethyl-dTTPs**	**Therminator IX**	**65** °C	**Yes**	**>90%**

h

Sequence ID Number	Oligonucleotide name	Sequence	Modification
1	Primer	5’-GCGACAGGTGACTGCAGC-3’	TAMRA at 5’-end
2	Template	5’-CACATCACGTCGTAGTC**X**GCTGCAG TCACCTGTCGC-3’	X = 2’-deoxyinosine

Figure 6

a



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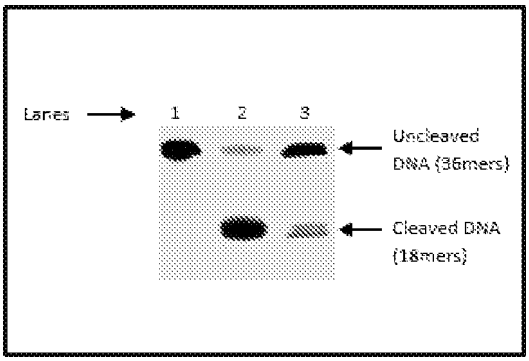
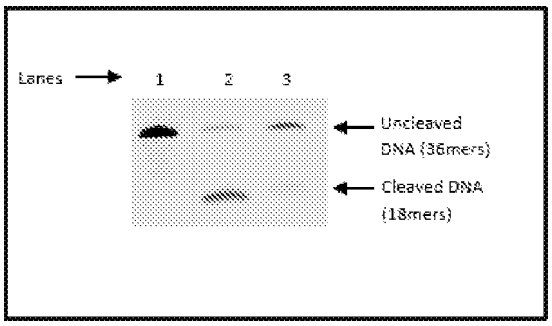


Sample Lanes	Content
1	Reference control Template (15pmols) only.
2	Positive control Template + Control (15pmols)
3	Test sample: Template + Primer with T **No helper strand included.**

c



Sample Lanes	Content
1	Reference control Template (15pmols) only.
2	Positive control Template + Control (15pmols)
3	Test sample: Template + Primer with T **No helper strand included.**

d

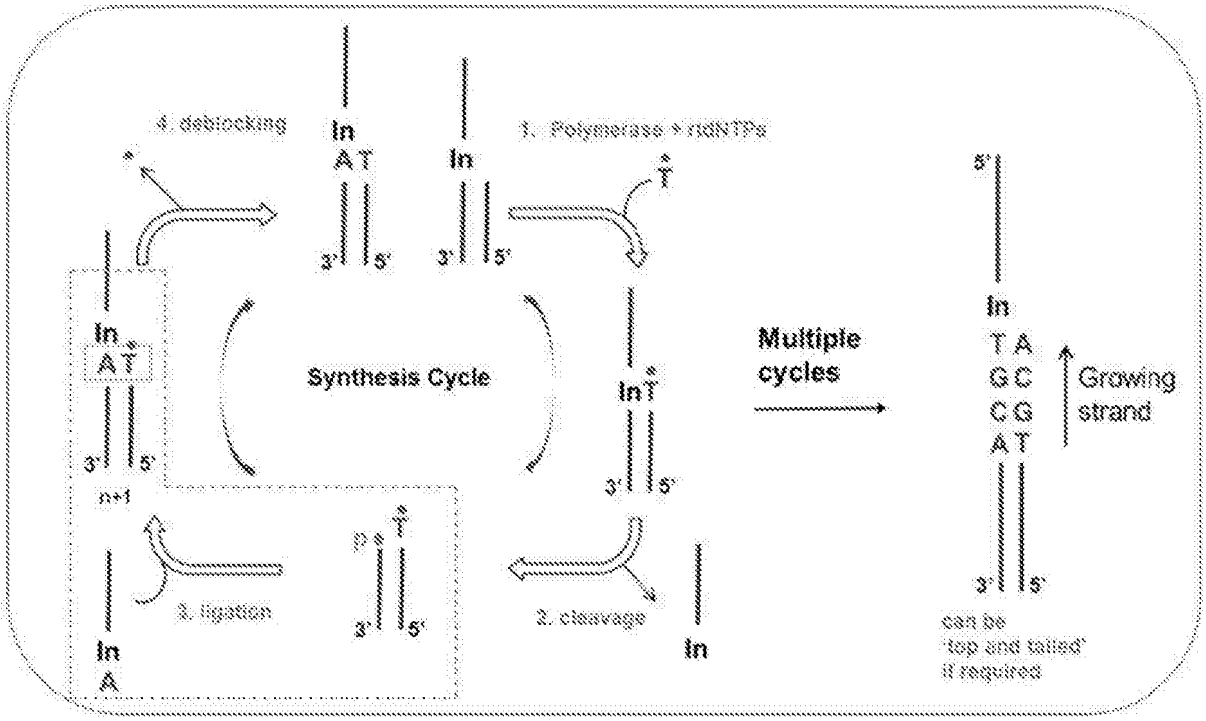
Gel	Cleavage reaction combination	% Conversion of cleaved DNA without a helper strand
A	hAAG/ Chemical base	< 10
B	hAAG/Endo VIII	< 7

e

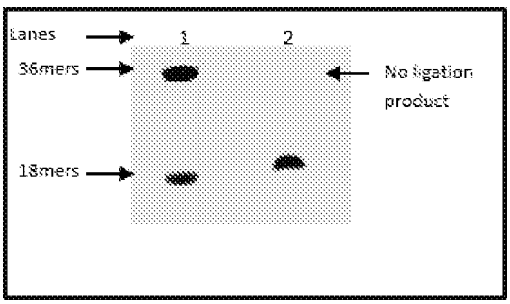
SEQ ID NO.	Oligonucleotide name	Sequence	Modification
3	Template	5'-CACATCACGTCGTAGTC**X**GCT GCAGTCACCTGTCGC-3'	TAMRA at 5'-end **X** = deoxyinosine
4	Primer with T	5'-GCGACAGGTGACTGCAGCT-3'	None
5	Control	5'-GCGACAGGTGACTGCAGCTGA CTACGACGTGATGTG-3'	None

Figure 7

a



b



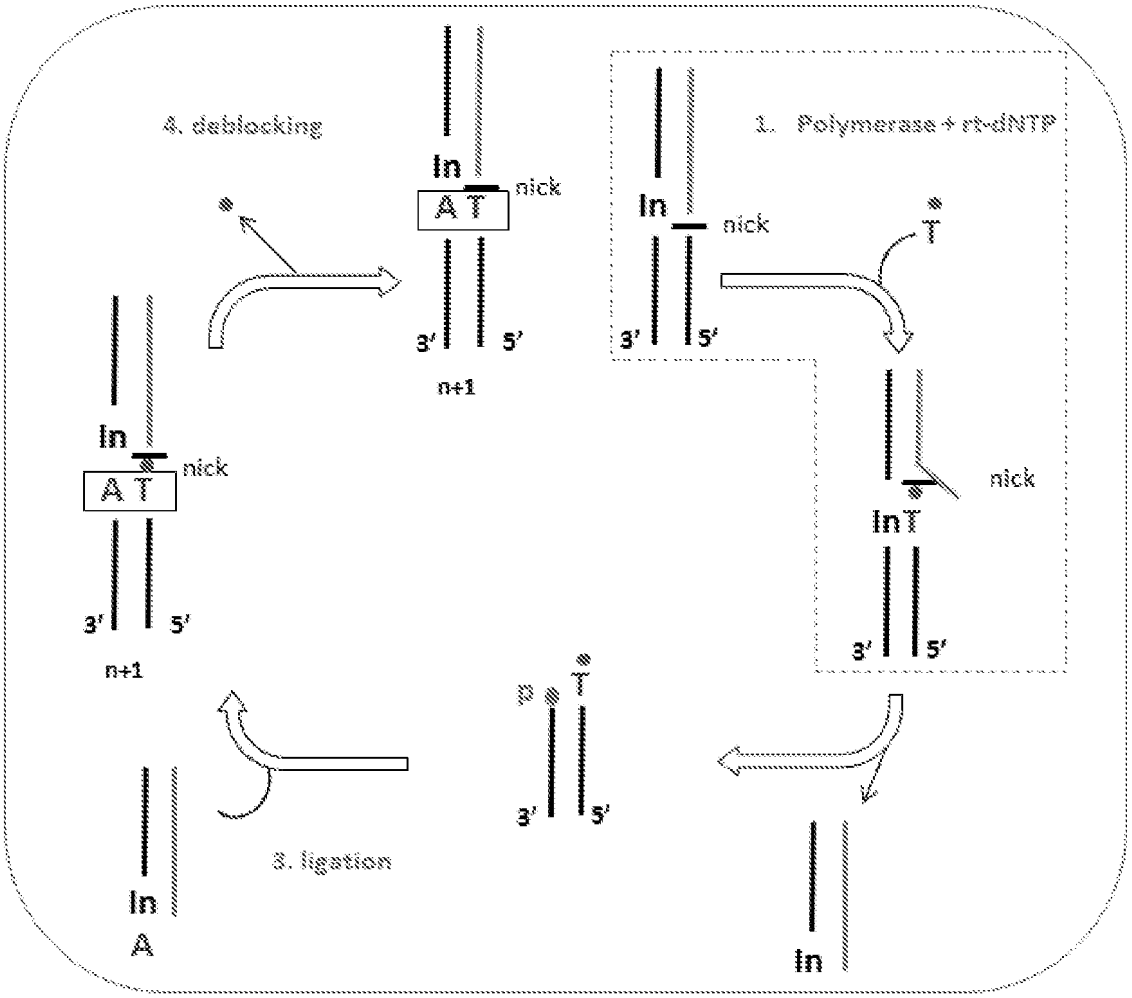
Sample Lanes	Content
1	Reference ladder bands: Ligation standard and phosphate strand (15pmols).
2	Test Sample: Phosphate strand, primer T and Inosine strand (15pmol). No helper strand included.

c

Seq ID No	Oligonucleotide name	Sequence	Modification
6	Ligation standard	5'-CACATCACGTCGTAGT**X**A GCTGCAGTCACCTGTCGC-3'	**X** = 2'-deoxyinosine
7	Phosphate strand	5'-GCTGCAGTCACCTGTCGC-3'	TAMRA at 3'-end Phosphate at 5' end
8	Primer with T	5'-GCGACAGGTGACTGCAGCT-3'	None
9	Inosine strand	5'-CACATCACGTCGTAGT**X**A-3'	**X** = 2'-deoxyinosine

Figure 8

a

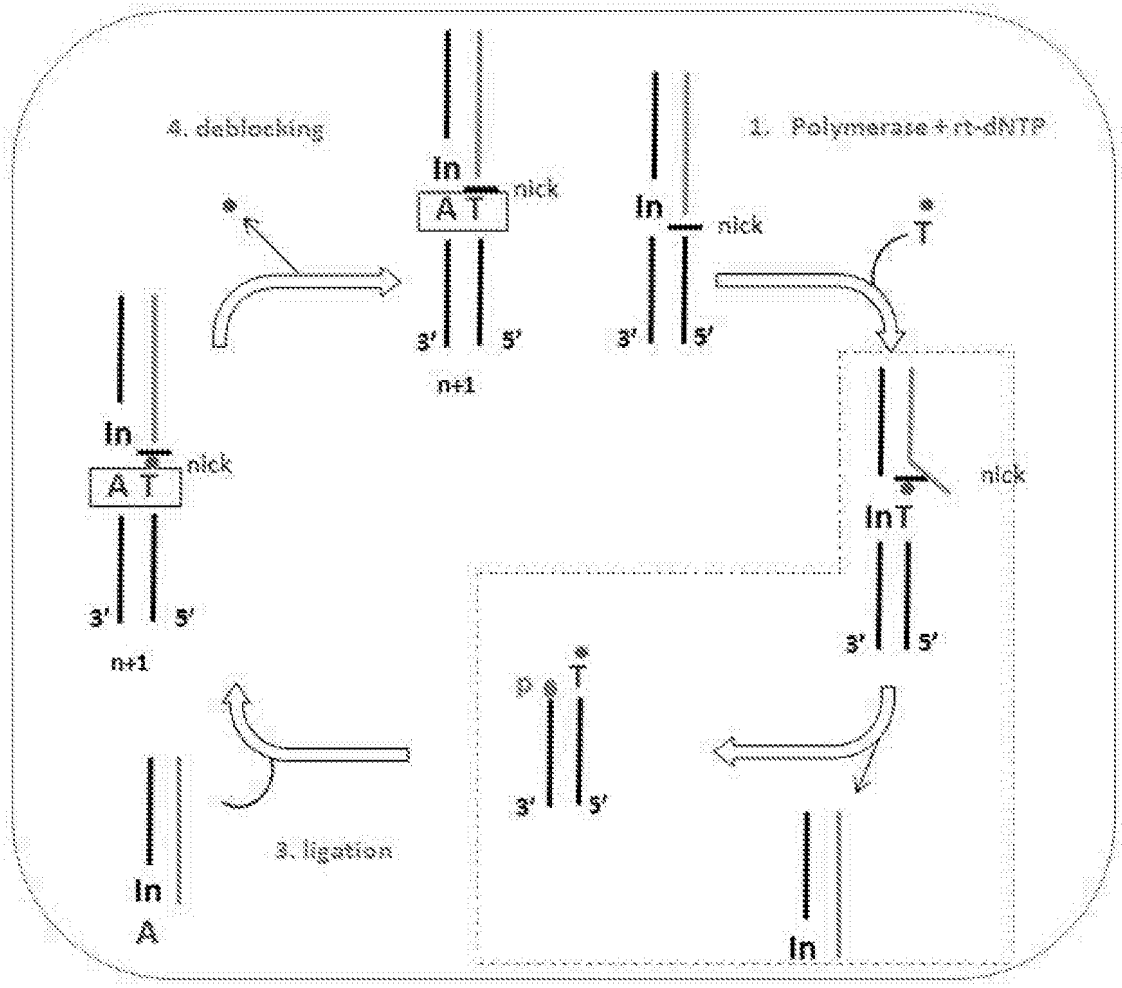


b

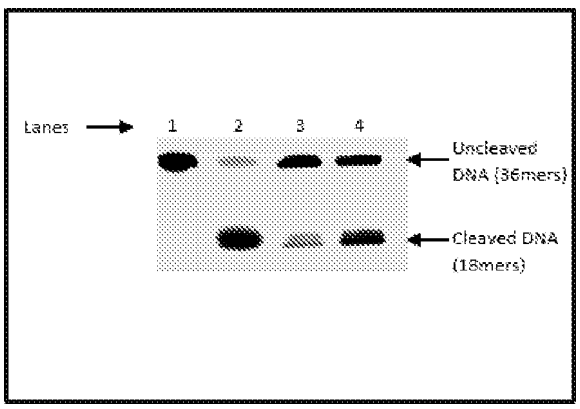
Sequence ID Number	Oligonucleotide name	Sequence	Modification
10	Primer	5′-GCGACAGGTGACTGCAGC-3′	TAMRA at 5′-end
11	Template	5′-CACATCACGTCGTAGTC**X**GCTGCAG TCACCTGTCGC-3′	X = 2′-deoxyinosine
12	Helper strand	5′-CGACTACGACGTGATGTG-3′	None

Figure 9

a

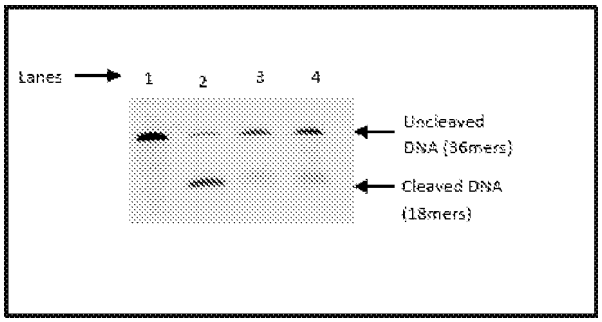


b



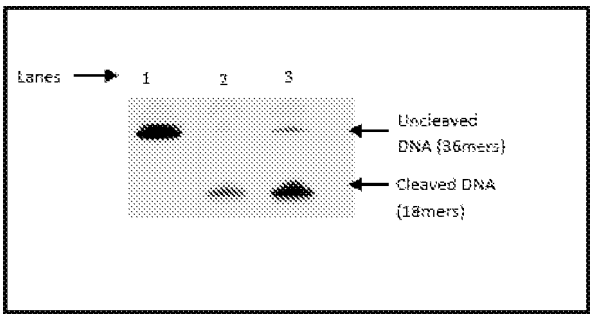
Sample Lanes	Content
1	Reference control Template (15pmols) only.
2	Positive control Template + Control (15pmols)
3	Test sample I: Template + Primer with T No helper strand included (15pmols)
4	Test Sample II : Template + Primer with T + **Helper strand** (15pmols)

c



Sample Lanes	Content
1	Reference control Template (15pmols) only.
2	Positive control Template + Control (15pmols)
3	Test sample I: Template + Primer with T **No helper strand included.** (15pmols)
4	Test Sample II: Template + Primer with T + **Helper strand** (15pmols).

d



Sample Lanes	Content
1	Reference control Template (15pmols) only.
2	Positive control Template + Control (15pmols)
3	Test Sample I: Template + Primer with T + **Helper strand** (15pmols).

e

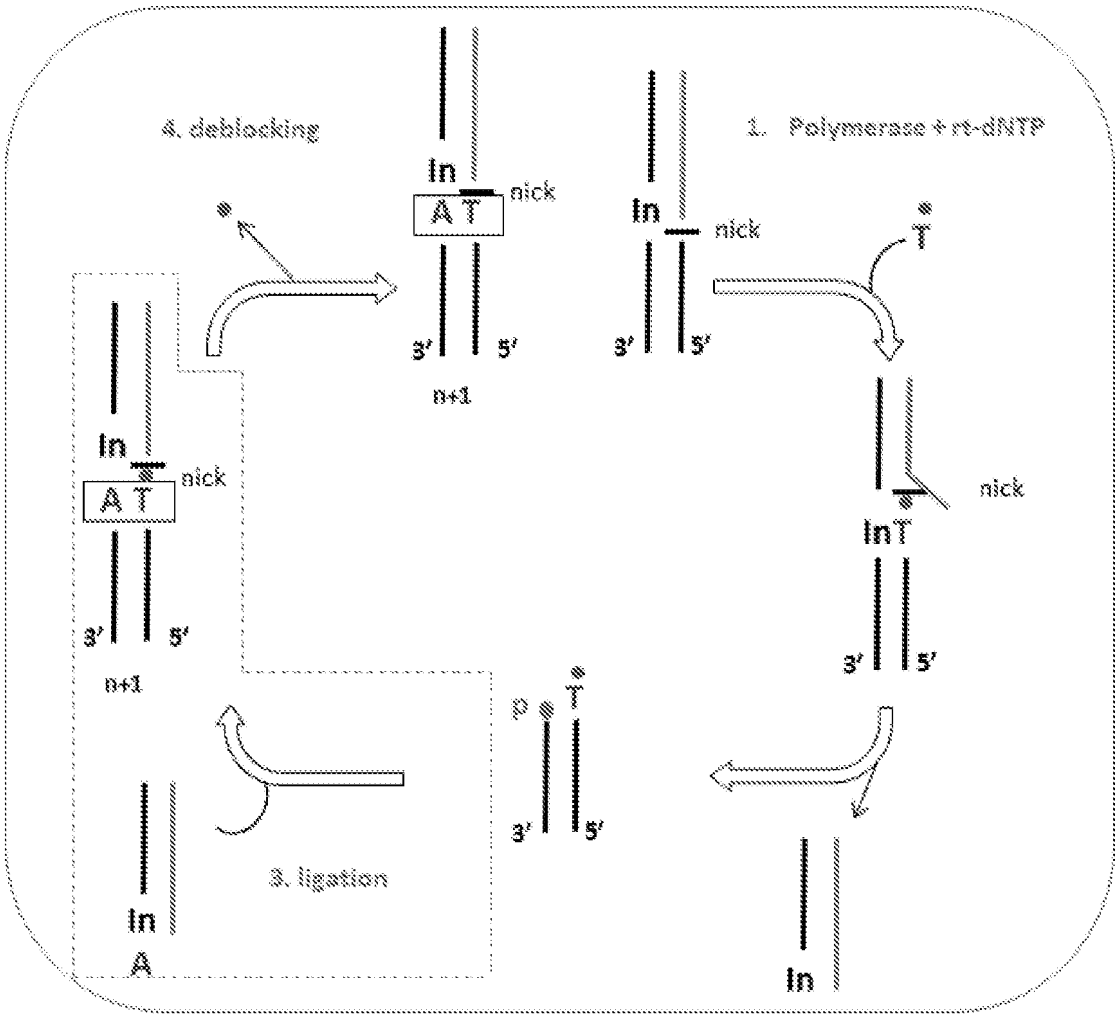
Gel	Cleavage reaction combination	% Conversion of cleaved DNA **with** a **helper strand**
A	hAAG/ Chemical base	50
B	hAAG/Endo VIII	< 10
C	hAAG/Alternative chemical base	90*

f

Sequence ID Number	**Oligonucleotide name**	**Sequence**	**Modification**
13	Template	5'-CACATCACGTCGTAGTC**X**GCT GCAGTCACCTGTCGC-3'	TAMRA at 5'-end **X** = deoxyinosine
14	Primer with T	5'-GCGACAGGTGACTGCAGCT-3'	None
15	Control	5'-GCGACAGGTGACTGCAGCTGA CTACGACGTGATGTG-3'	None
16	Helper Strand	5'-TGACTACGACGTGATGTG-3'	None

Figure 10

a



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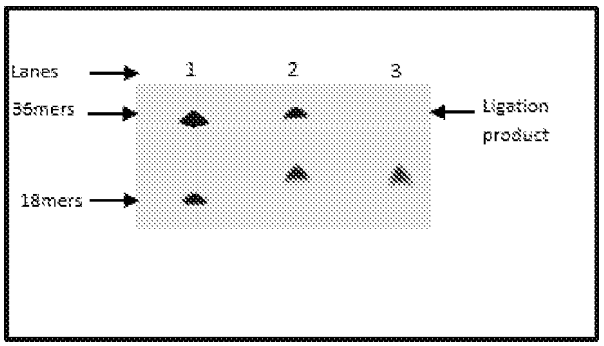
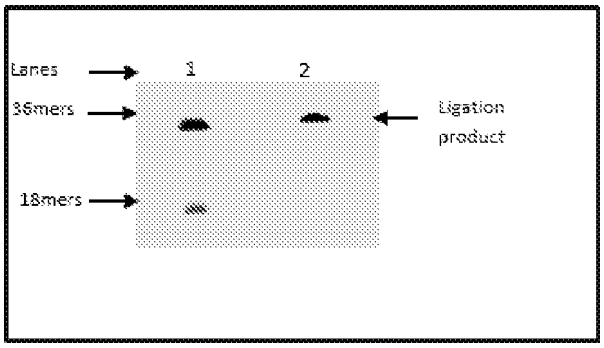


Sample Lanes	Content
1	Reference ladder bands: Ligation standard and phosphate strand (15pmols).
2	Test Sample I: Phosphate strand, primer T and inosine strand + **With helper strand** (15pmol)
3	Test Sample II: Phosphate strand, primer T and inosine strand + without helper strand (15pmol) (Sample after **1hr incubation**

c



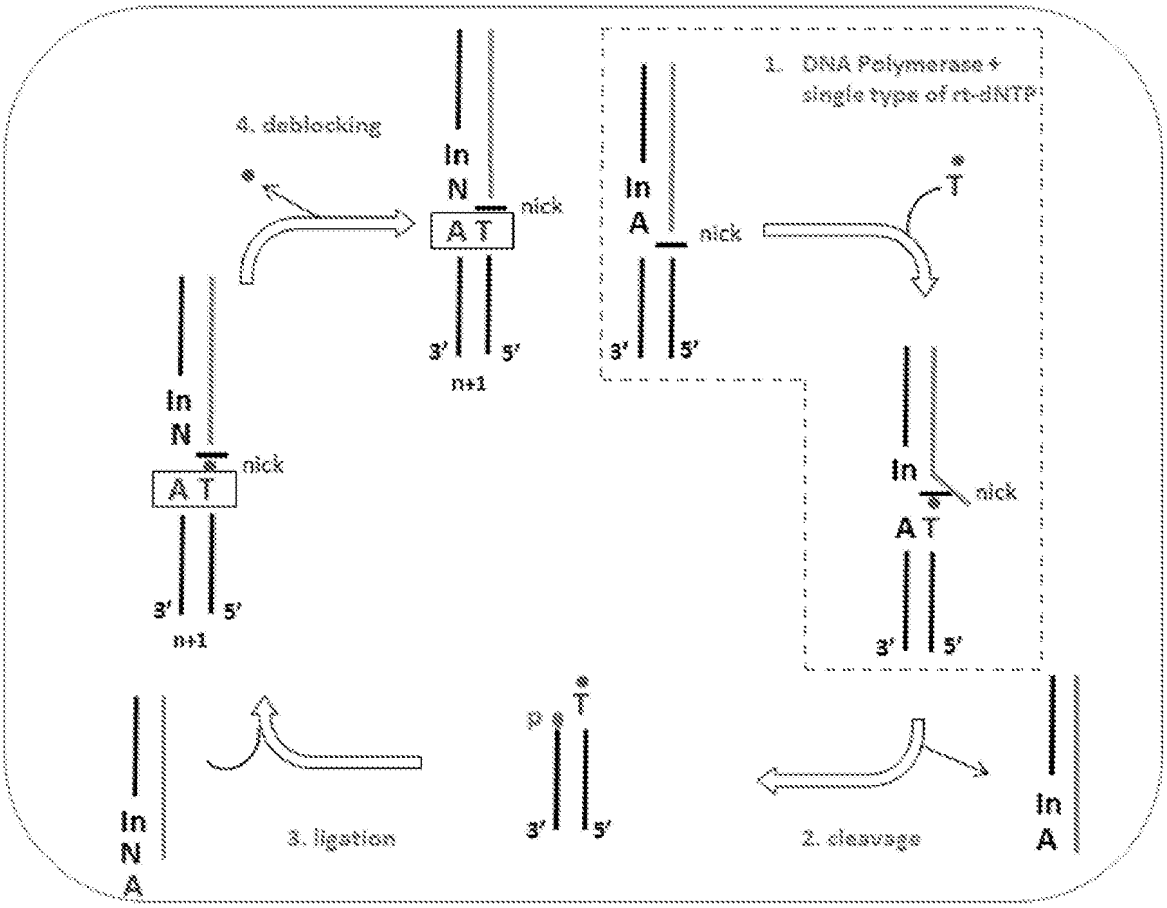
Sample Lanes	Content
1	Reference ladder bands: Ligation standard and phosphate strand (15pmols).
2	Test Sample I: Phosphate strand, primer T and Inosine strand + **With helper strand** (15pmol)

d

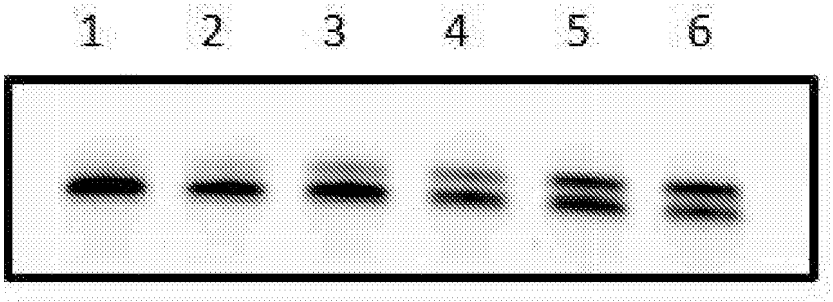
SEQ ID NO:	Oligonucleotide name	Sequence	Modification
17	Ligation standard	5'-CACATCACGTCGTAGT**X**A GCTGCAGTCACCTGTCGC-3'	**X** = 2'-deoxyinosine
18	Phosphate strand	5'-GCTGCAGTCACCTGTCGC-3'	TAMRA at 3'-end Phosphate at 5' end
19	Primer with T	5'-GCGACAGGTGACTGCAGCT-3'	None
20	Inosine strand	5'-CACATCACGTCGTAGT**X**A-3'	**X** = 2'-deoxyinosine
21	Helper strand	5'-CACTACGACGTGATGTG-3'	None

Figure 11

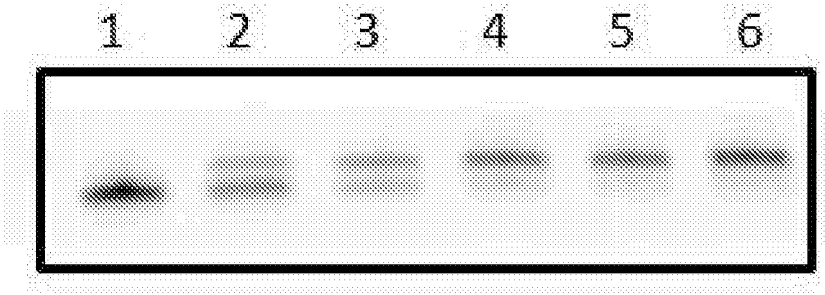
a


4. deblocking
In
N
nick
A T
3'
5'
n+1
In
N
nick
A T
3'
5'
n+1
1. DNA Polymerase + single type of rt-dNTP
In
A
nick
T
3'
5'
In
nick
A T
3'
5'
In
N
A
3. ligation
P
T
3'
5'
2. cleavage
In
A

b


1
2
3
4
5
6

c



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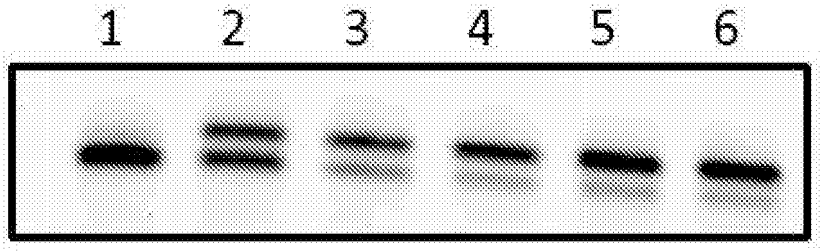

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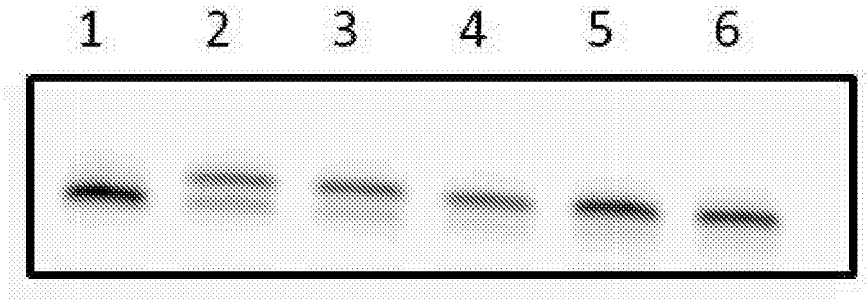

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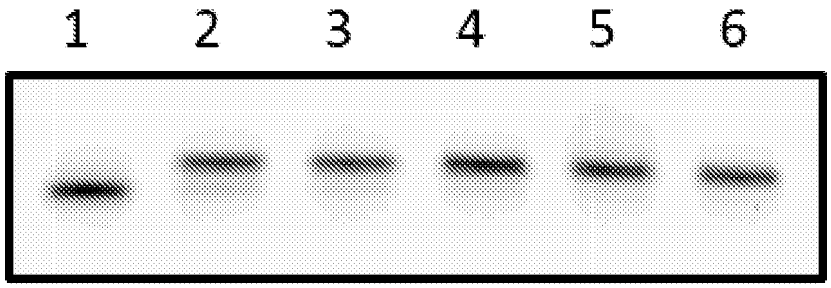

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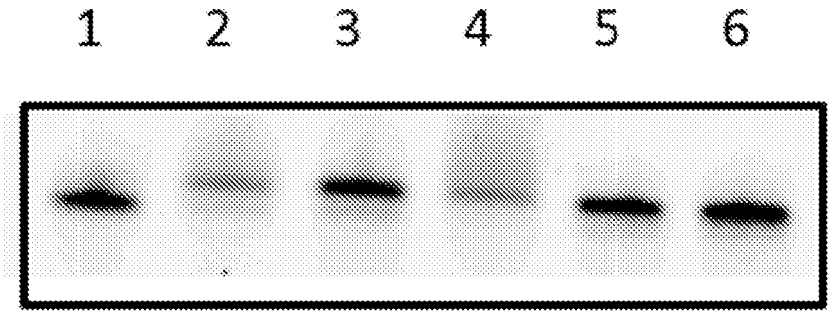
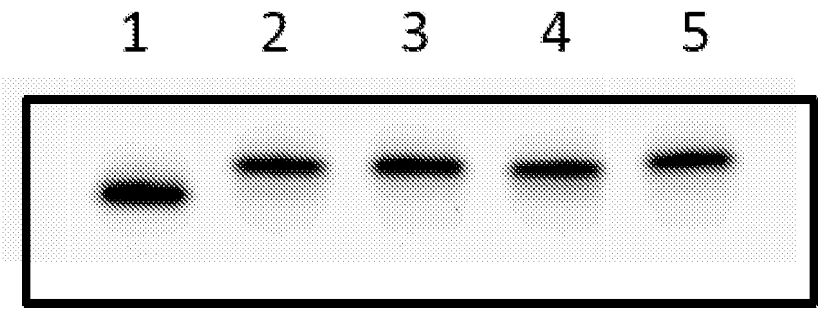


h

	0 min	1 min	2 min	5 min	10 min	20 min
27°C without Mn^{2+}	0	5	10	20	35	40
37°C without Mn^{2+}	0	30	58	90	90	90
47°C without Mn^{2+}	0	30	65	90	90	90
27°C with Mn^{2+}	0	70	85	92	95	95
37°C with Mn^{2+}	0	85	90	>96	>96	>96
47°C with Mn^{2+}	0	85	90	>96	>96	>96

i

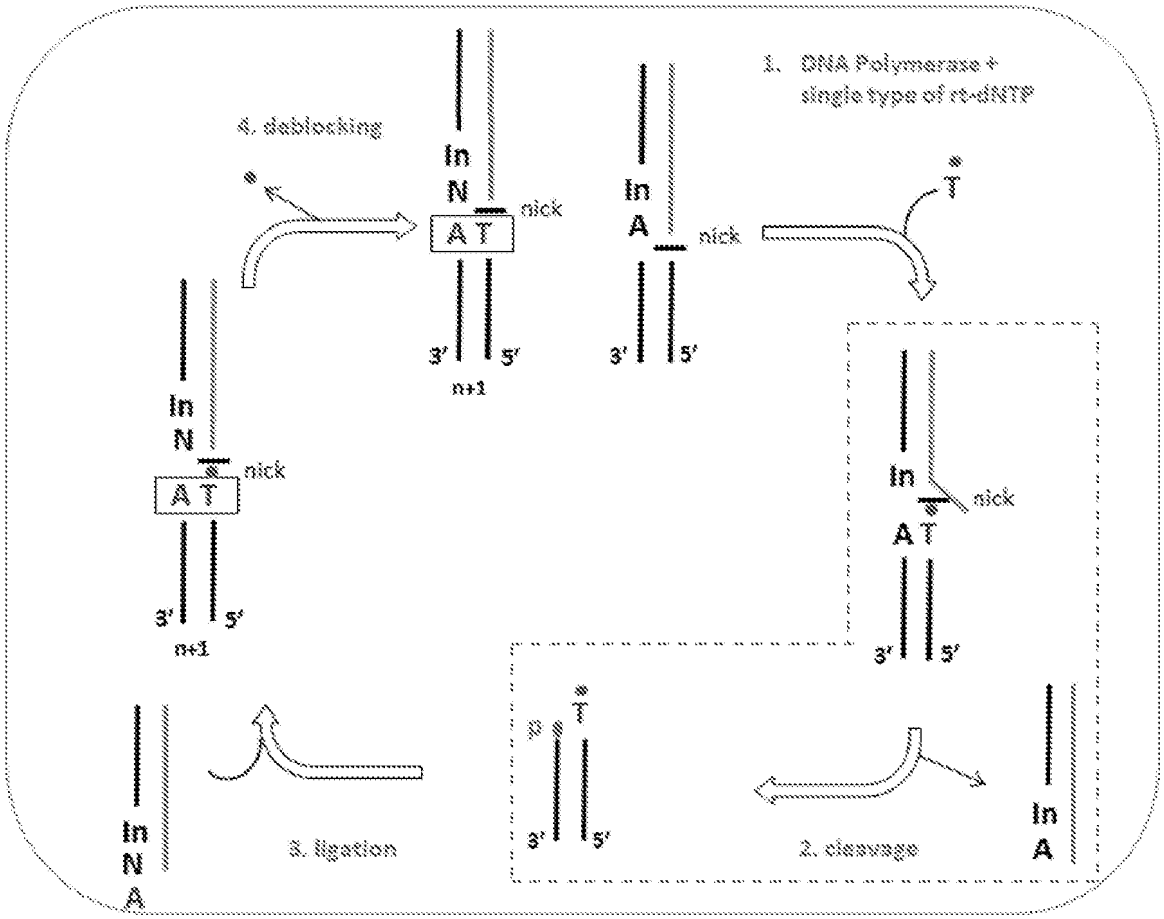


j

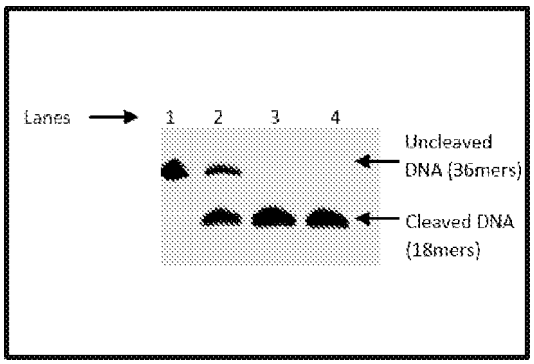
SEQ ID NO:	Oligonucleotide name	Sequence	Modification
22	Primer	5'-GCGACAGGTGACTGCAGC-3'	TAMRA at 5'-end
23	Template-A	5'-CACATCACGTCGTAGTCXAGCTGCAG TCACCTGTCGC-3'	X = 2'-deoxyinosine
24	Template-G	5'-CACATCACGTCGTAGTCXGGCTGCAG TCACCTGTCGC-3'	X = 2'-deoxyinosine
25	Template-T	5'-CACATCACGTCGTAGTCXAGCTGCAG TCACCTGTCGC-3'	X = 2'-deoxyinosine
26	Template-C	5'-CACATCACGTCGTAGTCXAGCTGCAG TCACCTGTCGC-3'	X = 2'-deoxyinosine
27	Helper strand-T	5'-TCGACTACGACGTGATGTG-3'	None
28	Helper strand-C	5'-CCGACTACGACGTGATGTG-3'	None
29	Helper strand-A	5'-ACGACTACGACGTGATGTG-3'	None
30	Helper strand-G	5'-GCGACTACGACGTGATGTG-3'	None

Figure 12

a



b



Sample Lanes	Content
1	Reference control: Template (15pmols) only.
2	Positive control : Template + Control (15pmols)
3	Test sample I: Template + Primer with T (15pmols) **No helper strand included.**
4	Test sample II: Template + Primer with T **Helper strand included** (15pmols).

c

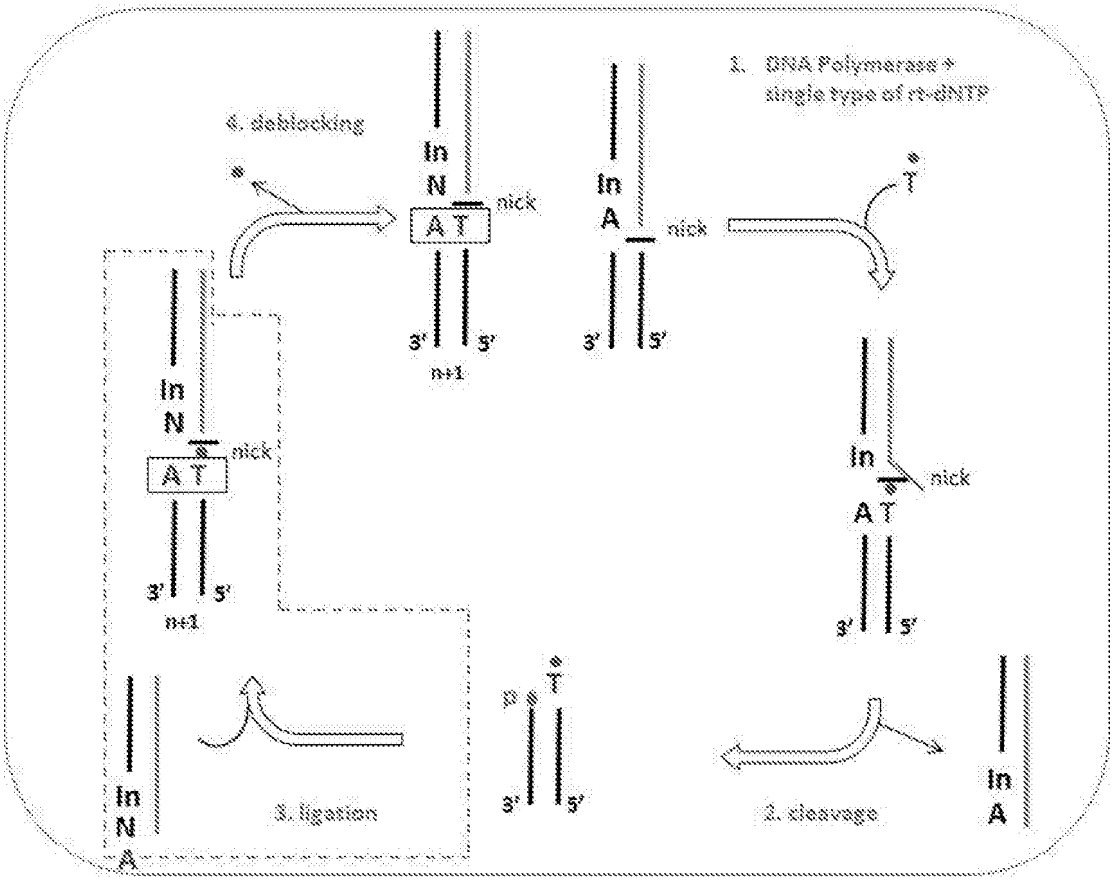
Cleavage reaction combination	% Conversion of cleaved DNA
Full length positive control	80
Presence of helper strand	>99
Absence of helper strand	>99

d

Sequence ID Number	Oligonucleotide name	Sequence	Modification
31	Template	5'-CACATCACGTCGTAGTCXAGCT GCAGTCACCTGTCGC-3'	TAMRA at 5'-end **X** = deoxyinosine
32	Primer with T	5'-GCGACAGGTGACTGCAGCT-3'	None
33	Control	5'-GCGACAGGTGACTGCAGCTGA CTACGACGTGATGTG-3'	None
34	Helper strand	5'-TCGACTACGACGTGATGTG-3'	None

Figure 13

a

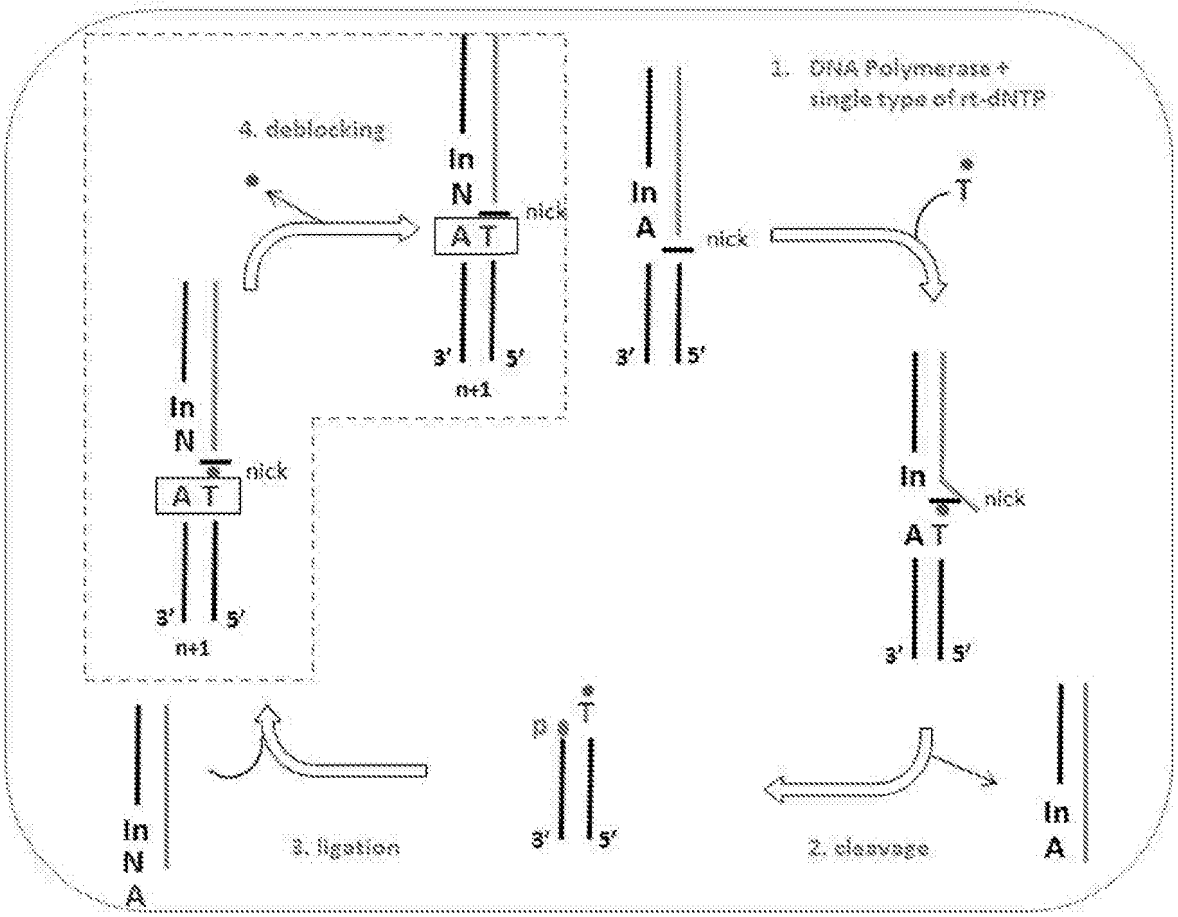


b

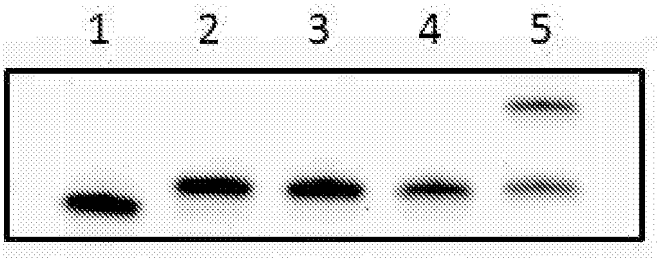
Sequence ID Number	Oligonucleotide name	Sequence	Modification
35	Phosphate strand	5'-GCTGCAGTCACCTGTCGC-3'	TAMRA at 3'-end Phosphate at 5' end
36	Primer with T	5'-GCGACAGGTGACTGCAGCT-3'	None
37	Inosine strand	5'-CACATCACGTCGTAGTXGA-3'	**X** = 2'-deoxyinosine
38	Helper strand	5'-CCACTACGACGTGATGTG-3'	None

Figure 14

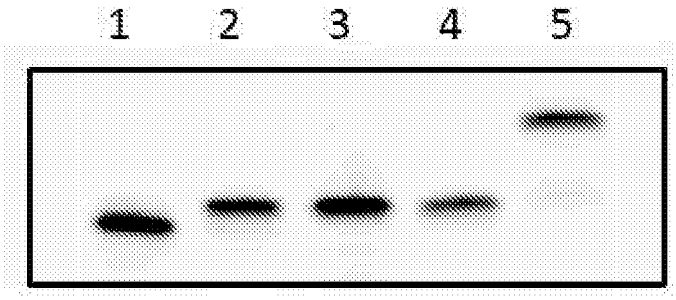
a


1. DNA Polymerase + single type of rt-dNTP
2. cleavage
3. ligation
4. deblocking
In N
In A
nick
A T
3'
5'
n+1

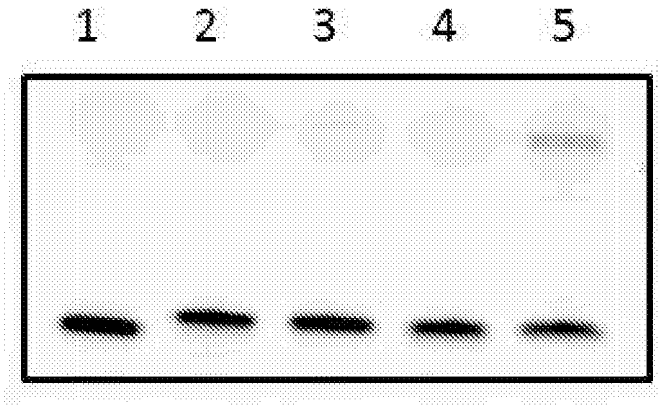
b


1
2
3
4
5

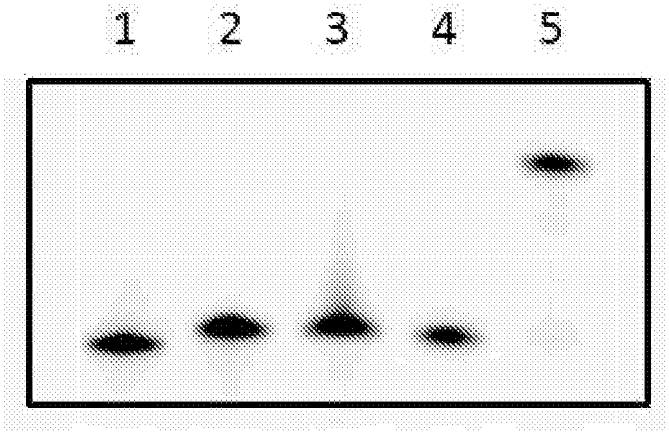
c



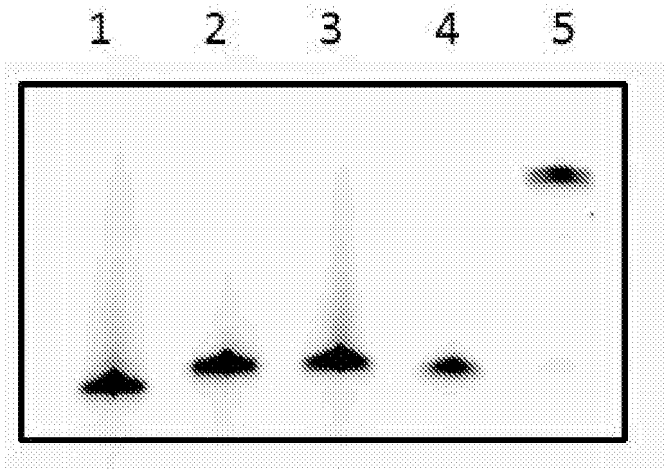
d



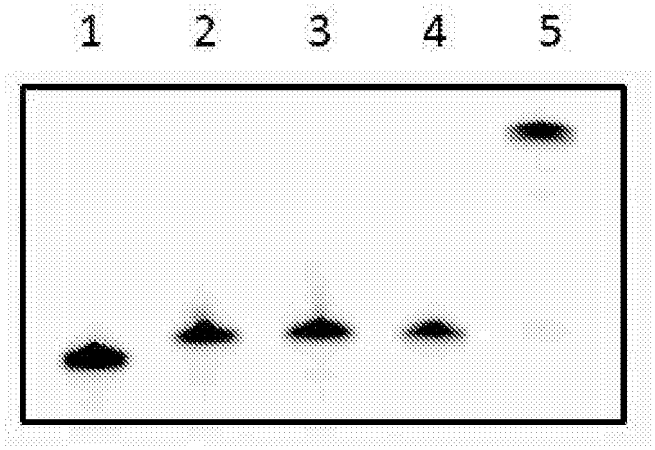
e



f



g



h

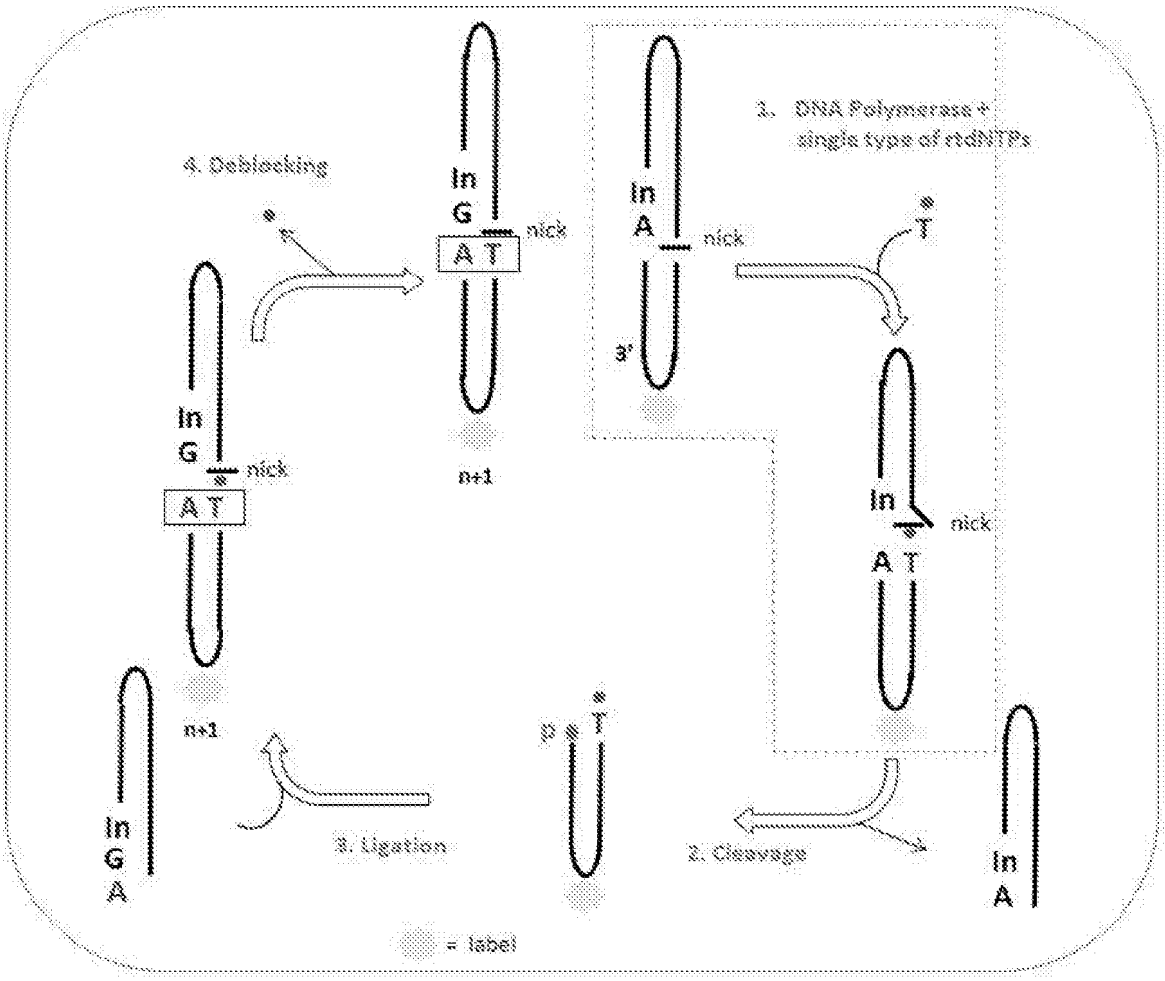
Incorporation	Deprotection by 50 mM TCEP	Deprotection by 300 mM TCEP
3'-*O*-azidomethyl-dTTP	50%	95%
3'-*O*-azidomethyl-dCTP	20%	95%
3'-*O*-azidomethyl-dATP	Not tested	95%
3'-*O*-azidomethyl-dGTP	Not tested	95%

i

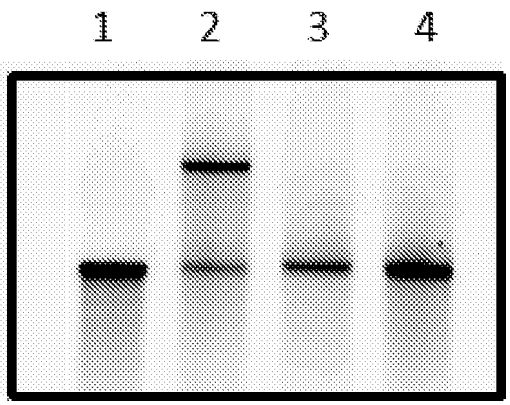
Sequence ID Number	Oligonucleotide name	Sequence	Modification
39	Primer	5'-GCGACAGGTGACTGCAGC-3'	TAMRA at 5'-end
40	Template-A	5'-CACATCACGTCGTAGTC**A**GCT GCAGTCACCTGTCGC-3'	None
41	Template-G	5'-CACATCACGTCGTAGTC**G**GCT GCAGTCACCTGTCGC-3'	None
42	Template-T	5'-CACATCACGTCGTAGTC**T**GCT GCAGTCACCTGTCGC-3'	None
43	Template-C	5'-CACATCACGTCGTAGTC**C**GCT GCAGTCACCTGTCGC-3'	None

Figure 15

a


4. Deblocking
In G
nick
A T
n+1
In G
nick
A T
n+1
In G A
3. Ligation
1. DNA Polymerase + single type of ntdNTPs
In A
nick
3'
T
In A T
nick
p
T
2. Cleavage
In A
= label

b

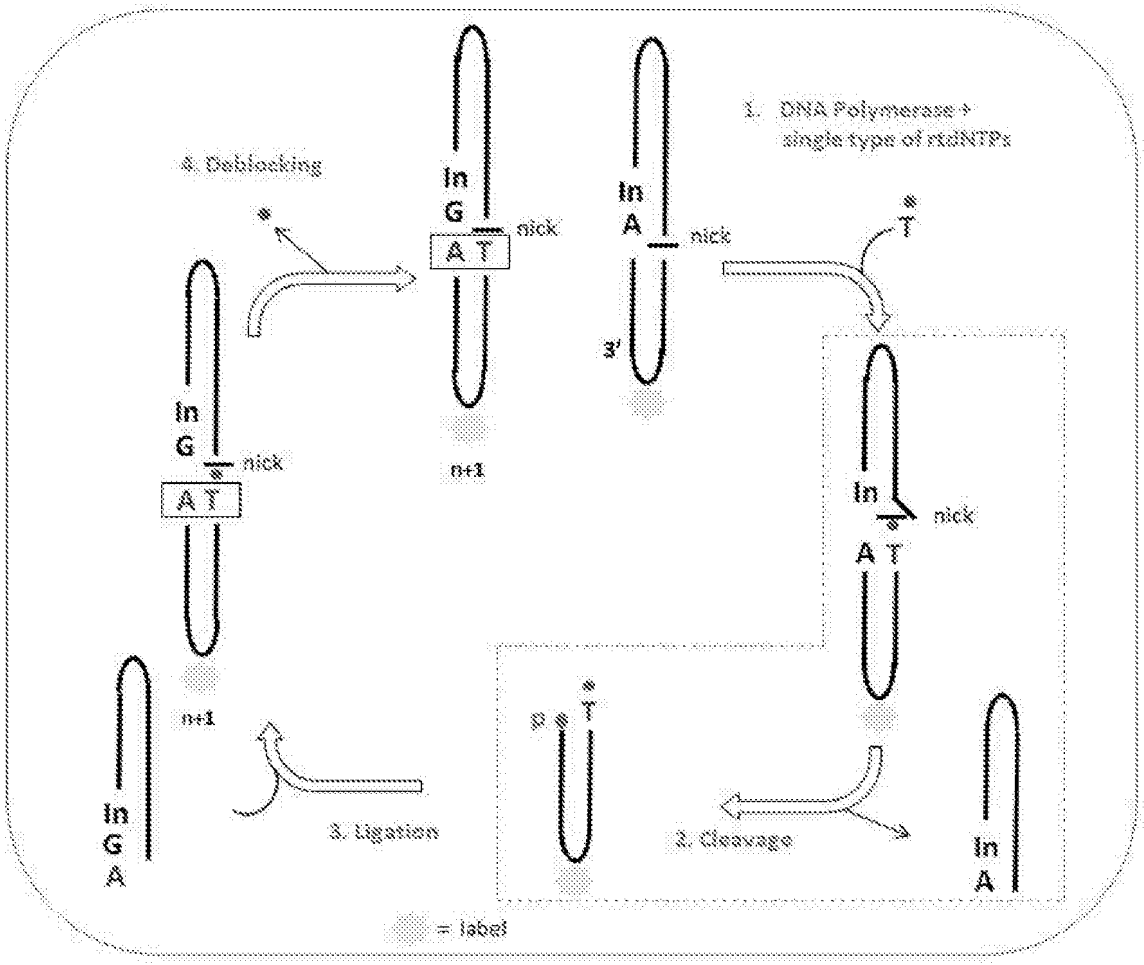

1
2
3
4

C

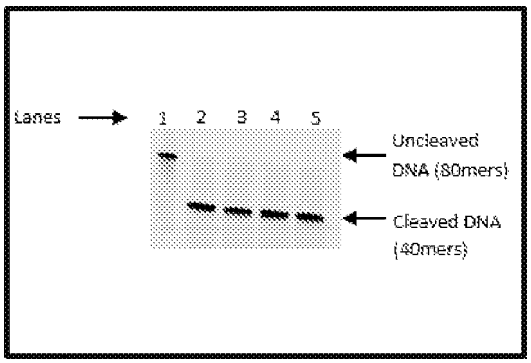
Sequence ID Number	Oligonucleotide name	Sequence	Modification
44	Dual hairpin model for incorporation	5'- TCGACTACGACGTGACTTTTAGTCAC GTCGTAGTC**X**AGCTGCAGTCACCTGCT GCTTYTTGCAGCAGGTGACTGCAGC-3'	X = 2'- deoxyinosine Y = Tamra-dT

Figure 16

a


4. Deblocking
1. DNA Polymerase + single type of rtdNTPs
In G
A T
nick
n+1
In A
3'
In G
A T
nick
n+1
In A
T
nick
In G A
3. Ligation
2. Cleavage
In A
= label

b



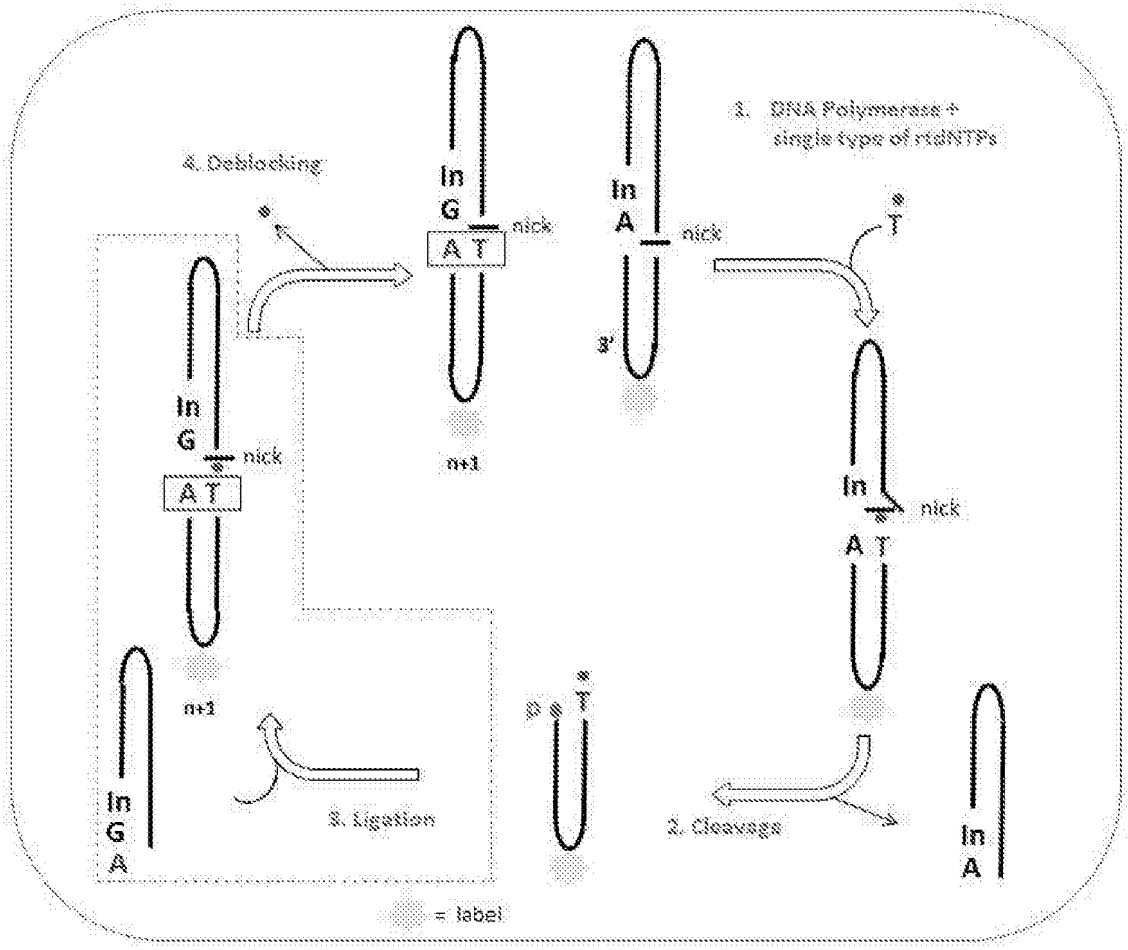
Sample Lanes	Content
1	Reference control: Template (15pmols) only.
2	Test Sample I : Cleaved hairpin template after 5mins incubation (15pmols)
3	Test sample I: Cleaved hairpin template after 10mins incubation (15pmols)
4	Test sample I: Cleaved hairpin template after 30mins incubation (15pmols)
5	Test sample I: Cleaved hairpin template after 1hr incubation (15pmols)

c

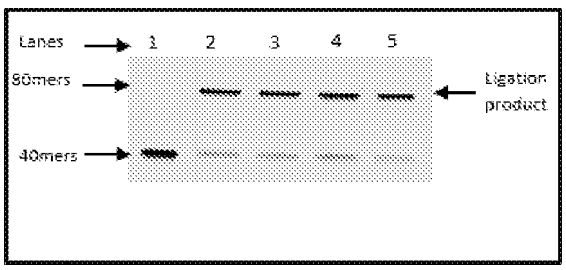
Sequence ID Number	Oligonucleotide name	Sequence	Modification
45	Dual hairpin model for incorporation	5'- TCGACTACGACGTGACTTTTAGTCAC GTCGTAGTCXAGCTGCAGTCACCTGCTG CTTYTTGCAGCAGGTGACTGCAGCT-3'	X = 2'-deoxyinosine Y = Tamra-dT

Figure 17

a



b



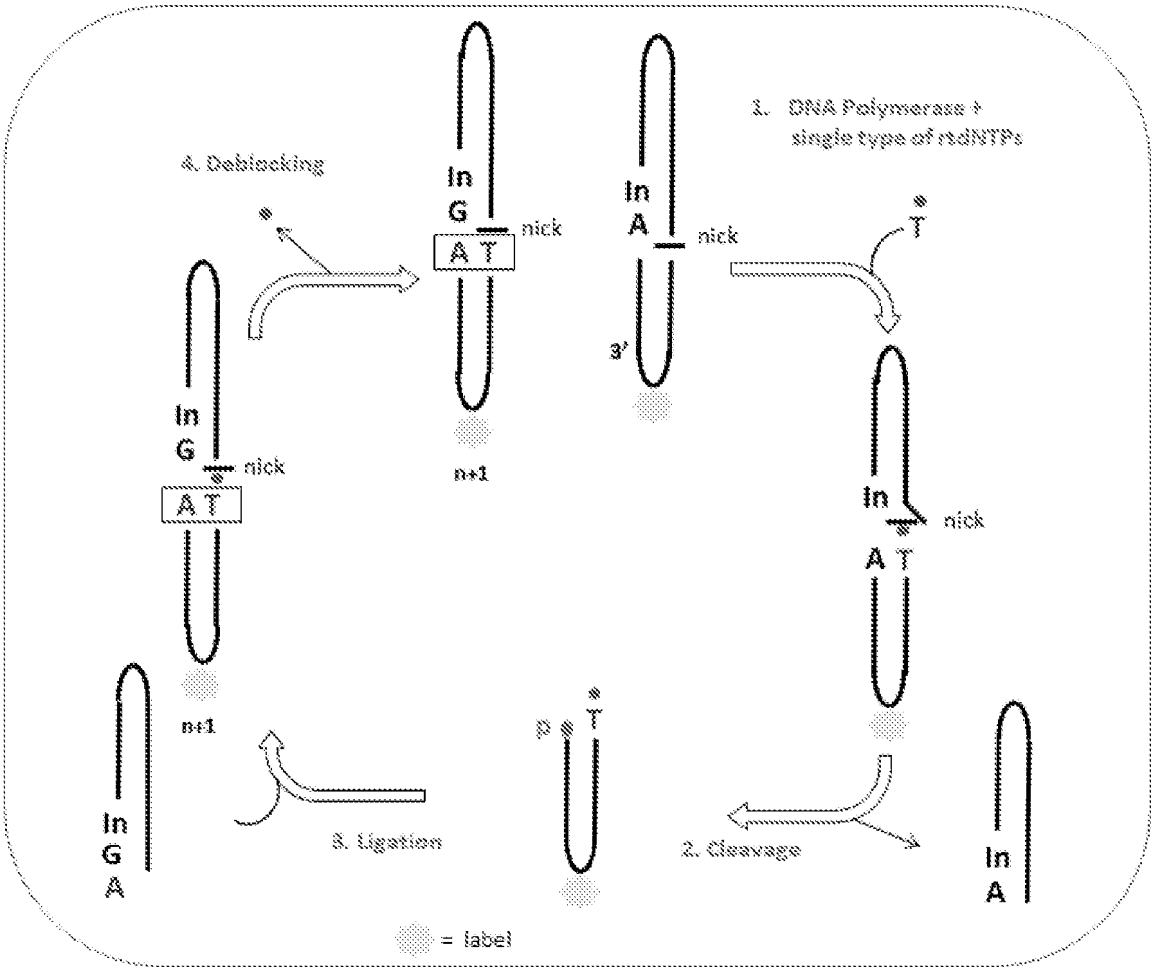
Sample Lanes	Content
1	Reference control: Phosphate Template (15pmols) only.
2	Test Sample I : Ligated hairpin product after 1min incubation (15pmols)
3	Test sample II: Ligated hairpin product after 2min incubation (15pmols)
4	Test sample II: Ligated hairpin product after 3mins incubation (15pmols)
5	Test sample II: Ligated hairpin product after 4mins incubation (15pmols)

C

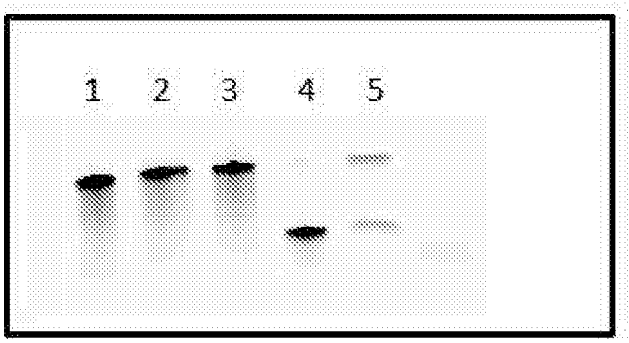
Sequence ID Number	Oligonucleotide name	Sequence	Modification
46	Phosphate hairpin	5'-GCTGCAGTCACCTGCTGCTTYT TGCAGCAGGTGACTGCAGCT-3'	Y = Tamra-dT phosphate
47	Inosine Hairpin	5'- CCGACTACGACGTGACTTTTAGT CACGTCGTAGTCXGA-3'	X = 2-deoxyinosine

Figure 18

a


4. Deblocking
In G
nick
A T
n+1
In A
nick
3'
1. DNA Polymerase + single type of rdNTPs
T
In A
nick
A T
2. Cleavage
In A
p T
3. Ligation
In G A
= label

b

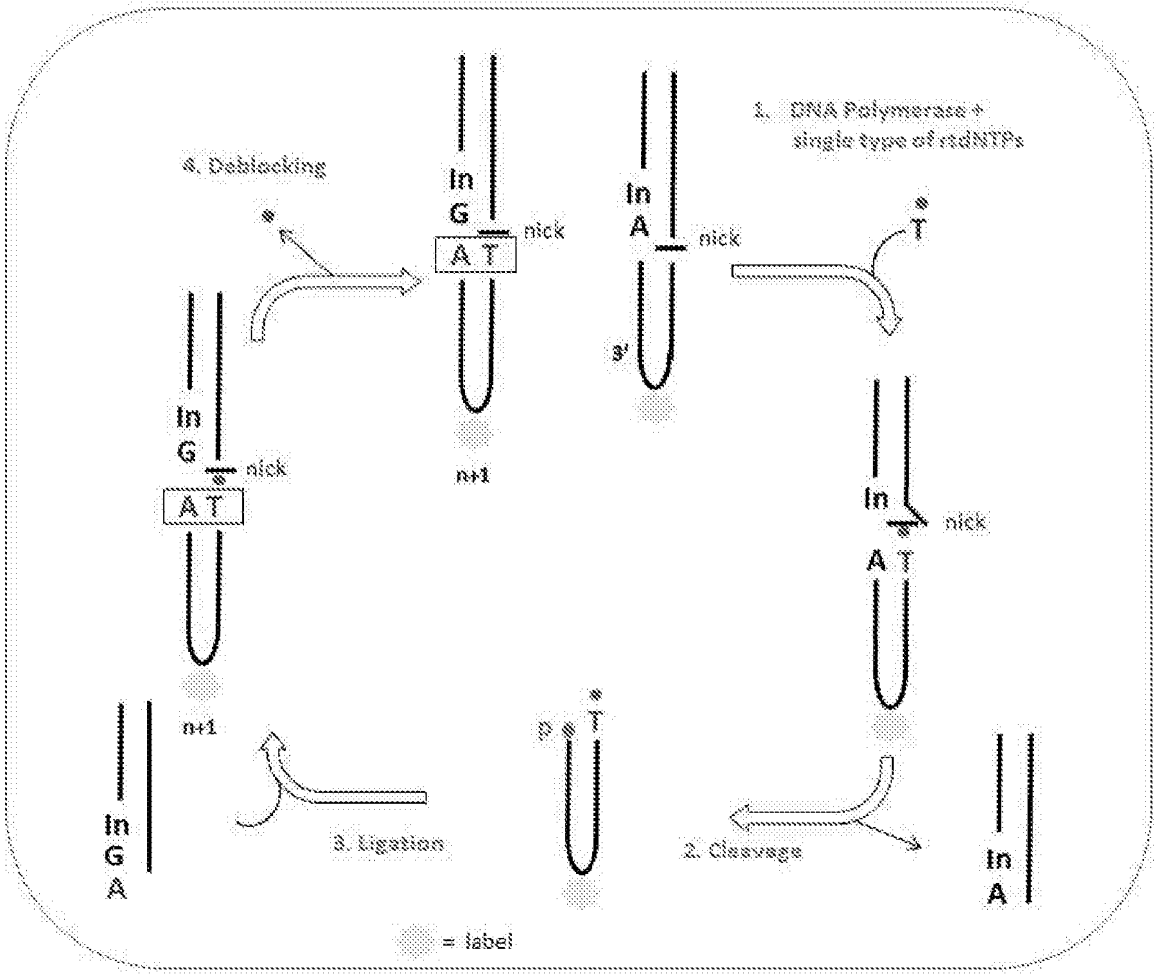

1
2
3
4
5

C

SEQ ID NO	Oligonucleotide name	Sequence	Modification
48	Double hairpin model	5'- TCGACTACGACGTGACTTTTAGTCAC GTCGTAGTCXAGCTGCAGTCACCTGCTGC TTYTTGCAGCAGGTGACTGCAGC-3'	X = 2'-deoxyinosine Y = Tamra-dT
49	Strand for ligation	5'- CCGACTACGACGTGACTTTTAGTCAC GTCGTAGTCXGA-3'	X = 2'-deoxyinosine

Figure 19

a

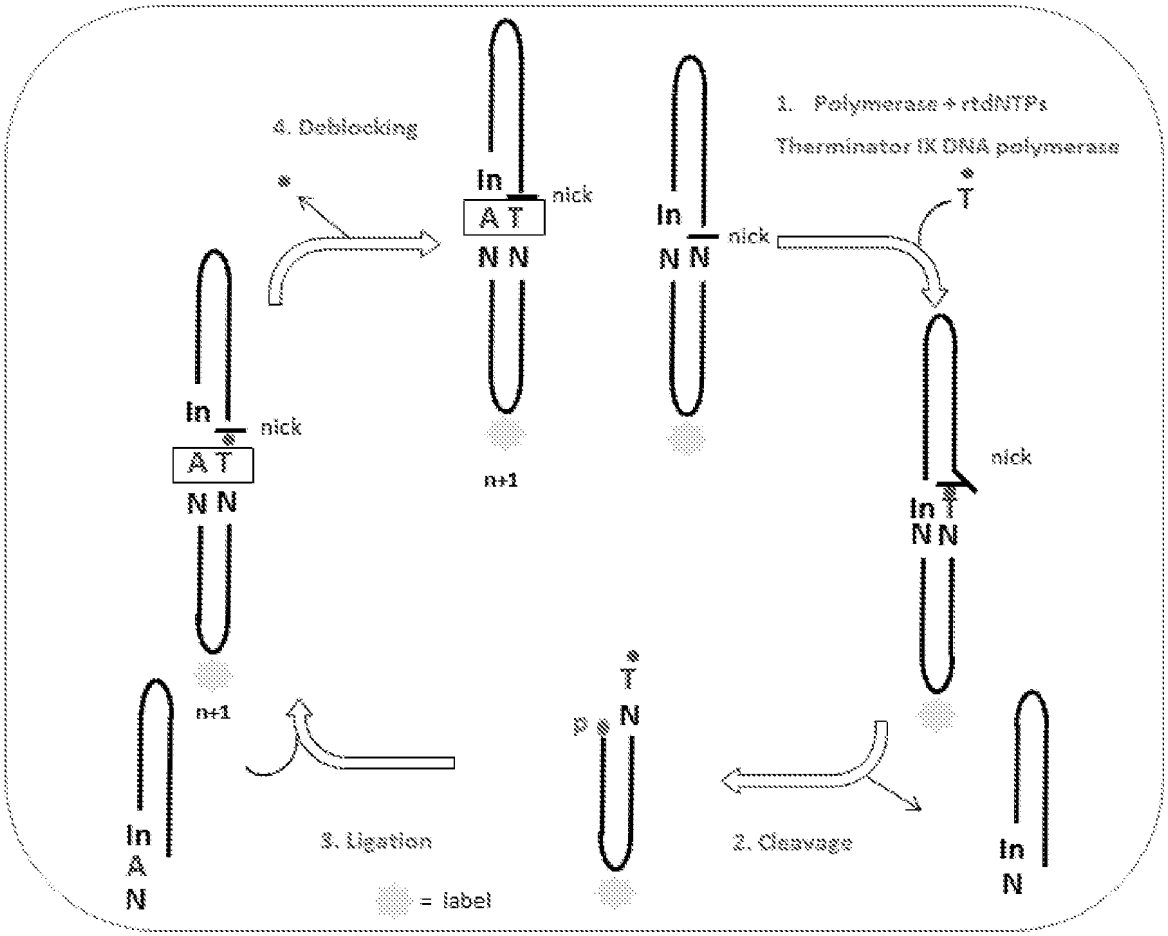


b

SEQ ID NO:	Oligonucleotide name	Sequence	Modification
50	Single hairpin model for incorporation	5'- AGTCACGTCGTAGTCXAGCTGCAGTC ACCTGCTGCTTYTTGCAGCAGGTGACTGC AGC-3'	X = 2'-deoxyinosine Y = Tamra-dT
51	Helper strand	5'- TCGACTACGACGTGACT-3'	
52	Strand for ligation	5'- AGTCACGTCGTAGTCXGA	X = 2'-deoxyinosine
53	Helper strand for ligation	5'- CCGACTACGACGTGACT-3'	

Figure 20

a

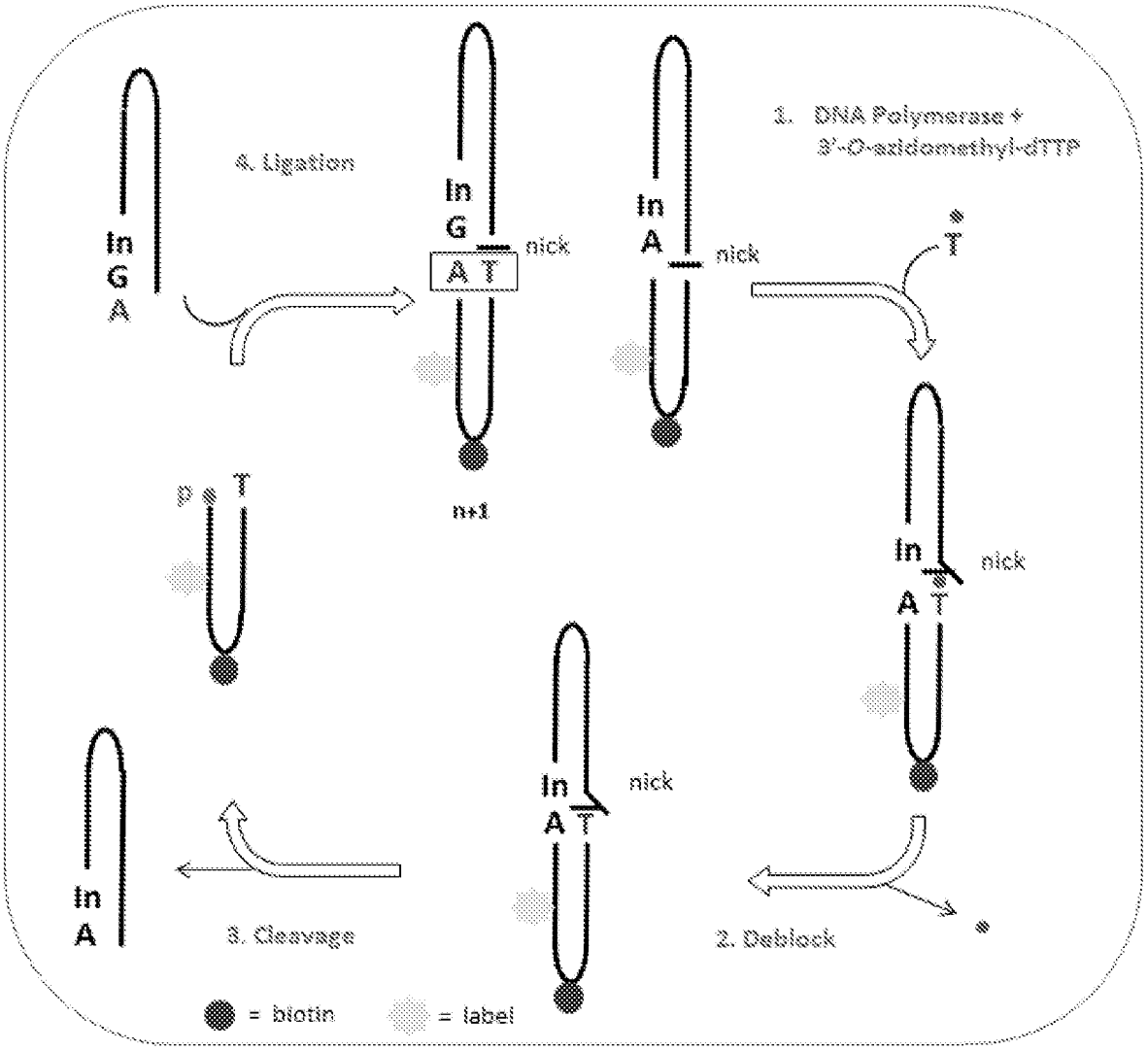


b

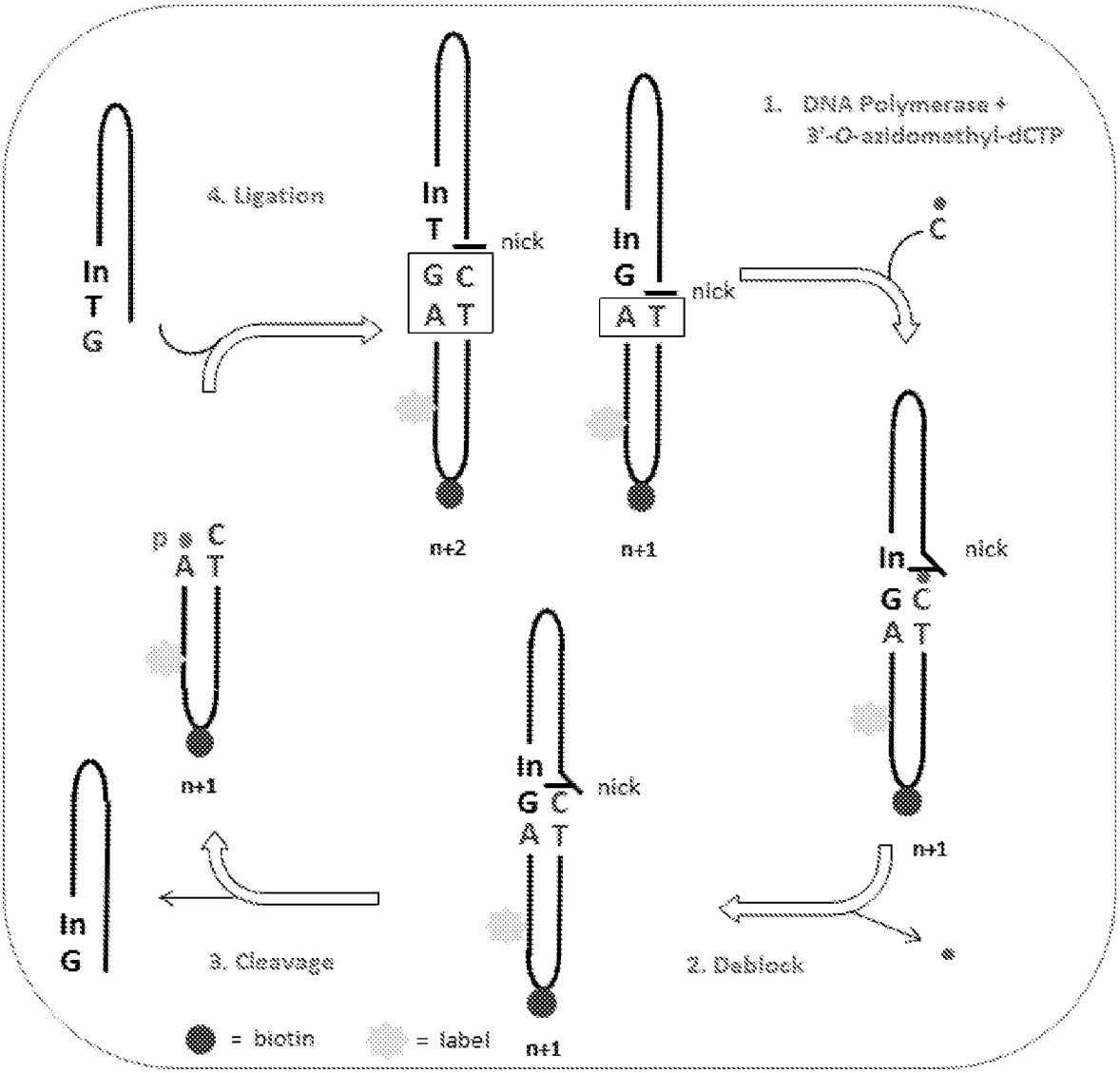
SEQ ID NO:	Oligonucleotide name	Sequence	Modification
54	Double hairpin model	5'- CGACTACGACGTGACTTTTAGTCAC GTCGTAGTCXAGCTGCAGTCACCTGCTGC TTYTTGCAGCAGGTGACTGCAGCT-3'	X = 2'-deoxyinosine Y = Tamra-dT
55	Strand for ligation	5'- CGACTACGACGTGACTTTTAGTCAC GTCGTAGTCXAA-3'	X = 2'-deoxyinosine

Figure 21

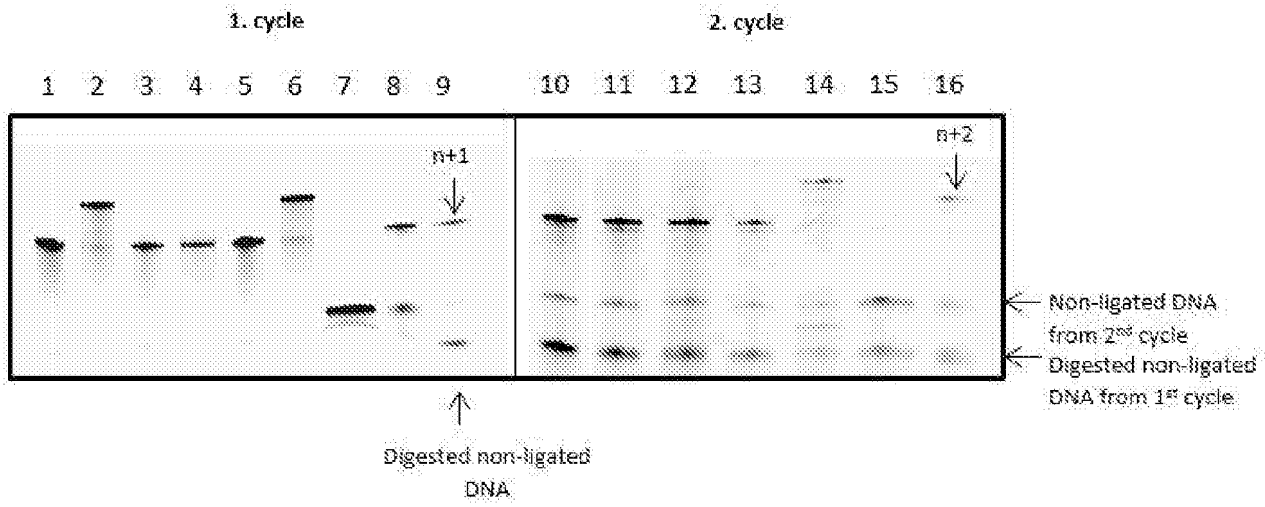
a


4. Ligation
In G A
In G
A T
nick
n+1
In A
nick
1. DNA Polymerase + 3'-O-azidomethyl-dTTP
T
In A T
nick
In A T
nick
2. Deblock
In A
3. Cleavage
P T
= biotin
= label

b


1. DNA Polymerase + 3'-O-azidomethyl-dCTP
2. Deblock
3. Cleavage
4. Ligation
nick
n+1
n+2
= biotin
= label

c



d

Sequence ID Number	Oligonucleotide name	Sequence	Modification
56	Double hairpin model	5'- CGACTACGACGTGACTTTTAGTCAC GTCGTAGTCXAGCTGCAGTCACCTGCYGCTT ZTTGCAGCAGGTGACTGCAGCT-3'	X = 2'-deoxyinosine Y = Tamra-dT Z = Biotin-dT
57	Strand for ligation in the 1st cycle	5'- CCGACTACGACGTGACTTTTAGTCAC GTCGTAGTCXGA-3'	X = 2'-deoxyinosine
58	Strand for ligation in the 2nd cycle	5'- ACGAGTGACCTGGTTTTTTTTTTTTTT TTTTTTTTTTTTTTTTTTTACCAGGTCACT C[I]TG-3'	X = 2'-deoxyinosine

Figure 22

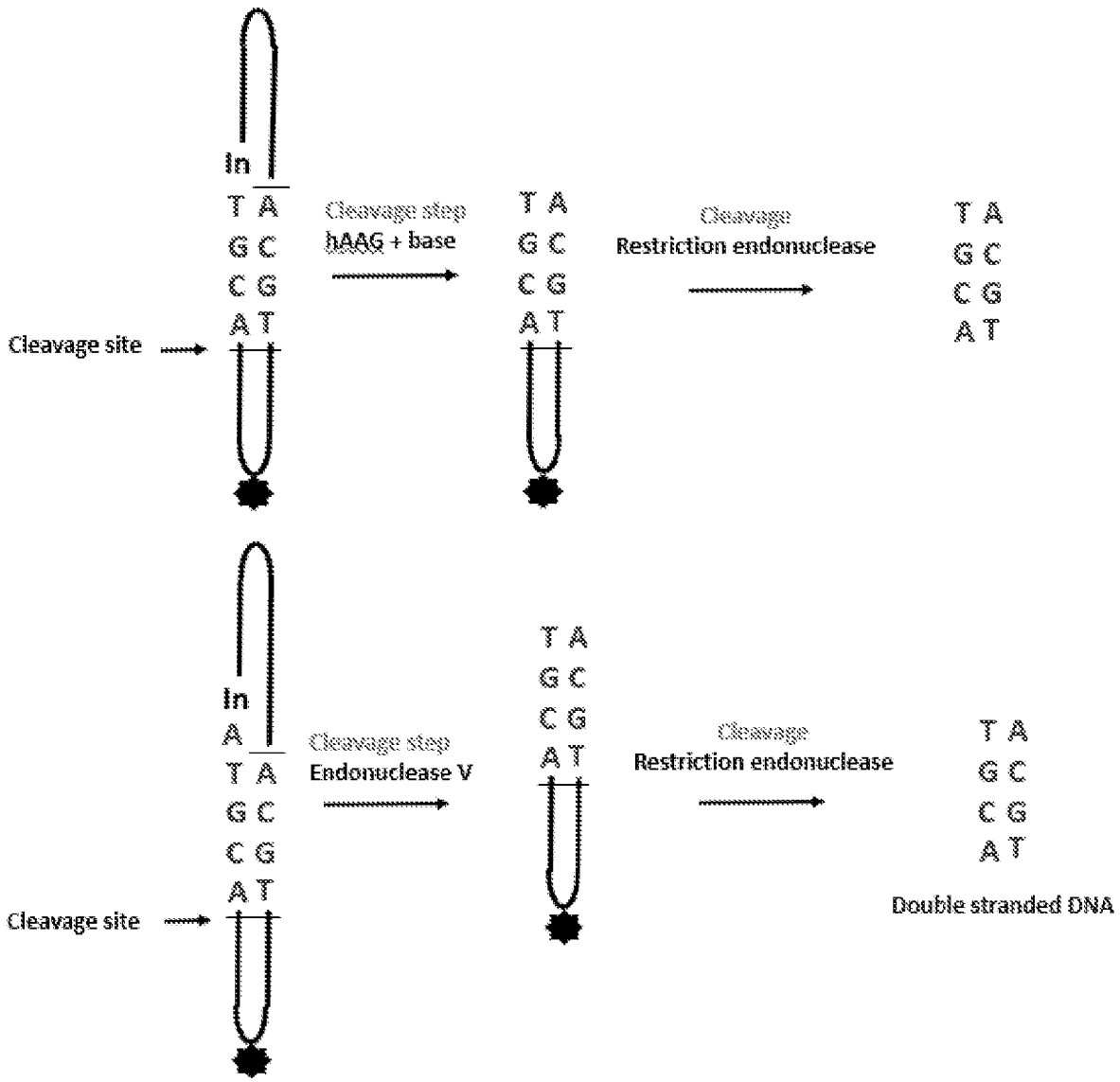

In
T A
G C
C G
A T
Cleavage site
Cleavage step
hAAG + base
T A
G C
C G
A T
Cleavage
Restriction endonuclease
T A
G C
C G
A T
In
A
T A
G C
C G
A T
Cleavage site
Cleavage step
Endonuclease V
T A
G C
C G
A T
Cleavage
Restriction endonuclease
T A
G C
C G
A T
Double stranded DNA

Figure 23

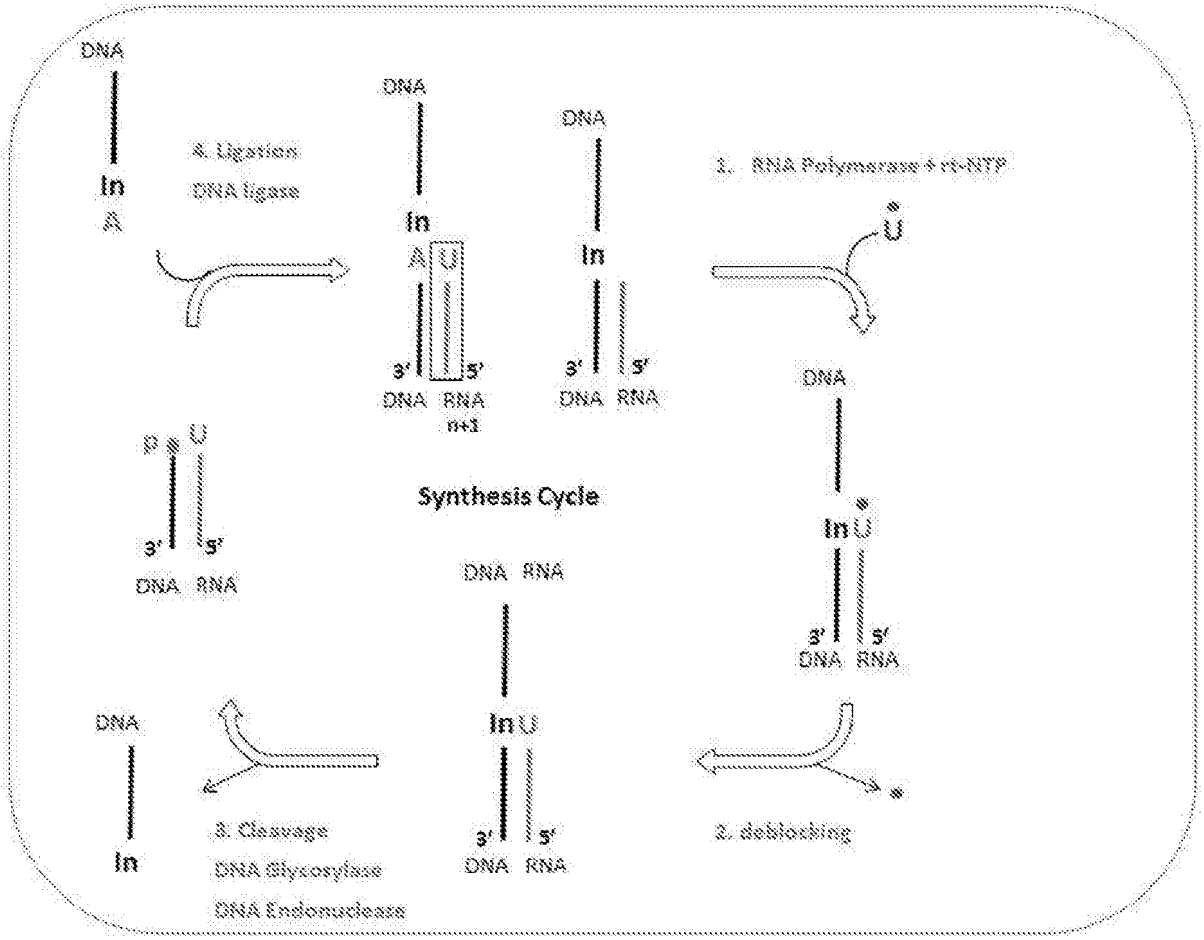

DNA
In
A
4. Ligation
DNA ligase
DNA
In
A U
3'
5'
DNA RNA
n+1
DNA
In
3'
5'
DNA RNA
1. RNA Polymerase + rt-NTP
U
DNA
In U
3'
5'
DNA RNA
2. deblocking
Synthesis Cycle
DNA RNA
In U
3'
5'
DNA RNA
3. Cleavage
DNA Glycosylase
DNA Endonuclease
DNA
In
U
3'
5'
DNA RNA

Figure 24

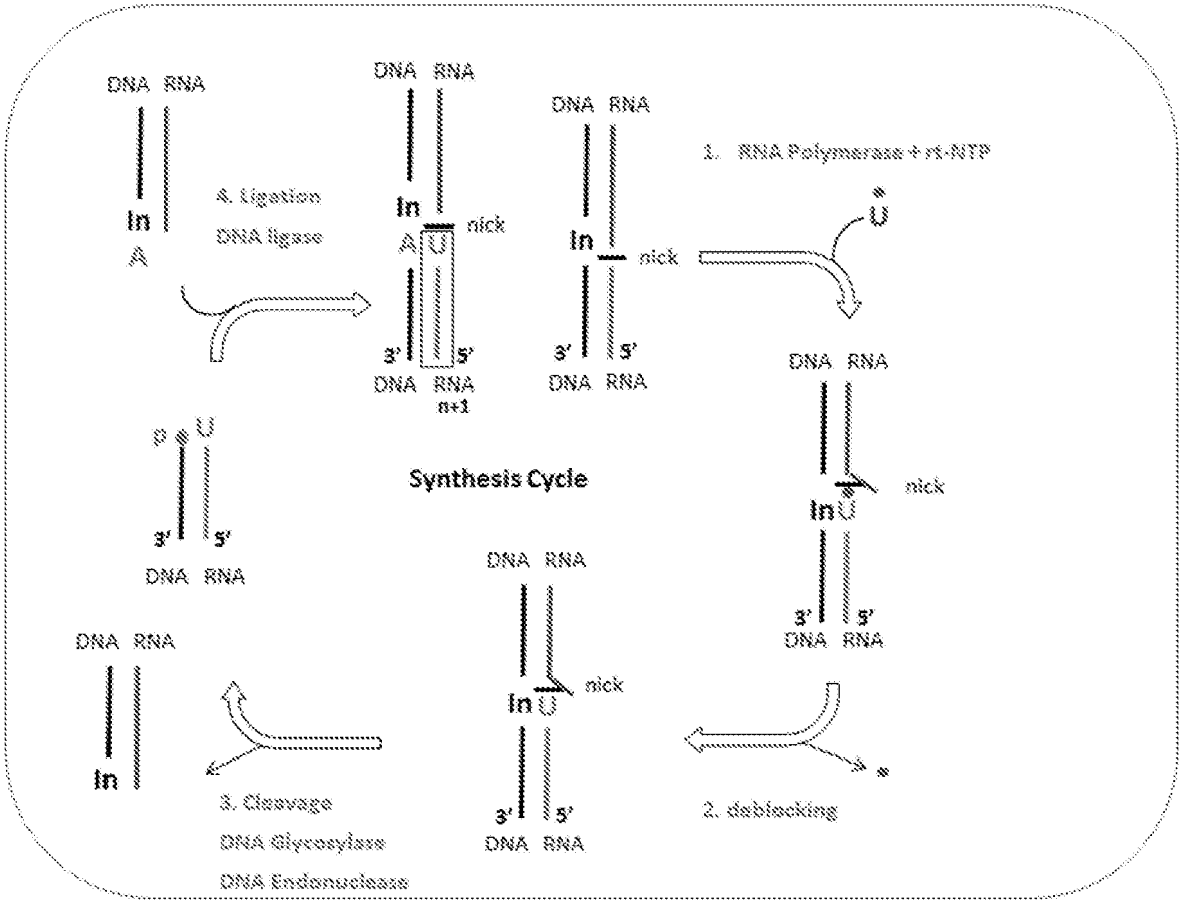
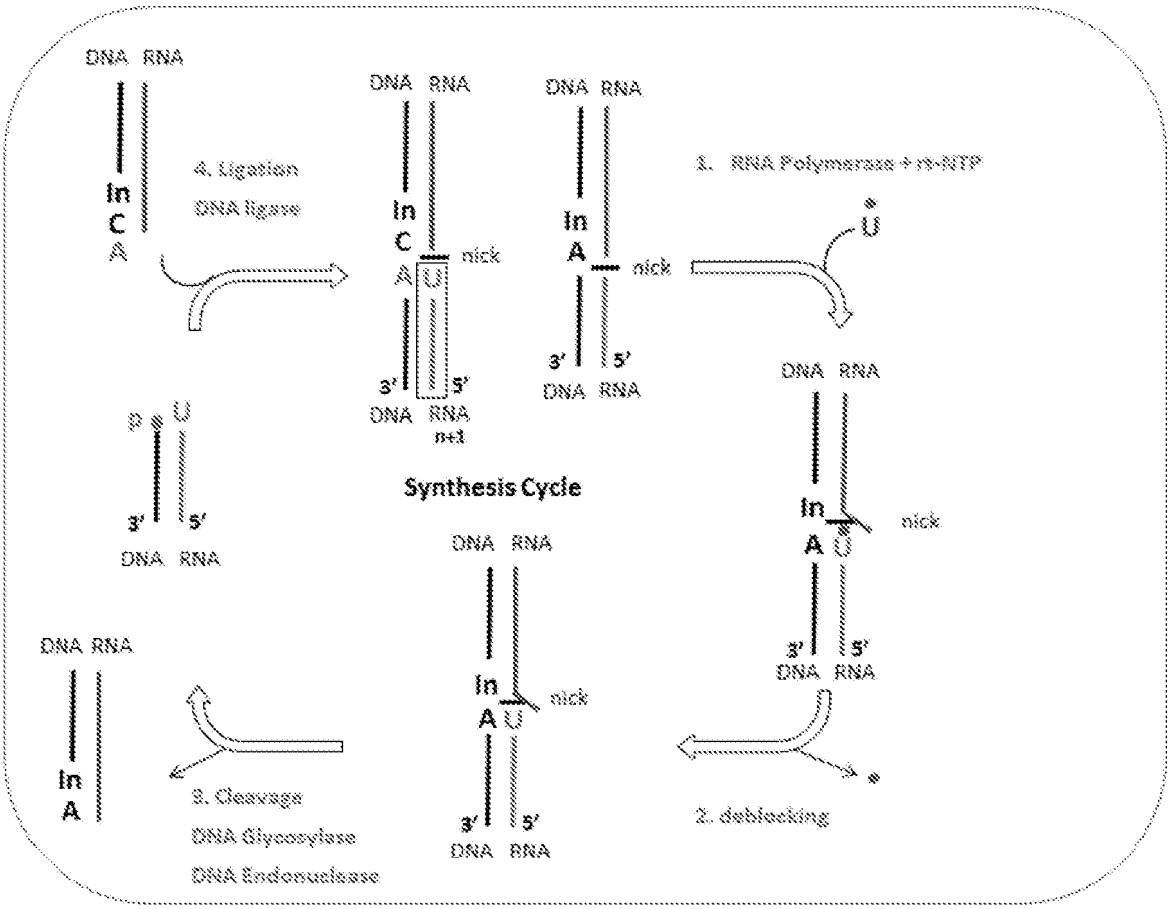

DNA RNA
In
A
4. Ligation
DNA ligase
nick
3'
5'
n+1
1. RNA Polymerase + rt-NTP
Synthesis Cycle
2. deblocking
3. Cleavage
DNA Glycosylase
DNA Endonuclease

Figure 25


DNA RNA
In C A
4. Ligation
DNA ligase
nick
3' 5'
DNA RNA
n+1
1. RNA Polymerase + rN-NTP
In A
Synthesis Cycle
2. deblocking
3. Cleavage
DNA Glycosylase
DNA Endonuclease

DOUBLE STRANDED POLYNUCLEOTIDE SYNTHESIS METHOD, KIT AND SYSTEM

Field of the invention

The invention relates to new methods for synthesizing 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 for performing the synthesis and assembly methods.

Background to the invention

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

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.

The Polymerase Synthetic approach uses a polymerase to synthesize 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 synthesize 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 synthesized *de novo* by this method, it requires a DNA template from which a copy is made. (Kosuri and Church, Nature Methods, 2014, 11, 499-507).

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

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

Summary of the invention

The invention provides an in vitro method for synthesising a double-stranded polynucleotide having a predefined sequence, preferably DNA, the method comprising performing cycles of synthesis wherein in each cycle, a first strand is extended by the incorporation of a nucleotide of the predefined sequence and the second strand which is hybridized to the first strand is extended by the incorporation of a nucleotide thereby forming a nucleotide pair with the incorporated nucleotide of the first strand; wherein each cycle comprises extending the first strand by incorporating the nucleotide of the predefined sequence together with an attached reversible terminator group followed by extending the second strand; further wherein in each cycle the nucleotides are incorporated into a scaffold polynucleotide and wherein each cycle comprises:

(1) providing a scaffold polynucleotide;

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

(3) cleaving the scaffold polynucleotide at a cleavage site;

(4) ligating a ligation polynucleotide to the cleaved scaffold polynucleotide, the ligation polynucleotide comprising a partner nucleotide for the nucleotide of the predefined sequence, wherein upon ligation the nucleotide of the predefined sequence pairs with the partner nucleotide; and

(5) removing the reversible terminator group from the nucleotide of the predefined sequence after step (4) or removing the reversible terminator group from the nucleotide of predefined sequence after step (2) and before step (3), or after step (3) and before step (4).

The scaffold polynucleotide may comprise a synthesis strand and a support strand hybridized thereto, wherein the synthesis strand comprises a primer strand portion and a helper strand portion.

The synthesis strand may be the first strand and the support strand may be the second strand.

The support strand, may be extended by ligating to the second strand a ligation polynucleotide, wherein in each cycle of synthesis the ligation polynucleotide comprises the nucleotide forming the nucleotide pair with the incorporated nucleotide of the first strand in that cycle.

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

In methods wherein the ligation polynucleotide is double-stranded, the ligation polynucleotide may preferably comprise a support strand and a helper strand.

The invention provides a method as described above, wherein step (1) comprises providing a scaffold polynucleotide comprising a synthesis strand and a support strand hybridized thereto, wherein the synthesis strand comprises a primer strand portion, and the support strand comprises a universal nucleotide; wherein step (3) comprises cleaving the scaffold polynucleotide at a cleavage site, the site defined by a sequence comprising the universal

nucleotide in the support strand, wherein cleavage comprises cleaving the support strand and removing the universal nucleotide; and wherein in step (4) the ligation polynucleotide comprises a support strand comprising a universal nucleotide which defines a cleavage site for use in the next cycle, and wherein the ligation polynucleotide is ligated to the support strand of the cleaved scaffold polynucleotide.

The invention provides a method as described above, comprising:

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

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

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

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

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

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

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

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

(9) removing the reversible terminator group from the next nucleotide after step (8) and before step (10), or after step (6) and before step (7), or after step (7) and before step (8); and

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

In such methods the universal nucleotide occupies position n in: (a) the support strand of the scaffold polynucleotide in steps 1 and 6 and (b) the support strand of the ligation polynucleotide in steps 4 and 8, and wherein the support strand of the scaffold polynucleotide is cleaved between positions n and n-1 in steps 3 and 7; wherein position n

is the nucleotide position in the support strand which is opposite the position in the synthesis strand which will be occupied by the nucleotide of the predefined sequence upon its incorporation in the respective next incorporation step, and wherein position n-1 is the next nucleotide position in the support strand relative to the position occupied by the universal nucleotide in the direction distal to the helper strand.

Alternatively, in such methods the universal nucleotide occupies position n+1 in: (a) the support strand of the scaffold polynucleotide in steps 1 and 6 and (b) the support strand of the ligation polynucleotide in steps 4 and 8, and wherein the support strand of the scaffold polynucleotide is cleaved between positions n and n-1 in steps 3 and 7; wherein position n is the nucleotide position in the support strand which is opposite the position in the synthesis strand which will be occupied by the nucleotide of the predefined sequence upon its incorporation in the respective next incorporation step, wherein position n-1 is the next nucleotide position in the support strand relative to position n in the direction distal to the helper strand, and wherein position n+1 is the next nucleotide position in the support strand relative to position n in the direction proximal to the helper strand.

Alternatively still, in such methods the universal nucleotide occupies position n in: (a) the support strand of the scaffold polynucleotide in steps 1 and 6 and (b) the support strand of the ligation polynucleotide in steps 4 and 8, and wherein the support strand of the scaffold polynucleotide is cleaved between positions n-1 and n-2 in steps 3 and 7; wherein position n is the nucleotide position in the support strand which is opposite the position in the synthesis strand which will be occupied by the nucleotide of the predefined sequence upon its incorporation in the respective next incorporation step, wherein position n-1 is the next nucleotide position in the support strand relative to the position occupied by the universal nucleotide in the direction distal to the helper strand, and wherein position n-2 is the next nucleotide position in the support strand relative to position n-1 in the direction distal to the helper strand.

The invention provides methods as described above, wherein:

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

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

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

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

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

ii. the universal nucleotide is positioned next to the terminal nucleotide of the support strand (position n);

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

The invention provides methods as described above, wherein:

a) in step (1) the scaffold polynucleotide is provided in the support strand with a nucleotide (position n) which is the partner nucleotide for the first nucleotide of

step (2), and the universal nucleotide in the support strand is positioned next to the partner nucleotide (position n+1, in the direction proximal to the helper strand);

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

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

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

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

ii. the universal nucleotide is positioned next to the penultimate nucleotide of the support strand (position n);

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

iv. the terminal nucleotide of the support strand (position n-1) overhangs the terminal nucleotide of the helper strand and is a partner nucleotide for the first nucleotide of step (2), or is a partner

nucleotide for the newly-incorporated nucleotide of step (6) of the current synthesis cycle.

The invention provides methods as described above, wherein:

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

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

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

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

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

ii. the universal nucleotide is positioned next to the penultimate nucleotide of the support strand; and

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

The invention also provides a method of assembling a polynucleotide having a predefined sequence, the method comprising performing any of the synthesis methods described herein to synthesize a first polynucleotide having a predefined sequence and one or more additional polynucleotides having a predefined sequence and joining together the first and one or more additional polynucleotides.

The invention additionally provides a polynucleotide synthesis system for carrying out any of the synthesis and assembly methods described 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 synthesized double-stranded polynucleotide from the scaffold polynucleotide. Such a system may further comprise means for providing the reaction reagents in droplets and means for delivering the droplets to the scaffold polynucleotide in accordance with the synthesis cycles.

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

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

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

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

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

Figure 1. Scheme of Exemplary Method Version 1.

Scheme showing a first synthesis cycle according to exemplary method version 1, comprising a cycle of provision of a scaffold polynucleotide, incorporation, cleavage, ligation and deprotection.

Figure 2. Scheme of Exemplary Method Version 2.

Scheme showing a first synthesis cycle according to exemplary method version 2, comprising a cycle of provision of a scaffold polynucleotide, incorporation, cleavage, ligation and deprotection.

Figure 3. Scheme of Exemplary Method Version 3.

Scheme showing a first synthesis cycle according to exemplary method version 3, comprising a cycle of provision of a scaffold polynucleotide, incorporation, cleavage, ligation and deprotection.

Figure 4. Scheme Showing Surface Immobilization of Scaffold Polynucleotides.

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

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

Figure 5. Absence of Helper Strand: Step 1 – Incorporation.

a) Scheme showing incorporation step highlighted in dashed box.

b) Evaluation of DNA polymerases for incorporation of 3'-*O*-modified-dTTPs opposite inosine. The figure depicts a gel showing results of incorporation of 3'-*O*-modified-dTTPs by various DNA polymerases (Bst, Deep Vent (Exo-), Therminator I and Therminator IX) in presence of Mn^{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-dTTPs 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.

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

d) Evaluation of the temperature on the incorporation using Therminator IX DNA polymerase. The figure depicts a gel showing results of incorporation of 3'-modified-dTTP opposite inosine in presence of Mn^{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.

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

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

g) Evaluation of the presence of Mn^{2+} on the incorporation using Therminator IX DNA polymerase. Results of incorporation in presence and absence of Mn^{2+} ions.

h) Oligonucleotides used for study of the incorporation step.

Figure 6. Absence of Helper Strand: Step 2 – Cleavage.

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

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

c) Gel showing cleavage of oligonucleotide with hAAG and Endo VIII at 37°C. Lane 2 which was a positive control that contained both full length strands showed a higher yield of cleaved to uncleaved DNA ratio of ~ 90% : 10%. Lane 3 which included the cleavage

reaction without a helper strand showed a low percentage yield of cleaved to uncleaved DNA ratio of ~7% : 93%.

d) A summary of cleavage of oligonucleotide with hAAG/Endo VIII and hAAG/Chemical base.

e) Oligonucleotides used for study of the cleavage step.

Figure 7. Absence of Helper Strand: Step 3 – Ligation.

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

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

c) Oligonucleotides used for study of the ligation step.

Figure 8. Version 1 Chemistry with Helper Strand: Step 1 – Incorporation.

a) Scheme showing incorporation step highlighted in dashed box.

b) Oligonucleotides applicable for study of the incorporation step.

Figure 9. Version 1 Chemistry with Helper Strand: Step 2 – Cleavage.

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

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

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

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

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

f) Oligonucleotides used for study of the cleavage step.

Figure 10. Version 1 Chemistry with Helper Strand: Step 3 – Ligation.

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

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

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

d) Oligonucleotides used for study of the ligation step.

Figure 11. Version 2 Chemistry with Helper Strand: Step 1 – Incorporation.

a) Scheme showing incorporation step highlighted in orange dashed box

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%.

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%.

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 1 minute, conversion 30%. Lane 3: Incorporation after 2 minutes, conversion 65%. Lane 4: Incorporation after 5 minutes, conversion 90%. Lane 5: Incorporation after 10 minutes, conversion 90%. Lane 6: Incorporation after 20 minutes, conversion 90%.

e) Gel showing results of incorporation of 3'-*O*-modified-dTTPs by 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%.

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%.

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: Incorporation after 10 minutes, conversion 96%. Lane 6: Incorporation after 20 minutes, conversion 96%.

h) Summary of incorporation of 3'-*O*-azidomethyl-dTTP at various temperatures and presence of Mn^{2+} ions.

i) Gel showing results of incorporation of 3'-*O*-modified-dNTPs opposite complementary base by Therminator IX DNA polymerase in presence of Mn^{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.

j) Oligonucleotides used for study of the incorporation step.

Figure 12. Version 2 Chemistry with Helper Strand: Step 2 – Cleavage.

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

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

c) A summary of cleavage study with Endonuclease V.

d) Oligonucleotides used for study of the cleavage step.

Figure 13. Version 2 Chemistry with Helper Strand: Step 3 – Ligation.

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

b) Oligonucleotides for study of the ligation step.

Figure 14. Version 2 Chemistry with Helper Strand: Step 3 – Deprotection.

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

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

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

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

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

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

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

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

i) Oligonucleotides used for study of the cleavage step.

Figure 15. Version 2 Chemistry with Double Hairpin Model: Step 1 – Incorporation.

a) Scheme showing incorporation step highlighted in dashed box.

b) Evaluation of DNA polymerases for incorporation of 3'-*O*-modified-dTTPs opposite its natural counterpart. The figure depicts a gel showing results of incorporation of 3'-*O*-modified-dTTPs by 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.

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

Figure 16. Version 2 Chemistry with Double Hairpin Model: Step 2 – Cleavage.

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

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

c) Oligonucleotides used for study of the cleavage step.

Figure 17. Version 2 Chemistry with Double Hairpin Model: Step 3 – Ligation.

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

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

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

Figure 18. Version 2 Chemistry - Complete Cycle on Double Hairpin Model.

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

b) Evaluation of DNA polymerases for incorporation of 3'-*O*-modified-dTTPs 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.

c) Oligonucleotides applicable for study of the incorporation step.

Figure 19. Version 2 Chemistry - Complete Cycle on Single Hairpin Model using Helper Strand.

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

b) Oligonucleotides applicable for study of the incorporation step.

Figure 20. Version 3 Chemistry - Complete Cycle on Double-Hairpin Model.

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

b) Oligonucleotides applicable for study of the incorporation step.

Figure 21. Version 2 Chemistry – Complete Two-Cycle on Double-Hairpin Model.

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

b) Scheme showing the second full cycle, following the first full cycle, involving enzymatic incorporation, deprotection, cleavage and ligation steps.

c) The figure depicts a gel showing full two-cycle experiment comprising: incorporation, deprotection, cleavage and ligation steps.

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.

d) Oligonucleotides used for study.

Figure 22.

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

Figure 23.

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.

Figure 24.

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.

Figure 25.

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.

Detailed description of the invention

The present invention provides methods for the *de novo* synthesis of polynucleotide molecules according to a predefined nucleotide sequence. Synthesized 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; and synthesis is not dependent upon the copying of a pre-existing template strand comprising the predefined nucleotide sequence.

The present inventors have determined that the use of a "universal nucleotide", as defined herein, allows in certain cases the correct pairing of a newly-incorporated nucleotide with its partner nucleotide during each cycle of synthesis. More particularly, the use of a universal nucleotide allows for the creation of a cleavage site within the region of *de novo* synthesis, which facilitates cleavage and repeat cycles of synthesis. The invention provides versatile methods for synthesizing polynucleotides, and for assembling large fragments comprising such synthesized polynucleotides.

The synthesis methods of the invention will be described in more general detail herein by reference to exemplary methods including three method versions. These three method versions are also described in specific detail in the Examples. It is to be understood that all exemplary methods, including the three method versions, are not intended to be limiting.

Reaction conditions

The invention provides a method for synthesising a double-stranded polynucleotide having a predefined sequence.

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 nucletides 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)).

Incorporation of nucleotides into polynucleotides can be carried out under suitable conditions, for example using Therminator IX polymerase to incoprorate 3'-*O*-modified-dNTPs at ~65°C in the presence of a suitable buffered solution such as comprising 2 mM Tris-HCl, 1 mM $(NH_4)_2SO_4$, 1 mM KCl, 0.2 mM $MgSO_4$ and 0.01% Triton® X-100.

Cleavage of polynucleotides can be carried out under suitable conditions, for example using endonuclease at 37°C in the presence of a suitable buffered solution such as comprising 5 mM potassium acetate, 2 mM Tris-acetate, 1 mM magnesium acetate and 0.1 mM DTT.

Ligation of polynucleotides can be carried out under suitable conditions, for example using T4 DNA ligase at room temperature in the presence of a suitable buffered solution such as comprising 4.4 mM Tris-HCl, 7mM $MgCl_2$, 0.7mM dithiothreitol, 0.7mM ATP, 5% polyethylene glycol (PEG6000).

Deprotection can be carried out under suitable conditions, for example using TCEP in Tris buffer at a final concentration of 300mM.

Anchor polynucleotide and scaffold polynucleotide

Double-stranded polynucleotides having a predefined sequence are synthesized in methods of the invention by incorporation of nucleotides into a pre-existing polynucleotide, which may be attached to or capable of being attached to a surface as described herein. Such a pre-existing polynucleotide may be referred to as an anchor polynucleotide. An anchor polynucleotide is any polynucleotide structure upon which or within which a double-stranded polynucleotide having a predefined sequence may be synthesized.

In certain exemplary methods of the invention involving steps of nucleotide incorporation, cleavage and ligation, the anchor polynucleotide may be referred to as a scaffold polynucleotide. A scaffold polynucleotide is a double-stranded polynucleotide which acts as a support structure to accommodate the synthetic polynucleotide having a predefined sequence during and after synthesis. A scaffold polynucleotide as described herein is an example of an anchor polynucleotide.

In one embodiment a scaffold polynucleotide comprises a synthesis strand hybridized to a complementary support strand. The synthesis strand comprises a polymerase primer strand portion and a helper strand portion separated by a single-strand break or "nick" (e.g.

Figures 1 to 3). Both the primer strand portion and the helper strand portion of the synthesis strand may be provided hybridized to the complementary support strand. Alternatively, the helper strand portion of the synthesis strand may be provided separately. The primer strand portion of the synthesis strand may be provided first, followed by the support strand and helper strand. Alternatively components of the scaffold polynucleotide may be provided separately. For example, the support strand may be provided first, followed by the primer strand portion of the synthesis strand and then the helper strand. The support strand may be provided first, followed by the helper strand portion of the synthesis strand and then the primer strand. The helper strand portion may be provided before a cleavage step. Upon mixing of the components in suitable conditions the scaffold polynucleotide forms upon hybridization of the separate components.

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

A scaffold polynucleotide may be provided with each strand of the polynucleotide unconnected at adjacent ends. A scaffold polynucleotide may be provided with both strands of the polynucleotide 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

strands of the polynucleotide connected at adjacent ends, such as via a hairpin loop, at one end of the scaffold polynucleotide.

Scaffold polynucleotides with or without hairpins may be immobilized to a solid support or surface as described in more detail herein (see Figure 4).

The terms "hairpin" or "hairpin loop" are commonly used in the current technical field. The term "hairpin loop" is also often referred to as a "stem loop". Such terms refer to a region of secondary structure in a polynucleotide comprising a loop of unpaired nucleobases which form when one strand of a polynucleotide molecule hybridizes with another section of the same strand due to intramolecular base pairing. Thus hairpins can resemble U-shaped structures. Examples of such structures are shown in Figure 4.

Nucleotides

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

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

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

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.

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

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

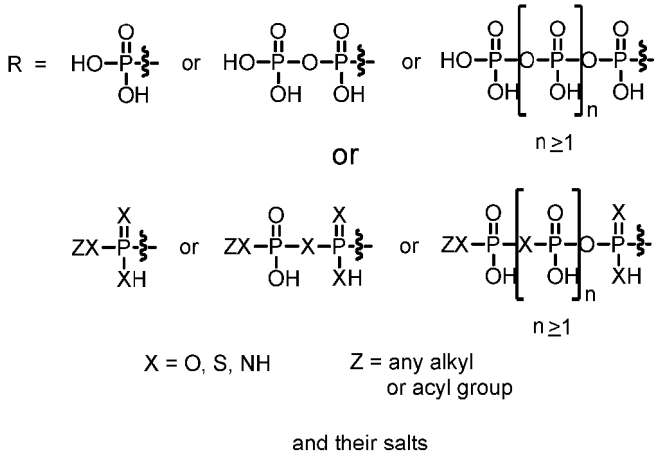
A non-natural nucleotide may comprise a modified sugar and/or a modified nucleobase. Modified sugars include but are not limited to 2'-*O*-methylribose sugar. Modified nucleobases include but are not limited to methylated nucleobases. Methylation of nucleobases is a recognised form of epigenetic modification 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.

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.

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

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 synthesized polynucleotide overall is double-stranded and wherein the first strand is attached to the second strand by hybridization.

Nucleotides and nucleotide analogues may preferably be provided as nucleoside triphosphates. Thus in any of the methods of the invention in order to synthesize DNA polynucleotides, nucleotides may be incorporated from 2'-deoxyribonucleoside-5'-O-triphosphates (dNTPs), e.g. via the action of a DNA polymerase enzyme. In any of the methods of the invention in order to synthesize RNA polynucleotides, nucleotides may be incorporated 2'-ribonucleoside-5'-O-triphosphates (NTPs), e.g. via the action of a RNA polymerase enzyme. Triphosphates can be substituted by tetraphosphates or pentaphosphates (generally oligophosphate). These oligophosphates can be substituted by other alkyl or acyl groups:



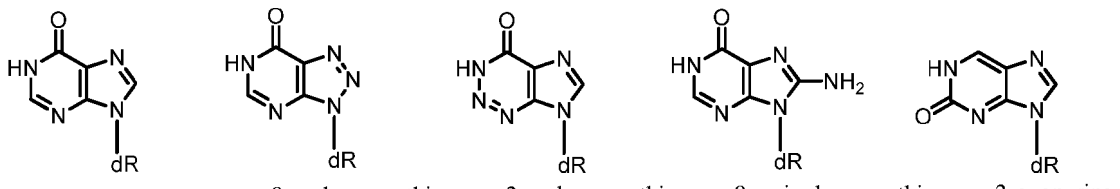
Universal nucleotides

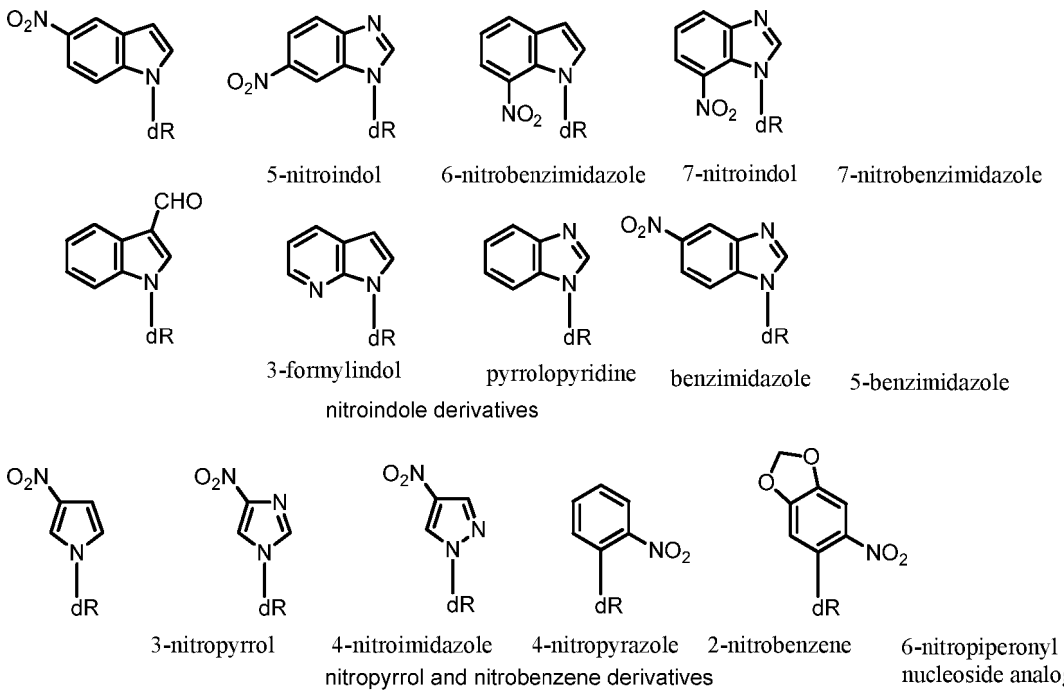
Methods of the invention may use a universal nucleotide. A universal nucleotide is one wherein the nucleobase will bond, e.g. hydrogen bond, to some degree to the nucleobase of any nucleotide of the predefined sequence. A universal nucleotide is preferably one which

will bond, e.g. hydrogen bond, to some degree to nucleotides comprising the nucleosides adenosine (A), thymine (T), uracil (U), guanine (G) and cytosine (C). The universal nucleotide may bond more strongly to some nucleotides than to others. For instance, a universal nucleotide (I) comprising the nucleoside, 2'-deoxyinosine, will show a preferential order of pairing of I-C > I-A > I-G approximately = I-T.

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

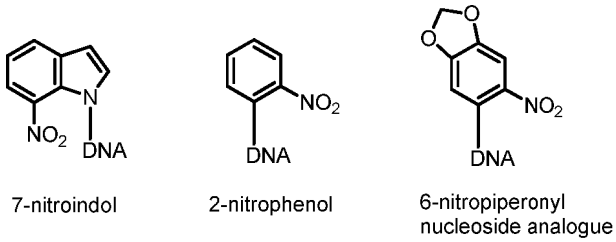
Some examples of universal bases are shown below:



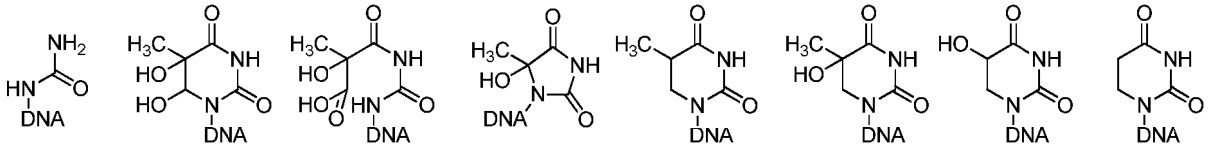


Universal nucleotides incorporating cleavable bases may also be used, including photo- and enzymatically-cleavable bases, some examples of which are shown below.

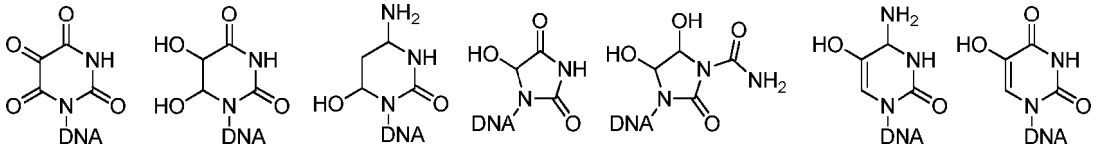
Photocleavable bases:



Base analogues cleavable by Endonuclease III:

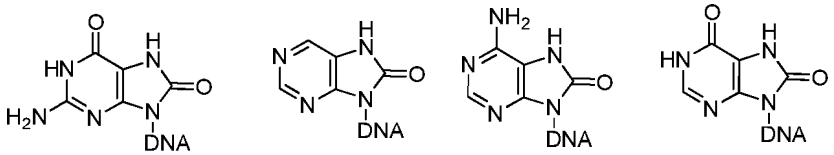


urea | thymine glycol (*cis* & *trans*) | methyl tartonyl urea | 5-hydroxy-5-methyhydantoin | 5,6-dihydro thymine | 5-hydroxy-6-hydrothymine | 5-hydroxy-6-hydrouracil | 5,6-dihydro-uracil

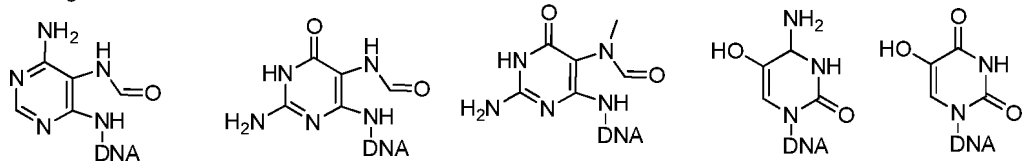


alloxan | uracil glycol (*cis* & *trans*) | 6-hydroxy-5,6-dihydrocytosine | 5-hydroxy hydantoin | *trans*-1-carbamoyl-2-oxo-4,5-dihydroxy-imidazolidine | 5-hydroxy-cytosine | 5-hydroxy-uracil

Base analogues cleavable by Formamidopyrimidine DNA glycosylase (Fpg):

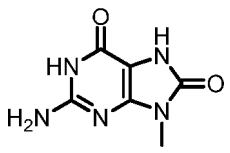


7,8-dihydro-8-oxo-guanine | 7,8-dihydro-8-oxo-inosine | 7,8-dihydro-8-oxo-adenine | 7,8-dihydro-8-oxo-nebularine



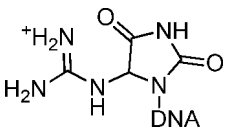
4,6-diamino-5-formamidopyrimidine (Fapy-adenine) | 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy-guanine) | 2,6-diamino-4-hydroxy-5-*N*-methylformamido-pyrimidine | 5-hydroxycytosine | 5-hydroxyuracil

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

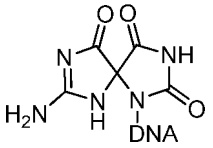


8-oxoguanine

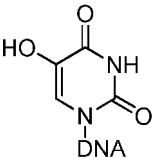
Base analogues cleavable by hNeil1:



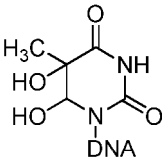
guanidinohydantoin
Gh



spiroiminodihydantoin
Sp

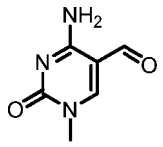


5-hydroxy-
uracil

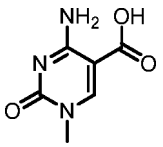


thymine glycol
(*cis* & *trans*)

Base analogues cleavable by Thymine DNA glycosylase (TDG):

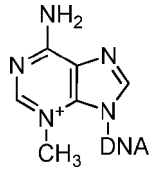


5-formylcytosine

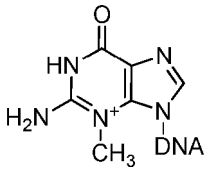


5-carboxycytosine

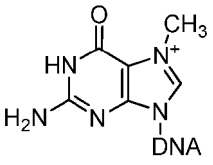
Base analogues cleavable by Human Alkyladenine DNA glycosylase (hAAG):



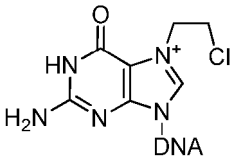
3-methyladenine



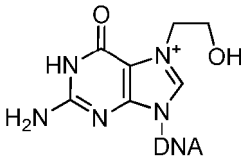
3-methylguanine



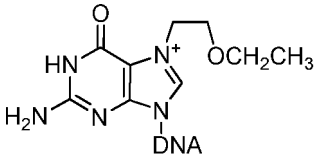
7-methylguanine



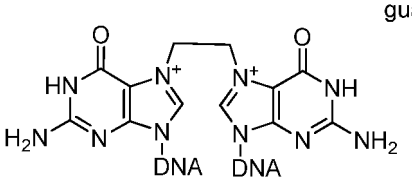
7-(2-chloroethyl)-
guanine



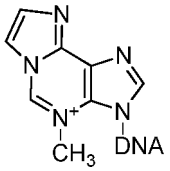
7-(2-hydroxyethyl)-
guanine



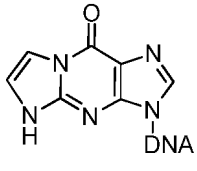
7-(2-ethoxyethyl)-
guanine



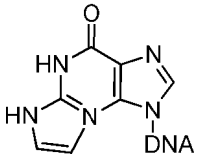
1,2-bis-(7-guanyl)-ethane



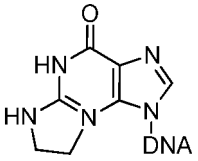
1,N^6-etheno-
adenine



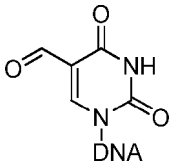
1,N^2-etheno-
guanine



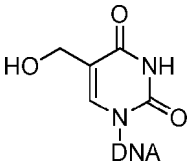
N^2,3-etheno-
guanine



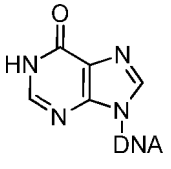
N^2,3-ethano-
guanine



5-formyluracil

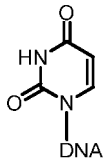


5-hydroxymethyl-
uracil



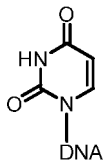
hypoxanthine

Bases cleavable by uracil DNA glycosylase:



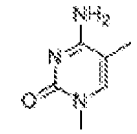
uracil

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



uracil

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



5-methylcytosine

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

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

Polymerase

Any suitable 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. Preferably, the polymerase will have strand displacement activity .

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. One such example of a modified polymerase is Therminator IX DNA polymerase available from New England BioLabs. This enzyme has an enhanced ability to incorporate 3'-*O*-modified dNTPs.

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

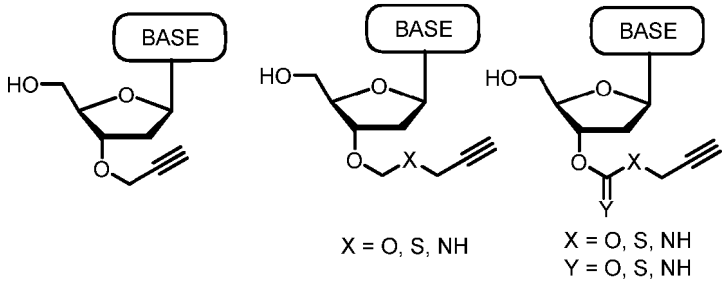
Examples of other polymerases that can be used for incorporation of reversible terminator NTPs in any of the methods of the invention are T3 RNA polymerase, T7 RNA polymerase, and SP6 RNA polymerase.

Reversible blocking groups

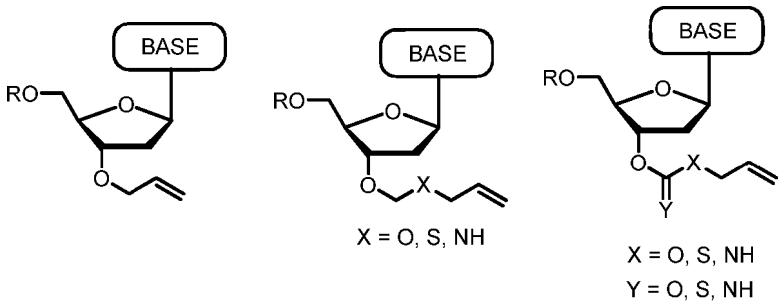
Any suitable reversible blocking group may be attached to a nucleotide to prevent further extension by polymerase following the incorporation of a nucleotide in a given cycle and

to limit incorporation to one nucleotide per cycle. In any the methods of the invention the reversible blocking group is preferably a reversible terminator group. Examples of reversible terminators are provided below.

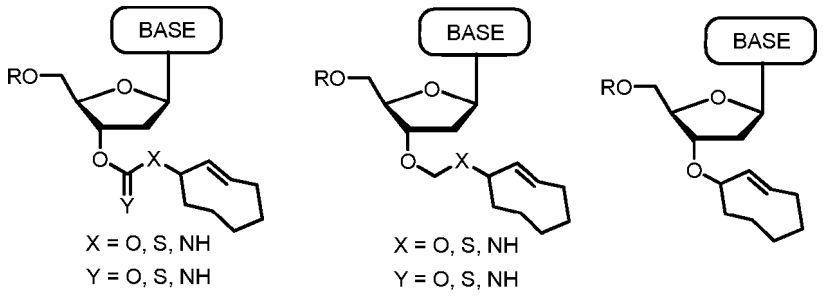
Propargyl reversible terminators:



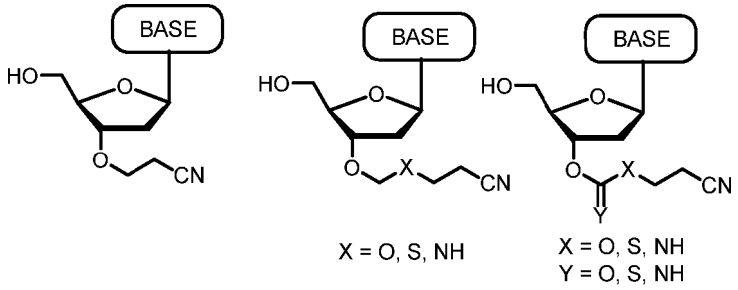
Allyl reversible terminators:



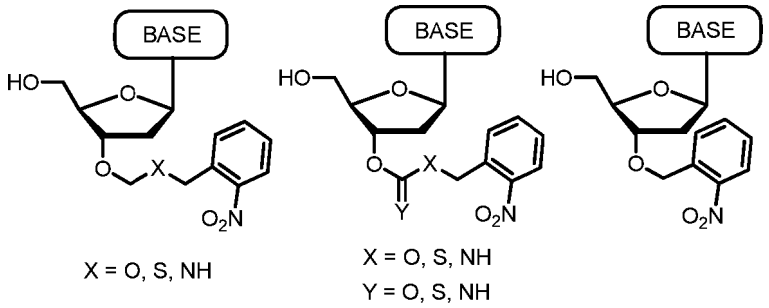
Cyclooctene reversible terminators:



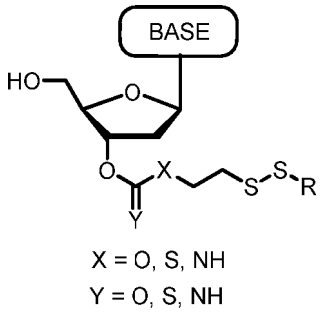
Cyanoethyl reversible terminators:



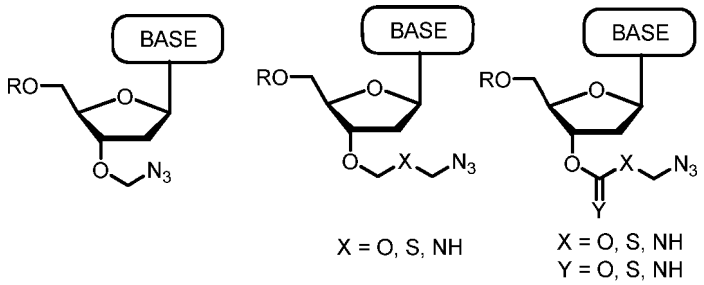
Nitrobenzyl reversible terminators:



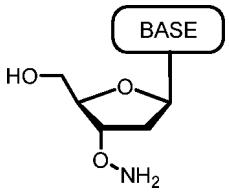
Disulfide reversible terminators:



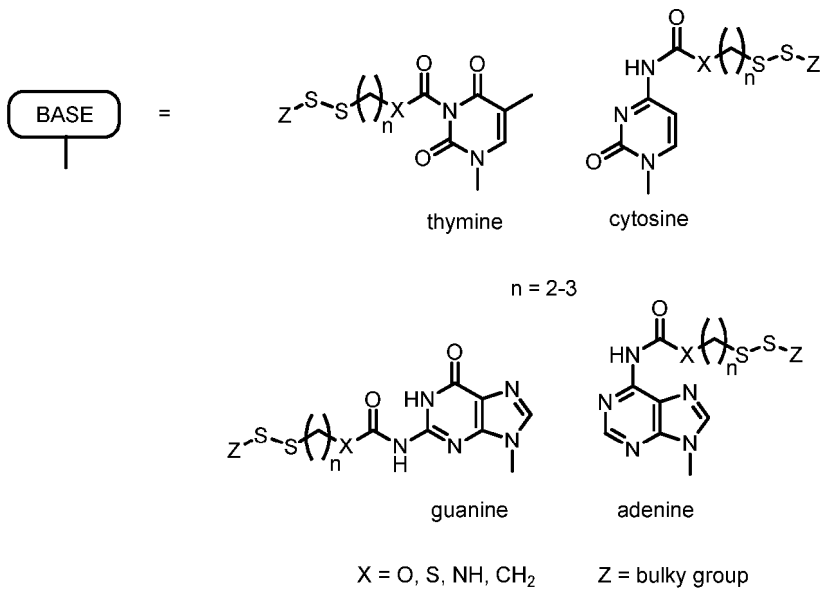
Azidomethyl reversible therminators:



Aminoalkoxy reversible therminators:



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

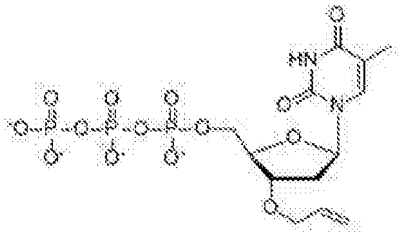


For synthesising DNA polynucleotides according to any of the methods of the invention preferred modified nucleosides are 3'-O-modified-2'-deoxyribonucleoside-5'-O-triphosphate. For synthesising RNA polynucleotides according to any of the methods of the invention preferred modified nucleosides are 3'-O-modified-2'-ribonucleoside-5'-O-triphosphate.

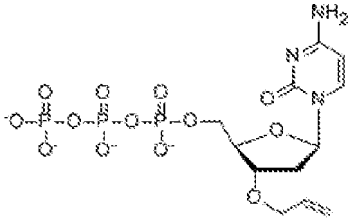
Preferred modified dNTPs are modified dNTPs which are 3'-*O*-allyl-dNTPs and 3'-*O*-azidomethyl-dNTPs.

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

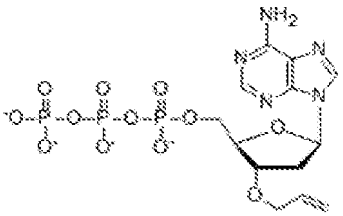
3'-*O*- allyl -dTTP:



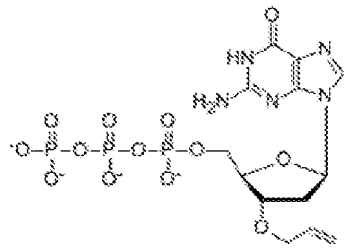
3'-*O*- allyl –dCTP:



3'-*O*- allyl -dATP:

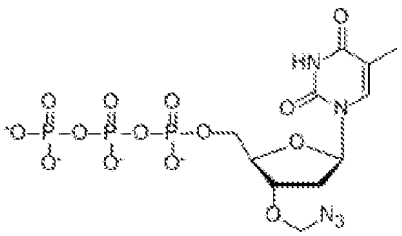


3'-*O*- allyl -dGTP:

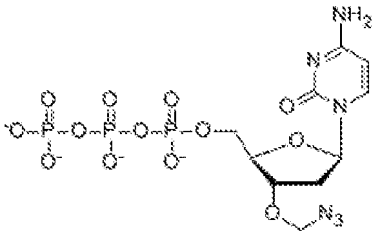


3'-*O*-azidomethyl-dNTPs are shown below.

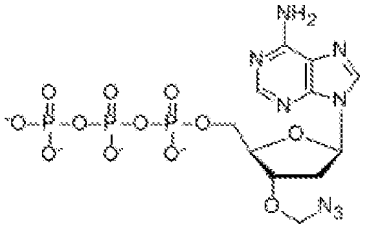
3'-*O*-azidomethyl-dTTP:



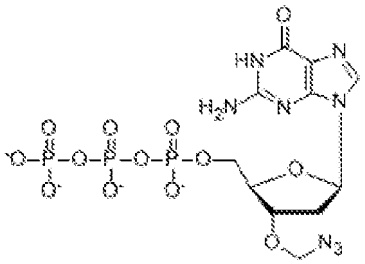
3'-*O*-azidomethyl-dCTP:



3'-*O*-azidomethyl-dATP:



3'-*O*-azidomethyl-dGTP:



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

A preferred deprotecting reagent is tris(carboxyethyl)phosphine (TCEP). TCEP may be used to remove reversible terminator groups from 3'-*O*-allyl-nucleotides (in conjunction with Pd^0) and 3'-*O*-azidomethyl- nucleotides following incorporation.

Examples of deprotecting reagents are provided below.

Propargyl reversible terminators:

Treatment by Pd catalysts – Na_2PdCl_4, $PdCl_2$.

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

Allyl reversible terminators:

Treatment by Pd catalysts – Na_2PdCl_4, $PdCl_2$.

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

Azidomethyl reversible terminators:

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

Cyanoethyl reversible terminators:

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

Nitrobenzyl reversible terminators:

Exposure to UV light

Disulfide reversible terminators:

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

Aminoalkoxy reversible terminators:

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

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

Synthetic polynucleotide

The polynucleotide having a predefined sequence synthesized according to the methods described herein is double-stranded. The synthesized 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.

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

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

Cleavage of scaffold polynucleotide

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

Some examples of DNA cleaving enzymes recognizing modified bases is shown in the Table below:

DNA glycosylase/ Endonuclease	**Main substrate**	**Cleavage site**	**Termini created from the cleavage**	
			5'-end	**3'-end**
APE1	AP site	1st phosphodiester bond 5' to the lesion	Deoxyribose-5'-phosphate	OH
Endonuclease III	AP site, thymine glycol	1st phosphodiester bond 3' to the lesion	phosphate	3´-phospho-α, β-unsaturated aldehyde
Endonuclease IV	AP site	1st phosphodiester bond 5' to the lesion	Deoxyribose-5'-phosphate	OH
Endonuclease V	Inosine	2nd phosphodiester bond 3' to the lesion	phosphate	OH
Endonuclease VIII	AP site, thymine glycol	1st phosphodiester bond 5' and 3' to the lesion	phosphate	phosphate
FpG	8-oxoguanine	1st phosphodiester bond 5' and 3' to the lesion	phosphate	phosphate
hOGG1	8-oxoguanine	1st phosphodiester bond 3' to the lesion	phosphate	3´-phospho-α, β-unsaturated aldehyde
hNeil1	Oxidized purines	1st phosphodiester bond 5' and 3' to the lesion	phosphate	phosphate

ROS1	5-methylcytosine	1st phosphodiester bond 5' and 3' to the lesion	phosphate	phosphate
Uracil DNA glycosylase	Uracil	*N*-glycosidic bond	AP site (no break)	
hSMUG	Uracil	*N*-glycosidic bond	AP site (no break)	
hAAG	Inosine	*N*-glycosidic bond	AP site (no break)	

Ligation polynucleotide

In methods requiring the presence of scaffold polynucleotides and steps of ligation following cleavage, the selection of the configuration and structure of the ligation molecule will also depend upon the particular method employed. For example, the requirement for a single- or double-nucleotide overhang in the support strand of the ligation end of the ligation molecule will depend upon the method employed. The appropriate structure can readily be achieved by reference to the exemplary methods described herein.

The ligation end of the ligation polynucleotide is typically provided with a non-phosphorylated terminal nucleotide in the helper strand adjacent the overhang. This prevents ligation of the helper strand portion of the synthesis strand to the primer strand portion of the synthesis strand and thus maintains the single-strand break in the synthesis strand. Alternative means for preventing ligation in the synthesis strands could be employed. For example blocking moieties could be attached to the terminal nucleotide in the helper strand.

Ligation

In methods of the invention which involve a ligation step, ligation may be achieved using any suitable means. Preferably, the ligation step will be performed by a ligase enzyme. The ligase may be a modified ligase with enhanced activity for single-base overhang

substrates. The ligase may be 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 both blunt-end and single-base overhang substrates. Molecules, enzymes, chemicals and methods for ligating (joining) single- and double-stranded polynucleotides are well known to the skilled person.

Solid phase synthesis

Synthetic polynucleotides in accordance with the invention may preferably be synthesized 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 solid planar surface, such as glass, or the surface of a microparticle such as a bead or functionalised quantum dot. 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-synthesized polynucleotide is immobilized.

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

Only one strand of such a double-stranded anchor/scaffold polynucleotide may be immobilized to the surface at the same end of the molecule. Alternatively both strands of a double-stranded anchor/scaffold polynucleotide may each be immobilized to the surface at

the same end of the molecule. A double-stranded anchor/scaffold polynucleotide may be provided with each strand connected at adjacent ends, such as via a hairpin loop at the opposite end to the site of initiation of new synthesis, and connected ends may be immobilized on a surface (for example as depicted schematically in Figure 4).

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 Figure 4(a) and (c). The synthesis strand comprising the primer strand portion and the portion of the support strand hybridized thereto may be connected at adjacent ends, such as via a hairpin loop, e.g. at the opposite end to the site of initiation of new synthesis, and connected ends may be tethered to a surface, as depicted in Figure 4(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 Figure 4(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

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

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.

Single-stranded oligonucleotides may be synthesized 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. Surfaces can be made of any suitable material, typically silicon, glass or polymeric material. 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).

Reversible immobilization

Synthetic polynucleotides having a predefined sequence can be synthesized 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.).

Surfaces can be provided in the form of microparticles, such as paramagnetic beads. Paramagnetic beads will only agglomerate under the influence of a magnetic field. For example, paramagnetic surfaces can be provided with chemical groups, e.g. carboxyl

groups, which in appropriate attachment conditions will act as binding moieties for nucleic acids, as described in more detail below. Nucleic acids can be eluted from such surfaces in appropriate elution conditions. Surfaces of microparticles and beads can be provided with UV-sensitive polycarbonate. Nucleic acids can be bound to the activated surface in the presence of a suitable immobilization buffer.

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

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

Microfluidic techniques and systems

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

Polynucleotides having a predefined sequence may be synthesized 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.

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.

Surface attachment chemistries

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

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

Some examples of chemistries suitable for attaching polynucleotides to surfaces are shown in Figure 4i and Figure 4j.

Microarrays

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

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

Other than the method of synthesising the polynucleotides of predefined sequence, microarray manufacture may be performed using techniques commonly used in this technical field, including techniques described herein. For example, anchor or scaffold polynucleotides may be tethered to surfaces using known surface attachment methods and chemistries, including those described herein.

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

Polynucleotides of predefined sequence may be provided at reaction sites in double-stranded form. Alternatively, following synthesis double-stranded polynucleotides may be separated and one strand removed, leaving single-stranded polynucleotides at reaction sites. Selective tethering of strands may be provided to facilitate this process. For 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

A polynucleotide having a predefined sequence synthesized by methods described herein may be joined to one or more other such polynucleotides to create larger synthetic polynucleotides.

Joining of multiple polynucleotides can be achieved by techniques commonly known in the art. A first polynucleotide and one or more additional polynucleotides synthesized 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 synthesized 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-synthesized polynucleotide as part of the predefined nucleotide sequence.

Assembly of polynucleotides is preferably performed using solid phase methods. For example, following synthesis a first polynucleotide may be subject to a single cleavage at a suitable position distal to the site of surface immobilization. The first polynucleotide will thus remain immobilized to the surface, and the single cleavage will generate a 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.

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

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

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

Examples of cleavage methods involving enzymatic cleavage are shown in Figure 22. 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-synthesized polynucleotide of predefined sequence.

Thus polynucleotides having a predefined sequence may be synthesized whilst immobilized to an electrowetting surface, as described above. Synthesized 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 synthesized polynucleotides for ligation with other cleaved synthesized polynucleotides. Polynucleotides can then be joined, for example by ligation. Using such techniques populations of different polynucleotides may

be synthesized 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.

Exemplary methods

Exemplary methods of the invention are described herein, including in the appended claims.

Three non-limiting exemplary methods are described below (see Figures 1 to 3). In step (1) of each of these exemplary methods a scaffold polynucleotide (see structure depicted in step 1 of each of Figures 1 to 3) is provided comprising a synthesis strand (see strand labelled "b" in structure depicted in step 1 of each of Figures 1 to 3) hybridized to a complementary support strand (see strand labelled "a" in structure depicted in step 1 of each of Figures 1 to 3). The scaffold polynucleotide is double-stranded and provides a support structure to accommodate the region of synthetic polynucleotide as it is synthesized *de novo*. The scaffold polynucleotide comprises a synthesis strand comprising a polymerase primer strand portion (see dotted portion of strand labelled "b" in structure depicted in step 1 of each of Figures 1 to 3) and a helper strand portion (see dashed portion of strand labelled "b" in structure depicted in step 1 of each of Figures 1 to 3) separated by a single-strand break or "nick". Both the primer strand portion and the helper strand portion of the synthesis strand are provided hybridized to a complementary support strand. The primer strand portion of the synthesis strand provides a primer sequence for use in the initiation of synthesis by a polymerase enzyme. Synthesis is initiated at the site of the single-strand break. Thus polymerase will act to extend the terminal nucleotide of the primer strand portion at the site of the single-strand break. This terminal nucleotide will therefore typically define a 3' terminus, of the primer strand portion to allow extension by polymerase enzymes which catalyze 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, and the terminal nucleotide of the support strand adjacent

the 5' terminus of the synthesis strand will consequently typically define the 3' terminus of the support strand.

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

The single-stranded break or "nick" between the helper strand portion and the primer strand portion of the synthesis strand is typically achieved by providing the (5') terminal nucleotide of the helper strand without a phosphate group. The break is typically achieved by assembling the scaffold polynucleotide from separate components comprising: (i) the support strand; (ii) the helper strand portion of the synthesis strand having a non-phosphorylated (5') terminal nucleotide; and (iii) the synthesis strand portion comprising the primer sequence. Upon mixing of the components in suitable conditions the scaffold polynucleotide forms upon hybridization of the separate components.

In step (2) of the methods a first nucleotide in the predefined nucleotide sequence is incorporated into the synthesis strand by the action of polymerase. The first nucleotide is provided with a reversible terminator group (depicted as the small triangle of the incorporated nucleotide in step 2 of each of Figures 1 to 3) which prevents further extension by the polymerase. Thus in step (2) only a single nucleotide is incorporated.

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 as described herein.

In each of the three methods a universal nucleotide (labelled "Un" in the structures depicted in each of Figures 1 to 3) is provided in the support strand which aids in the incorporation of a nucleotide of the predefined sequence and/or facilitates cleavage of the scaffold polynucleotide. The role of the universal nucleotide will be apparent from the detailed description of each method below.

Synthesis Method Version 1

In a first exemplary version of the synthesis method of the invention a new nucleotide is incorporated into a double-stranded scaffold polynucleotide opposite a universal nucleotide positioned in the support strand (steps 2 and 3 of Figure 1). In each cycle of synthesis the scaffold polynucleotide is cleaved at a cleavage site defined by a sequence comprising the universal nucleotide (step 3 of Figure 1). A single-nucleotide overhang comprising the newly-incorporated nucleotide is generated in the cleaved scaffold polynucleotide (see structure depicted in the middle of the lower part of Figure 1). Ligation of a ligation polynucleotide (see structure depicted at the far left of the lower part of Figure 1) to the cleaved scaffold polynucleotide incorporates a partner nucleotide into the scaffold polynucleotide and allows the newly-incorporated nucleotide to pair with the partner nucleotide (step 4 of Figure 1), thus completing a synthesis cycle.

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

In step (2) the first nucleotide of the predefined sequence is incorporated opposite the universal nucleotide such that the universal nucleotide pairs with the first nucleotide upon its incorporation. Thus in this configuration the universal nucleotide is positioned in the support strand in steps (1) and (2) at position "n" with respect to the incorporated first nucleotide in the synthesis strand, as depicted in Figure 1 (step 3).

During extension, polymerase will act to "invade" the helper strand and displace the terminal nucleotide of the helper strand. The incorporated first nucleotide will occupy the position previously occupied by the displaced terminal nucleotide of the helper strand (step 3 of Figure 1).

In step (3) of the method the scaffold polynucleotide is cleaved at a cleavage site. The cleavage site is defined by a sequence comprising the universal nucleotide in the support strand. Cleavage comprises cleaving the support strand to provide in the synthesis strand an overhanging end comprising the first nucleotide. Cleavage results in a double-stranded break in the scaffold polynucleotide. The synthesis strand is already provided with a single-stranded break or "nick", thus in this exemplary method only cleavage of the support strand is necessary to provide a double-stranded break in the scaffold polynucleotide.

In this exemplary method version, cleavage generates an overhang in the synthesis strand which overhangs the support strand. The overhanging end of the synthesis strand at the cleavage site comprises only a single unhybridized nucleotide which is the incorporated first nucleotide. Typically the overhanging first nucleotide will define a 3' terminus of the synthesis strand overhanging the 5' terminus of the support strand in the cleaved scaffold polynucleotide (see structure depicted in the middle of the lower part of Figure 1).

In this method the universal nucleotide occupies position "n" in the support strand. To obtain such a single-nucleotide overhang when the universal nucleotide occupies position "n" in the support strand, the support strand is cleaved at a specific position relative to the universal nucleotide. The support strand of the scaffold polynucleotide is cleaved between nucleotide positions "n" and "n-1".

By "n" it is meant the nucleotide position in the support strand which is occupied by, or has been occupied by, the universal nucleotide paired with the nucleotide of the predefined sequence incorporated in that given cycle. Thus at the cleavage step, position "n" in the support strand is opposite the position occupied by the nucleotide of the predefined sequence incorporated in that cycle, i.e. the terminal nucleotide of the primer strand portion of the synthesis strand. By "n-1" it is meant the next nucleotide position in the support strand relative to the position which is occupied by, or has been occupied by, the universal nucleotide, in the direction distal to the helper strand (nucleotide labelled "z" at position n-1, as shown schematically in step 3 of Figure 1). Thus at the cleavage step position "n-1"

in the support strand is opposite the position occupied by the penultimate nucleotide of the primer strand portion of the synthesis strand (as depicted in step 3 of Figure 1).

Upon cleavage of the support strand between nucleotide positions n and n-1, the universal nucleotide, helper strand and portion of the support strand which is hybridized to the helper strand are removed from the remaining scaffold polynucleotide (see structure depicted at the far right of the lower part of Figure 1), thus generating a single-nucleotide overhang comprising the first nucleotide in the synthesis strand overhanging the support strand in the cleaved scaffold polynucleotide.

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

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

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

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

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

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

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

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

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

In the first cleavage step 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 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 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.

In the second cleavage step 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, an enzyme having abasic site lyase activity, such as Endonuclease VIII, may be used. Other enzymes, molecules or chemicals could be used provided that the support strand is cleaved at the abasic site as described.

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

In step (4) of the method a double-stranded ligation polynucleotide is ligated to the cleaved scaffold polynucleotide. The ligation polynucleotide comprises a support strand and a helper strand. The ligation polynucleotide further comprises a complementary ligation end comprising in the support strand a universal nucleotide and a single overhanging nucleotide which is the partner nucleotide for the first nucleotide of the predefined sequence. The ligation polynucleotide further comprises in the helper strand adjacent the overhang a terminal nucleotide lacking a phosphate group (see the position labelled "X" in the structure depicted at the far left of the lower part of Figure 1). The complementary ligation end is configured so that it will compatibly join with the overhanging end of the cleaved scaffold polynucleotide product of step (3) when subjected to suitable ligation conditions. Upon ligation of the support strands the first nucleotide becomes paired with its partner nucleotide.

Thus in step (4) of this exemplary method, in the complementary ligation end of the ligation polynucleotide the universal nucleotide in the support strand is positioned opposite the terminal nucleotide of the helper strand and is paired therewith. The universal nucleotide is positioned (position n) next to the terminal nucleotide of the support strand. By position n in the ligation polynucleotide it is meant that when the ligation end of the ligation polynucleotide is ligated to the cleaved scaffold polynucleotide the universal nucleotide will be positioned in the support strand such that it will pair with the next nucleotide to be incorporated in step (6), i.e. in the next synthesis cycle, as depicted in Figure 1. In the complementary ligation end of the ligation polynucleotide of step (4) the

terminal nucleotide of the support strand is a partner nucleotide for the first nucleotide of step (2) and overhangs the terminal nucleotide of the helper strand.

In the ligation polynucleotide the helper strand is provided such that the terminal nucleotide adjacent the overhang lacks a phosphate group. Typically, as described above, this non-phosphorylated terminal nucleotide of the helper strand will define the 5' terminus of the helper strand.

In step (4), upon ligation of the support strand of the ligation polynucleotide and the support strand of the cleaved scaffold polynucleotide the first nucleotide in the synthesis strand pairs with its partner nucleotide in the support strand.

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

Ligation may typically be performed by enzymes having ligase activity. For example, ligation may be performed with T4 DNA ligase. The use of such enzymes will result in the maintenance of the single-stranded break in the synthesis strand, since the terminal nucleotide of the helper strand cannot act as a substrate for ligase due to the absence of a terminal phosphate group.

Ligation of the ligation polynucleotide to the cleaved scaffold polynucleotide completes a first synthesis cycle whereupon the scaffold polynucleotide of step (1) is effectively re-constituted except that the first nucleotide of the predefined nucleotide sequence is incorporated into the polynucleotide opposite its partner nucleotide. In this exemplary method, at the end of a given synthesis cycle, during cycles of synthesis, the universal nucleotide will occupy position n+1 in the support strand relative to the position occupied by the universal nucleotide in the support strand in the previous cycle. At the same time, at the end of a given synthesis cycle the universal nucleotide will occupy position n in the support strand relative to the position in the synthesis strand which will be occupied by the

next nucleotide of the predefined nucleotide sequence to be incorporated in the next cycle. Thus at the end of a given synthesis cycle a modified scaffold molecule is provided for use in the next synthesis cycle, wherein the universal nucleotide is once again positioned in the support strand to facilitate incorporation of the next nucleotide of the predefined nucleotide sequence and cleavage of the support strand in the next synthesis cycle.

In this exemplary method version of the invention, as well as with versions 2 and 3, 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). This can be performed at various stages of the first cycle. Typically it will be performed as step (5) of the method, after ligation step (4), as shown in step 5 of Figure 1. However, the deprotection step could be performed at any step after incorporation of the new nucleotide. Regardless of which stage the deprotection step is performed, polymerase and residual unincorporated first nucleotides should first be removed in order to prevent multiple incorporation of first nucleotides. Polymerase and unincorporated first nucleotides are preferably removed prior to the cleavage step (step (3)). Thus, removal of the reversible terminator group from the first nucleotide could be performed prior to the cleavage step (step (3)), prior to the ligation step (step (4)), or after the ligation step (as step (5)).

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

In method version 1, second and subsequent synthesis cycles may be performed as described above for the first synthesis cycle.

Thus in step (6) the scaffold polynucleotide provided for the next synthesis cycle is the product of the ligation step (4) and deprotection step (5) of the first synthesis cycle. In step (6) the next nucleotide in the predefined nucleotide sequence is incorporated into the synthesis strand of the scaffold polynucleotide by the action of polymerase, as described above for step (2) of the first cycle. The next nucleotide also comprises a reversible terminator group which prevents further extension in that cycle by polymerase.

As in step (2) of the first synthesis cycle of method version 1, in step (6) the next nucleotide is incorporated opposite a universal nucleotide which is positioned in the support strand such that it pairs with the next nucleotide upon its incorporation. In this configuration the universal nucleotide is again positioned at position "n" relative to the incorporated next nucleotide in the synthesis strand. Furthermore, as described above for the first synthesis cycle, in step (6) of the next synthesis cycle the universal nucleotide will occupy position "n+1" in the support strand relative to the position occupied by the universal nucleotide in the support strand in step (2) of the previous cycle. This is achieved because in the ligation polynucleotide of the previous synthesis cycle the universal nucleotide was positioned to be opposite to and paired with the terminal non-phosphorylated nucleotide of the helper strand.

In step (7) the scaffold polynucleotide is cleaved at a cleavage site, the site defined by a sequence comprising the universal nucleotide in the support strand. Cleavage comprises cleaving the support strand and removing the universal nucleotide to provide in the synthesis strand a single-nucleotide overhanging end comprising the next nucleotide in the predefined nucleotide sequence as the terminal nucleotide of the overhang. The single-nucleotide overhang of the synthesis strand overhangs the terminal nucleotide of the support strand in the cleaved scaffold polynucleotide. The cleavage steps may be performed as described above for step (3) of the first cycle.

In step (8) of the next cycle a double-stranded ligation polynucleotide is ligated to the cleaved scaffold polynucleotide. The ligation polynucleotide comprises a support strand and a helper strand. The ligation polynucleotide further comprises a complementary ligation end comprising in the support strand a universal nucleotide and a single overhanging nucleotide which is a partner nucleotide for the next nucleotide of the predefined nucleotide sequence. The ligation polynucleotide further comprises in the helper strand adjacent the overhang a terminal nucleotide lacking a phosphate group. The complementary ligation end is configured so that it will compatibly join with the overhanging end of the cleaved scaffold polynucleotide product of step (7) when subjected

to suitable ligation conditions. Upon ligation of the support strands the next nucleotide of the predefined nucleotide sequence becomes paired with its partner nucleotide.

The ligation polynucleotide of step (8) of the next and subsequent synthesis cycles may be configured, and the ligation step may be performed, as described above for step (4) of the first synthesis cycle.

Thus in step (8) upon ligation the universal nucleotide in the support strand is positioned opposite the terminal nucleotide of the helper strand, and is paired therewith. The universal nucleotide in the support strand is positioned at position "n" with respect to the next nucleotide to be incorporated in the next cycle. Furthermore, as described above, following step (8) the universal nucleotide will occupy position "n+1" in the support strand relative to the position occupied by the universal nucleotide in the support strand prior to the commencement of step (6).

Deprotection of the reversible terminator group in the next cycle may be performed as described above with respect to the first synthesis cycle.

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

Synthesis Method Version 2.

In a second exemplary version of the synthesis method of the invention a new nucleotide is incorporated into a double-stranded scaffold polynucleotide opposite a complementary nucleotide positioned in the support strand (steps 2 and 3 of Figure 2). In each cycle of synthesis the scaffold polynucleotide is cleaved at a cleavage site defined by a sequence comprising the universal nucleotide (step 3 of Figure 2). A single-nucleotide overhang comprising the newly-incorporated nucleotide is generated in the cleaved scaffold polynucleotide (see structure depicted in the middle of the lower part of Figure 2). Ligation of a ligation polynucleotide to the cleaved scaffold polynucleotide incorporates a partner nucleotide into the scaffold polynucleotide and allows the newly-incorporated

nucleotide to pair with the partner nucleotide, thus completing a complete synthesis cycle. Ligation of the ligation polynucleotide (see structure depicted at the far left of the lower part of Figure 2) to the cleaved scaffold polynucleotide also incorporates into the scaffold polynucleotide a nucleotide which is capable of pairing with the next nucleotide to be incorporated in the next cycle (step 4 of Figure 2).

In the second exemplary version of the synthesis method of the invention a scaffold polynucleotide is provided in step (1) as described above. In this method a nucleotide which is capable of pairing with the first nucleotide of step (2) is provided in the support strand of the scaffold polynucleotide and is positioned opposite the terminal nucleotide of the helper strand at the single-strand break site, and is paired therewith. The complementary nucleotide is positioned in the support strand in at position "n" with respect to the incorporated first nucleotide in the synthesis strand, as depicted in step 1 of Figure 2.

In step (2) the first nucleotide of the predefined sequence is incorporated opposite the complementary nucleotide such that the complementary nucleotide pairs with the first nucleotide upon its incorporation.

In step (1) of the second version of the synthesis method the scaffold polynucleotide is also provided with a universal nucleotide in the support strand. In this method the universal nucleotide is positioned (position "n+1") in the support strand opposite to and paired with the penultimate nucleotide in the helper strand at the single-strand break site, i.e. typically at the 5' terminus of the helper strand (see structure depicted in step 1 of Figure 2).

During extension, polymerase will act to "invade" the helper strand and displace the terminal nucleotide of the helper strand and the incorporated first nucleotide will occupy the position previously occupied by the displaced terminal nucleotide of the helper strand. Following incorporation of the first nucleotide, the universal nucleotide will be positioned in the support strand at position "n+1" with respect to the first nucleotide in the synthesis strand at the single-stranded break site, as depicted in the structure of step 3 of Figure 2.

In step (3) of the method the scaffold polynucleotide is cleaved at a cleavage site. The cleavage site is defined by a sequence comprising the universal nucleotide in the support strand. Cleavage comprises cleaving the support strand to provide in the synthesis strand an overhanging end comprising the first nucleotide of the predefined nucleotide sequence. Cleavage results in a double-stranded break in the scaffold polynucleotide. The synthesis strand is already provided with a single-stranded break or "nick", thus only cleavage of the support strand is necessary to provide a double-stranded break in the scaffold polynucleotide.

In this exemplary method version cleavage generates an overhang in the synthesis strand which overhangs the support strand. The overhanging end of the synthesis strand comprises only a single unhybridized nucleotide which is the incorporated first nucleotide. Typically the overhanging first nucleotide will define a 3' terminus of the synthesis strand overhanging the 5' terminus of the support strand in the cleaved scaffold polynucleotide, as depicted in the structure shown in the middle of the lower part of Figure 2.

In this method the universal nucleotide occupies position "n+1" in the support strand. To obtain such a single-nucleotide overhang when the universal nucleotide occupies the "n+1" position in the support strand, the support strand is cleaved in step 3 between nucleotide positions "n" and "n-1". .

By "n" in exemplary method version 2 it is meant the nucleotide position in the support strand which is the next nucleotide position in the support strand relative to the position which is occupied by the universal nucleotide, in the direction distal to the helper strand. Thus at the cleavage step position "n" in the support strand is opposite the position occupied by the nucleotide of the predefined sequence incorporated in that cycle, i.e. the terminal nucleotide of the primer strand portion of the synthesis strand. By "n-1" it is meant the second nucleotide position in the support strand relative to the position which is occupied by the universal nucleotide, in the direction distal to the helper strand (nucleotide labelled "z" in Figure 2). Thus at the cleavage step position "n-1" in the support strand is opposite the position occupied by the penultimate nucleotide of the primer strand portion of the synthesis strand. In this configuration the universal nucleotide occupies position

"n+1". In this method the universal nucleotide at position "n+1" is opposite the penultimate nucleotide in the helper strand portion of the synthesis strand relative to the nick (as depicted in step 3 of Figure 2).

Upon cleavage of the support strand, the universal nucleotide, helper strand and portion of the support strand which is hybridized to the helper strand are removed from the remaining scaffold polynucleotide (see structure depicted at the far right of the lower part of Figure 2) thus generating a single-nucleotide overhang comprising the first nucleotide of the predefined nucleotide sequence in the synthesis strand overhanging the support strand in the cleaved scaffold polynucleotide (see structure depicted in the middle of the lower part of Figure 2).

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

Thus in method version 2 the support strand is cleaved between nucleotide positions n and n-1.

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

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

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

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

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

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 single-nucleotide overhang described above is formed.

In step (4) of the method a ligation polynucleotide is ligated to the cleaved scaffold polynucleotide. The ligation polynucleotide comprises a support strand and a helper strand. The ligation polynucleotide further comprises a complementary ligation end comprising in the support strand a universal nucleotide and an overhanging nucleotide which is the partner nucleotide for the first nucleotide. The ligation polynucleotide further comprises in the helper strand adjacent the overhang a terminal nucleotide lacking a phosphate group (see structure depicted at the far left of the lower part of Figure 2). The complementary ligation end is configured so that it will compatibly join with the overhanging end of the cleaved scaffold polynucleotide product of step (3) when subjected to suitable ligation conditions. Upon ligation of the support strands the first nucleotide becomes paired with its partner nucleotide.

In this method, the universal nucleotide in the support strand of the ligation polynucleotide is positioned in the complementary ligation end opposite the penultimate nucleotide of the helper strand at the site of the single-stranded break site, and is hybridized thereto. The universal nucleotide in the support strand is positioned in the ligation polynucleotide at position "n+1" with respect to the next nucleotide of the predefined nucleotide sequence to be incorporated into the synthesis strand of step (6), i.e. in the next synthesis cycle as

depicted schematically in Figure 2. In the complementary ligation end of the ligation polynucleotide the penultimate nucleotide of the support strand is a partner nucleotide for the next nucleotide of step (6) and is paired with the terminal nucleotide of the helper strand. The terminal nucleotide of the support strand is a partner nucleotide for the first nucleotide of step (2). The terminal nucleotide of the support strand overhangs the terminal nucleotide of the helper strand.

In the ligation polynucleotide the helper strand is provided such that the terminal nucleotide adjacent the overhang lacks a phosphate group. Typically, as described above, this non-phosphorylated terminal nucleotide of the helper strand will define the 5' terminus of the helper strand.

In step (4), upon ligation of the support strand of the ligation polynucleotide and the support strand of the cleaved scaffold polynucleotide, the first nucleotide in the synthesis strand becomes paired with its partner nucleotide in the support strand.

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

As with method version 1, ligation in method version 2 may typically be performed by enzymes having ligase activity. For example, ligation may be performed with T4 DNA ligase. The use of such enzymes will result in the maintenance of the single-stranded break in the synthesis strand, since the terminal nucleotide of the helper strand cannot act as a substrate for ligase due to the absence of a terminal phosphate group.

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

is positioned in the support strand and is paired with the terminal nucleotide of the helper strand, as depicted in Figure 2 (step 4). As in exemplary method version 1, in exemplary method version 2 at the end of a given synthesis cycle, during cycles of synthesis, the universal nucleotide will occupy position n+1 in the support strand relative to the position occupied by the universal nucleotide in the support strand in the previous cycle. At the same time, at the end of a given synthesis cycle the universal nucleotide will also occupy position n+1 in the support strand relative to the position in the synthesis strand which will be occupied by the next nucleotide of the predefined nucleotide sequence to be incorporated in the next cycle. Thus at the end of a given synthesis cycle a modified scaffold molecule is provided for use in the next synthesis cycle, wherein the universal nucleotide is once again positioned in the support strand to facilitate cleavage of the support strand in the next synthesis cycle.

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). This can be performed as described above for method version 1.

In exemplary method version 2, second and subsequent synthesis cycles may be performed as described above for the first synthesis cycle.

Thus in step (6) the scaffold polynucleotide provided for the next synthesis cycle is the product of the ligation step (4) and deprotection step, e.g. step (5) of the first synthesis cycle. In step (6) the next nucleotide in the predefined nucleotide sequence is incorporated into the synthesis strand of the scaffold polynucleotide by the action of polymerase, as described above for step (2) of the first cycle. The next nucleotide also comprises a reversible terminator group which prevents further extension in that cycle by polymerase.

As in step (2) of the first synthesis cycle of exemplary method version 2, in step (6) the next nucleotide of the predefined nucleotide sequence is incorporated opposite its partner nucleotide which is positioned in the support strand such that it pairs with the next nucleotide upon its incorporation. In this configuration the universal nucleotide is positioned at position "n+1" with respect to the incorporated next nucleotide in the

synthesis strand. Furthermore, as described above for the first synthesis cycle, in step (6) of the next synthesis cycle the universal nucleotide will also occupy position "n+1" in the support strand relative to the position occupied by the universal nucleotide in the support strand in step (2) of the previous cycle. This is achieved because in the ligation polynucleotide of the previous synthesis cycle the universal nucleotide was positioned to be opposite to and paired with the penultimate nucleotide of the helper strand.

In step (7) the scaffold polynucleotide is cleaved at a cleavage site, the site defined by a sequence comprising the universal nucleotide in the support strand. Cleavage comprises cleaving the support strand and removing the universal nucleotide to provide in the synthesis strand a single-nucleotide overhanging end comprising the next nucleotide as the terminal nucleotide of the overhang in the remaining scaffold polynucleotide. The single-nucleotide overhang of the synthesis strand overhangs the terminal nucleotide of the support strand in the remaining cleaved scaffold polynucleotide. The cleavage steps may be performed as described above for step (3) of the first cycle.

In step (8) of the next cycle a double-stranded ligation polynucleotide is ligated to the cleaved scaffold polynucleotide. The ligation polynucleotide comprises a support strand and a helper strand. The ligation polynucleotide further comprises a complementary ligation end comprising in the support strand a universal nucleotide and an overhanging nucleotide which is a partner nucleotide for the next nucleotide of the predefined nucleotide sequence. The ligation polynucleotide further comprises in the helper strand adjacent the overhang a terminal nucleotide lacking a phosphate group. The complementary ligation end is configured so that it will compatibly join with the overhanging end of the cleaved scaffold polynucleotide product of step (7) when subjected to suitable ligation conditions. Upon ligation of the support strands the next nucleotide of the predefined nucleotide sequence becomes paired with its partner nucleotide.

The ligation polynucleotide of step (8) of the next and subsequent synthesis cycles may be configured, and the ligation step may be performed, as described above for step (4) of the first synthesis cycle.

Thus in step (8) upon ligation the universal nucleotide in the support strand is positioned opposite the penultimate nucleotide of the helper strand, and is paired therewith. The universal nucleotide in the support strand is positioned at position "n+1" with respect to the next nucleotide to be incorporated in the next cycle. Furthermore, as described above, following step (8) the universal nucleotide will occupy position "n+1" in the support strand relative to the position occupied by the universal nucleotide in the support strand prior to the commencement of step (6).

Deprotection of the reversible terminator group in the next cycle may be performed as described above with respect to the first synthesis cycle.

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

Synthesis Method Version 3.

In a third exemplary version of the synthesis method of the invention a new nucleotide is incorporated into a double-stranded scaffold polynucleotide opposite a universal nucleotide positioned in the support strand (steps 2 and 3 of Figure 3). In each cycle of synthesis the scaffold polynucleotide is cleaved at a cleavage site defined by a sequence comprising the universal nucleotide (step 3 of Figure 3). A double-nucleotide overhang comprising the newly-incorporated nucleotide is generated in the cleaved scaffold polynucleotide (see structure depicted in the middle of the lower part of Figure 3). Ligation of a ligation polynucleotide (see structure depicted at the far left of the lower part of Figure 3) to the cleaved scaffold polynucleotide incorporates a partner nucleotide into the scaffold polynucleotide and thus allows the newly-incorporated nucleotide to pair with the partner nucleotide (step 4 of Figure 3), thus completing a complete synthesis cycle.

In the third exemplary version of the synthesis method of the invention a scaffold polynucleotide is provided in step (1) as described above. In this method the universal nucleotide in the support strand of the scaffold polynucleotide is positioned opposite the

terminal nucleotide of the helper strand at the single-strand break site, and is paired therewith (see structure depicted in step 1 of Figure 3).

In step (2) the first nucleotide is incorporated opposite a universal nucleotide which is positioned in the support strand such that it pairs with the first nucleotide upon its incorporation. In this configuration the universal nucleotide is positioned at position "n" with respect to the incorporated first nucleotide in the synthesis strand, as depicted in Figure 3 (step 3).

During extension, polymerase will act to "invade" the helper strand and displace the terminal nucleotide of the helper strand. The incorporated first nucleotide will occupy the position previously occupied by the displaced terminal nucleotide of the helper strand (step 3 of Figure 3).

In step (3) of the method the scaffold polynucleotide is cleaved at a cleavage site. The cleavage site is defined by a sequence comprising the universal nucleotide in the support strand. Cleavage comprises cleaving the support strand to provide in the synthesis strand an overhanging end comprising the first nucleotide. Cleavage results in a double-stranded break in the scaffold polynucleotide. The synthesis strand is already provided with a single-stranded break or "nick", thus only cleavage of the support strand is necessary to provide a double-stranded break in the scaffold polynucleotide.

In this exemplary method version cleavage generates an overhang in the synthesis strand which overhangs the support strand. The overhanging end of the synthesis strand comprises two unhybridized nucleotides. The first overhanging unhybridized nucleotide is the terminal nucleotide of the synthesis strand of the cleaved scaffold polynucleotide and is the incorporated first nucleotide of the predefined nucleotide sequence. The second unhybridized nucleotide is the nucleotide next to the first nucleotide in the synthesis strand. Typically the overhanging first nucleotide will define a 3' terminus of the synthesis strand overhanging the 5' terminus of the support strand in the cleaved scaffold polynucleotide, as (see structure depicted in the middle of the lower part of Figure 3).

In this method the universal nucleotide occupies position "n" in the support strand. To obtain such a double-nucleotide overhang when the universal nucleotide occupies position "n" in the support strand, the support strand is cleaved between positions "n-1" and "n-2".

By "n" it is meant the nucleotide position in the support strand which is occupied by the universal nucleotide paired with the nucleotide of the predefined sequence incorporated in that given cycle. Thus at the cleavage step position "n" in the support strand is opposite the position occupied by the nucleotide of the predefined sequence incorporated in that given cycle, i.e. the terminal nucleotide of the primer strand portion of the synthesis strand. By "n-1" it is meant the next nucleotide position in the support strand relative to the position which is occupied by the universal nucleotide, in the direction distal to the helper strand. Thus at the cleavage step position "n-1" in the support strand is opposite the position occupied by the penultimate nucleotide of the primer strand portion of the synthesis strand. By "n-2" it is meant the second nucleotide position in the support strand relative to the position which is occupied by the universal nucleotide, in the direction distal to the helper strand. (as depicted in step 3 of Figure 3).

Thus upon cleavage of the support strand, the universal nucleotide, helper strand and portion of the support strand which is hybridized to the helper strand are removed from the remaining scaffold polynucleotide (see structure depicted at the far right of the lower part of Figure 3) thus generating the double-nucleotide overhang comprising the first nucleotide in the synthesis strand overhanging the remaining support strand.

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

Thus the support strand is cleaved between nucleotide positions n-1 and n-2.

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

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

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

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

One mechanism of cleaving the support strand at a cleavage site defined by a sequence comprising a universal nucleotide occupying position n in the support strand in order to generate a double-nucleotide overhang is described in Example 7. The mechanism described is exemplary and other mechanisms could be employed, provided that the double-nucleotide overhang described above is achieved.

In this exemplified 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 single-nucleotide overhang described above is formed.

In step (4) of the method a double-stranded ligation polynucleotide is ligated to the cleaved scaffold polynucleotide. The ligation polynucleotide comprises a support strand and a helper strand. The ligation polynucleotide further comprises a complementary ligation end comprising in the support strand a universal nucleotide and an overhanging nucleotide which is the partner nucleotide for the first nucleotide. The ligation polynucleotide further comprises in the helper strand adjacent the overhang a terminal nucleotide lacking a phosphate group (see structure depicted at the far left of the lower part of Figure 3). The

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

In this method, the universal nucleotide in the support strand of the ligation polynucleotide is positioned in the complementary ligation end opposite the terminal nucleotide of the helper strand at the site of the single-strand break, and is paired therewith. The universal nucleotide in the support strand of the ligation polynucleotide is positioned at position "n" with respect to the next nucleotide of the predefined nucleotide sequence to be incorporated into the synthesis strand of step (6), i.e. in the next synthesis cycle, as depicted schematically in Figure 3. In the complementary ligation end of the ligation polynucleotide the penultimate nucleotide of the support strand is a partner nucleotide for the first nucleotide of step (2) and overhangs the terminal nucleotide of the helper strand.

In the ligation polynucleotide the helper strand is provided such that the terminal nucleotide adjacent the overhang lacks a phosphate group. Typically, as described above, this non-phosphorylated terminal nucleotide of the helper strand will define the 5' terminus of the helper strand.

In step (4), upon ligation of the support strand of the ligation polynucleotide and the support strand of the cleaved scaffold polynucleotide, the first nucleotide of the predefined nucleotide sequence in the synthesis strand becomes paired with its partner nucleotide in the support strand.

Ligation may typically be performed by enzymes having ligase activity. For example, ligation may be performed with T4 DNA ligase. The use of such enzymes will result in the maintenance of the single-stranded break in the synthesis strand, since the terminal nucleotide of the helper strand cannot act as a substrate for ligase due to the absence of a terminal phosphate group.

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

As with method versions 1 and 2, ligation in method version 3 may typically be performed by enzymes having ligase activity. For example, ligation may be performed with T4 DNA ligase. The use of ligase enzymes will result in the maintenance of the single-stranded break in the synthesis strand, since the terminal nucleotide of the helper strand cannot act as a substrate for ligase due to the absence of a terminal phosphate group.

Ligation of the ligation polynucleotide to the cleaved scaffold polynucleotide completes a first synthesis cycle whereupon the scaffold polynucleotide of step (1) is effectively re-constituted except that the first nucleotide of the predefined nucleotide sequence is incorporated into the polynucleotide opposite its partner nucleotide, as depicted in Figure 3. As in exemplary method versions 1 and 2, in exemplary method version 3 at the end of a given synthesis cycle, during cycles of synthesis, the universal nucleotide will occupy position n+1 in the support strand relative to the position occupied by the universal nucleotide in the support strand in the previous cycle. At the same time, and as in exemplary method version 1, at the end of a given synthesis cycle the universal nucleotide will also occupy position n in the support strand relative to the position in the synthesis strand which will be occupied by the next nucleotide of the predefined nucleotide sequence to be incorporated in the next cycle. Thus at the end of a given synthesis cycle a modified scaffold molecule is provided for use in the next synthesis cycle, wherein the universal nucleotide is once again positioned in the support strand to facilitate incorporation of the next nucleotide of the predefined nucleotide sequence and cleavage of the support strand in the next synthesis cycle.

To allow the next nucleotide of the predefined nucleotide sequence to be incorporated in the next synthesis cycle, the reversible terminator group must be removed from the first nucleotide (deprotection step). This can be performed as described above for method version 1.

In exemplary method version 3, second and subsequent synthesis cycles may be performed as described above for the first synthesis cycle.

Thus in step (6) the scaffold polynucleotide provided for the next synthesis cycle is the product of the ligation step (4) and deprotection step, e.g. step (5) of the first synthesis cycle. In step (6) the next nucleotide in the predefined nucleotide sequence is incorporated into the synthesis strand of the scaffold polynucleotide by the action of polymerase, as described above for step (2) of the first cycle. The next nucleotide also comprises a reversible terminator group which prevents further extension in that cycle by polymerase.

As in step (2) of the first synthesis cycle of exemplary method version 3, in step (6) the next nucleotide is incorporated opposite a universal nucleotide which is positioned in the support strand such that it pairs with the next nucleotide upon its incorporation. In this configuration the universal nucleotide is again positioned at position "n" with respect to the incorporated next nucleotide in the synthesis strand. Furthermore, as described above for the first synthesis cycle, in step (6) of the next synthesis cycle the universal nucleotide will occupy position "n+1" in the support strand relative to the position occupied by the universal nucleotide in the support strand in step (2) of the previous cycle. This is achieved because in the ligation polynucleotide of the previous synthesis cycle the universal nucleotide was positioned to be opposite to and paired with the terminal non-phosphorylated nucleotide of the helper strand.

In step (7) the scaffold polynucleotide is cleaved at a cleavage site, the site defined by a sequence comprising the universal nucleotide in the support strand. Cleavage comprises cleaving the support strand and removing the universal nucleotide to provide in the synthesis strand a double-nucleotide overhanging end comprising the next nucleotide as the terminal nucleotide of the overhang in the remaining scaffold polynucleotide. The double-nucleotide overhang of the synthesis strand overhangs the terminal nucleotide of the support strand in the remaining cleaved scaffold polynucleotide. The cleavage steps may be performed as described above for step (3) of the first cycle.

In step (8) of the next cycle a double-stranded ligation polynucleotide is ligated to the cleaved scaffold polynucleotide. The ligation polynucleotide comprises a support strand and a helper strand. The ligation polynucleotide further comprises a complementary ligation end comprising in the support strand a universal nucleotide and an overhanging nucleotide which is the partner nucleotide for the next nucleotide of the predefined nucleotide sequence. The ligation polynucleotide further comprises in the helper strand adjacent the overhang a terminal nucleotide lacking a phosphate group. The complementary ligation end is configured so that it will compatibly join with the overhanging end of the cleaved scaffold polynucleotide product of step (7) when subjected to suitable ligation conditions. Upon ligation of the support strands the next nucleotide of the predefined nucleotide sequence becomes paired with its partner nucleotide.

The ligation polynucleotide of step (8) of the next and subsequent synthesis cycles may be configured, and the ligation step may be performed, as described above for step (4) of the first synthesis cycle.

Thus in step (8) upon ligation the universal nucleotide in the support strand is positioned opposite the terminal nucleotide of the helper strand, and is paired therewith. The universal nucleotide in the support strand is positioned at position "n" with respect to the next nucleotide, as described above with respect to the first synthesis cycle. Furthermore, as described above, following step (8) the universal nucleotide will occupy position "n+1" in the support strand relative to the position occupied by the universal nucleotide in the support strand prior to the commencement of step (6).

Deprotection of the reversible terminator group in the next cycle may be performed as described above with respect to the first synthesis cycle.

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

Synthesis strand

In methods of the invention including, but not limited to, method versions 1, 2 and 3 as described above, the scaffold polynucleotide is provided with a synthesis strand. During cycles of synthesis each new nucleotide of the predefined sequence is incorporated into the synthesis strand. A polymerase enzyme can be used to catalyse incorporation of each new nucleotide, nucleotide analogue/derivative or non-nucleotide. The synthesis strand comprises a primer strand portion and preferably comprises a helper strand portion.

Helper strand

A helper strand may be provided in the scaffold polynucleotide to 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 synthesis strand is provided with a helper strand.

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 cleavage enzyme(s) at the cleavage step.

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

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

The length of the helper strand may be 10 bases or more. Optionally, the length of the helper strand may be 15 bases or more, preferably 30 bases or more. However, the length

of the helper strand may be varied, provided that the helper strand is capable of facilitating cleavage and/or ligation.

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

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

Primer strand

The primer strand should be of sufficient length and should possess a sequence and structure such that it is suitable to allow a polymerase enzyme to initiate synthesis, i.e. catalyse the incorporation of a new nucleotide at the terminal end of the primer strand at the site of the nick.

The primer strand may comprise a region of sequence which can act to prime new polynucleotide synthesis (e.g. as shown by the dotted line in the structures depicted in each

of Figures 1 to 3). 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.

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.

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

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

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

The primer strand must be hybridized to the corresponding region of the support strand. It is not essential that the entirety of the primer strand is hybridized to the corresponding region of the support strand, provided that the primer strand is capable of 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.

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

Support strand

In methods of the invention including, but not limited to, method versions 1, 2 and 3, 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 and, if included, the helper strand portion of the synthesis strand, as described above.

RNA synthesis

Methods described for DNA synthesis may be adapted for the synthesis of RNA. In one adaptation the synthesis steps described for method versions 1-3 may be adapted. Thus in each of method versions 1-3 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, is preferably an RNA strand. The helper strand, if present, may be a DNA strand.

Nucleotides may be incorporated from 2'-ribonucleoside-5'-*O*-triphosphates (NTPs) which may be modified to comprise a reversible terminator group, as described above. Preferably 3'-*O*-modified-2'-ribonucleoside-5'-*O*-triphosphates are used. Modified nucleotides are incorporated by the action of RNA polymerase.

Thus the above descriptions relating to method versions 1-3 may be applied mutatis mutandis for RNA synthesis but adapted as described. Exemplary adapted reaction schemes relating to method versions 1 and 2 are shown in Figures 23 to 25. Method version 3 can be adapted in the same way. In any of the adapted methods for RNA

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

The following Examples illustrate the invention.

Example 1. Absence of Helper Strand.

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.

Step1: Incorporation

The first step describes controlled addition of 3'-*O*-protected single nucleotide to oligonucleotide by enzymatic incorporation by DNA polymerase (Figure 5a).

Materials and Methods

Materials

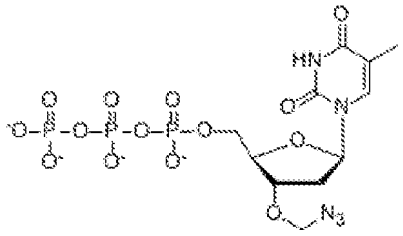
1. 3'-*O*-modified dNTPs were synthesized in-house according to the protocol described in PhD thesis Jian Wu: Molecular Engineering of Novel Nucleotide Analogues for DNA Sequencing by Synthesis, Columbia University, 2008. The protocol for synthesis is also described in patent: J. William Efcavitch, Juliesta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

2. Oligonucleotides were designed in house and obtained from Sigma-Aldrich (Figure 5h). The stock solutions were prepared at a concentration of 100 µM.

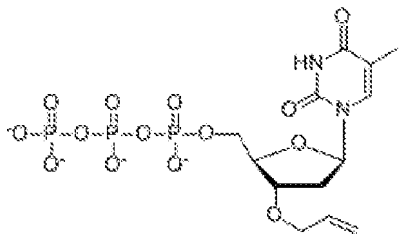
3. Therminator IX DNA polymerase was used that has been engineered by New England BioLabs with enhanced ability to incorporate 3-*O*-modified dNTPs. However, any DNA polymerase that could incorporate modified dNTPs could be used.

Two types of reversible terminators were tested:

3'-*O*-azidomethyl-dTTP:



3'-*O*-allyl-dTTP:



Methods

1. 2 µl of 10x Thermopol® buffer (20 mM Tris-HCl, 10 mM $(NH_4)2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton® X-100, pH 8.8, New England BioLabs) was mixed with 12.25 µl of sterile deionized water (ELGA VEOLIA) in 1.5ml Eppendorf tube.

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

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

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

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

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

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

Gel Electrophoresis and DNA Visualization:

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

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

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

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

Results

Customised engineered 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 (Figure 5b-c).

Efficient incorporation opposite inosine occurred at a temperature of 65°C (Figure 5d-e).

Incorporation of 3'-*O*-modified-dTTPs opposite inosine requires the presence of Mn^{2+} ions (Figure 5f-g). Successful conversion is marked in bold in Figures 5 c, e, g and h.

Conclusion

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^{2+} ions and at a temperature at 65°C.

Step2: Cleavage

The second step describes a two-step cleavage of polynucleotides with either hAAG/Endo VIII or hAAG/chemical base (Figure 6a).

Materials and Methods

Materials

1. Oligonucleotides utilized in Example 1 were designed in-house and synthesized by Sigma Aldrich (see table in Figure 6(e) for sequences).

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

Methods

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

1. A pipette (Gilson) was used to transfer 41µl sterile distilled water (ELGA VEOLIA) into a 1.5ml Eppendorf tube.

2. 5µl of 10X ThermoPol® reaction buffer NEB (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton® X-100, pH 8.8) were then added into the same Eppendorf tube.

3. 1µl each of oligonucleotides (Figure 6e); template (SEQ ID: No 3) or any fluorescently tagged long oligo strand, primer with T (SEQ ID: No 4) and control (SEQ ID: No 5) all at 5pmols were added into the same tube.

4. 1µl of Human Alkyladenine DNA Glycosylase (hAAG) NEB (10units/µl) was added into the same tube.

5. Reaction mixture was then gently mixed by resuspension with a pipette, centrifuged at 13,000rpm for 5 seconds and incubated at 37°C for 1 hour.

6. Typically after incubation time had elapsed, reaction was terminated by enzymatic heat inactivation (i.e. 65°C for 20 minutes).

Purification under ambient conditions. The sample mixture was purified using the protocol outlined below:

1. 500 µl of buffer PNI QIAGEN (5M guanidinium chloride) was added to the sample and mixed by gentle resuspension with pipette.

2. The mixture was transferred into a QIAquick spin column (QIAGEN) and centrifuged for 1 min at 6000 rpm.

3. After centrifugation, flow-through was discarded and 750 µl of buffer PE QIAGEN (10mM Tris-HCl pH 7.5 and 80% ethanol) was added into the spin column and centrifuged for 1min at 6000 rpm.

4. The flow-through was discarded and the spin column was centrifuged for an additional 1 min at 13000 rpm to remove residual PE buffer.

5. The spin column was then placed in a sterile 1.5ml Eppendorf tube.

6. For DNA elution, 50 µl of Buffer EB QIAGEN (10mM Tris.CL, pH 8.5) was added to the centre of the column membrane and left to stand for 1 min at room temperature.

7. The tube was then centrifuged at 13000 rpm for 1 minutes. Eluted DNA concentration was measured and stored at -20°C for subsequent use.

Measurement of purified DNA concentration was determined using the protocol below:

1. NanoDrop one (Thermo Scientific) was equilibrated by adding 2 µl of sterile distilled water (ELGA VEOLIA) onto the pedestal.

2. After equilibration, the water was gently wiped off using a lint-free lens cleaning tissue (Whatman).

3. NanoDrop one was blanked by adding 2 µl of Buffer EB QIAGEN (10mM Tris.CL, pH 8.5). Then step 2 was repeated after blanking.

4. DNA concentration was measured by adding 2 µl of the sample onto the pedestal and selecting the measure icon on the touch screen.

Cleavage of the generated abasic site was carried out using the procedure below:

1. 2 µl (10-100ng/µl) DNA was added into a sterile 1.5ml Eppendorf tube.

2. 40 µl (0.2M) NaOH or 1.5 µl Endo VIII NEB (10units/µl) and 5 µl 10X Reaction Buffer NEB (10 mM Tris-HCl, 75 mM NaCl, 1 mM EDTA , pH 8 @ 25°C) was also added into the same tube and gently mixed by resuspension and centrifugation at 13000 rpm for 5 sec.

3. The resulting mixture was incubated at room temperature for 5 minutes for the NaOH treated sample while Endo VIII reaction mixture was incubated at 37°C for 1hr.

4. After incubation time had elapsed, the reaction mixture was purified using steps 1-7 of purification protocol as outlined above.

Gel Electrophoresis and DNA Visualization:

1. 5 µl of DNA and TBE-Urea sample buffer (Novex) was added into a sterile 1.5 ml Eppendorf tube and heated to 95°C for 2 minutes using a heat thermoblock (Eppendorf).

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

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

4. Detection and visualization of DNA in the gel was carried out with ChemiDoc MP (BioRad) using Cy3 LEDS. Visualization and analysis was carried out on the Image lab 2.0 platform.

Results and Conclusion

The cleavage reaction without a helper strand showed a low percentage yield of cleaved to uncleaved DNA ratio of ~7% : 93% (Figure 6b-d).

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 chemical base for cleavage of abasic site was less time-consuming compared to EndoVIII cleavage.

Step 3: Ligation

The third step describes ligation of polynucleotides with DNA ligase in the absence of a helper strand. A diagrammatic illustration is shown in Figure 7.

Materials and Methods

Materials

1. Oligonucleotides utilized in Example 1 were designed in-house and synthesized by Sigma Aldrich (see table in Figure 7c for sequences).

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

Methods

Ligation reaction on oligonucleotides was carried out using the procedure below:

1. A pipette (Gilson) was used to transfer 16µl sterile distilled water (ELGA VEOLIA) into a 1.5ml Eppendorf tube.

2. 10µl of 2X Quick Ligation Reaction buffer NEB (132 mM Tris-HCl, 20mM $MgCl_2$, 2mM dithiothreitol, 2mM ATP, 15% Polyethylene glycol (PEG6000) and pH 7.6 at 25°C) was then added into the same Eppendorf tube.

3. 1µl each of oligonucleotides (Figure 7c); 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.

4. 1µl of Quick T4 DNA Ligase NEB (400units/µl) was added into the same tube.

5. The reaction mixture was then gently mixed by resuspension with a pipette, centrifuged at 13,000rpm for 5 seconds and incubated at room temperature for 20 minutes.

6. Typically after incubation time had elapsed, reaction was terminated with the addition of TBE-Urea sample Buffer (Novex).

7. The reaction mixture was purified using protocol outlined in purification steps 1-7 as described above.

Measurement of purified DNA concentration was determined using the protocol below:

1. NanoDrop one (Thermo Scientific) was equilibrated by adding 2 µl of sterile distilled water (ELGA VEOLIA) onto the pedestal.

2. After equilibration, the water was gently wiped off using a lint-free lens cleaning tissue (Whatman).

3. NanoDrop one was blanked by adding 2 µl of Buffer EB QIAGEN (10mM Tris.CL, pH 8.5), then step 2 was repeated after blanking.

4. DNA concentration was measured by adding 2 µl of the sample onto the pedestal and selecting the measure icon on the touch screen.

5. Purified DNA was run on a polyacrylamide gel and visualized in accordance with the procedure in steps 5-8 described above. No change in conditions or reagents was introduced.

Results and Conclusion

In this specific example, and based on the specific reagents used, ligation of oligonucleotides with DNA ligase, in this particular case quick T4 DNA ligase, at room temperature (24°C) in the absence of a helper strand results in a reduced amount of ligation product (Figure 7b).

Example 2. Version 1 Chemistry with Helper Strand.

This example describes the synthesis of polynucleotides using 4 steps: incorporation of 3'-*O*-modified dNTPs from a nick site, cleavage, ligation and deprotection, with the first step taking place opposite a universal nucleotide, in this particular case inosine. The method uses a helper strand which improves the efficiency of the ligation and cleavage steps.

Step1: Incorporation

The first step describes controlled addition of 3'-*O*-protected single nucleotide to oligonucleotide by enzymatic incorporation using DNA polymerase (Figure 8a).

Materials and Methods

Materials

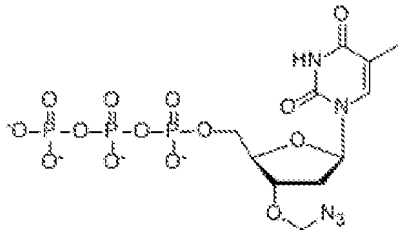
1. 3'-*O*-modified dNTPs were synthesized in-house according to the protocol described in PhD thesis Jian Wu: Molecular Engineering of Novel Nucleotide Analogues for DNA Sequencing by Synthesis. Columbia University, 2008. The protocol for synthesis is also described in patent: J. William Efcavitch, Juliesta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

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 Figure 8b.

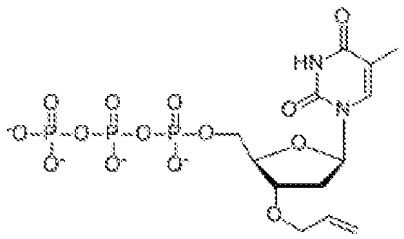
3. Therminator IX DNA polymerase was used that has been engineered by New England BioLabs with enhanced ability to incorporate 3-*O*-modified dNTPs.

Two types of reversible terminators were tested:

3'-*O*-azidomethyl-dTTP:



3'-*O*-allyl-dTTP:



Methods

1. 2 µl of 10x Thermopol® buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton® X-100, pH 8.8, New England BioLabs) was mixed with 10.25 µl of sterile deionized water (ELGA VEOLIA) in 1.5ml Eppendorf tube.

2. 0.5 µl of 10 µM primer (5 pmol, 1 equiv) (SEQ ID: No 10, Table in Figure 8(b)), 0.75 µl of 10 µM template (6 pmol, 1.5equiv) (SEQ ID: No 11, Table in Figure 8(b)), 2 µl of 10 µM of helper strand (SEQ ID: No 12, Table in Figure 8(b)) were added to the reaction mixture.

3. 3'-*O*-modified-dTTP (2 µl of 100 µM) and $MnCl_2$ (1 µl of 40 mM) were added.

4. 1.5 µl of Therminator IX DNA polymerase (15 U, New England BioLabs) was then added.

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

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

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

Gel Electrophoresis and DNA Visualization:

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

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

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

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

The incorporation step can be studied according to the protocol described above.

Step 2: Cleavage

The second step describes a two-step cleavage of polynucleotides with either hAAG/Endo VIII or hAAG/chemical base (x2) (Figure 9a).

Materials and Methods

Materials

1. Oligonucleotides utilized in Example 2 were designed in-house and synthesized by Sigma Aldrich (see Figure 9f for sequences).

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

Methods

Cleavage reaction on oligonucleotides was carried out using the procedure below:

1. A pipette (Gilson) was used to transfer 41µl sterile distilled water (ELGA VEOLIA) into a 1.5 ml Eppendorf tube.

2. 5µl of 10X ThermoPol® Reaction buffer NEB (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton® X-100, pH 8.8) was then added into the same Eppendorf tube.

3. 1µl each of oligonucleotides (Figure 9f); 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.

4. 1µl of Human Alkyladenine DNA Glycosylase (hAAG) NEB (10 units/µl) was added into the same tube.

5. In the reaction using alternative base, 1µl of Human Alkyladenine DNA Glycosylase (hAAG) NEB (100 units/µl) was added.

6. Reaction mixture was then gently mixed by resuspension with a pipette, centrifuged at 13,000 rpm for 5 seconds and incubated at 37°C for 1 hour.

7. Typically after incubation time had elapsed, reaction was terminated by enzymatic heat inactivation (i.e. 65°C for 20 minutes).

Purification under ambient conditions. The sample mixture was purified using the protocol outlined below:

1. 500 µl of buffer PNI QIAGEN (5M guanidinium chloride) was added to the sample and mixed by gentle resuspension with pipette.

2. The mixture was transferred into a QIAquick spin column (QIAGEN) and centrifuged for 1 min at 6000 rpm.

3. After centrifugation, flow-through was discarded and 750 µl of buffer PE QIAGEN (10mM Tris-HCl pH 7.5 and 80% ethanol) was added into the spin column and centrifuged for 1 min at 6000 rpm.

4. The flow-through was discarded and the spin column was centrifuged for an additional 1 min at 13000 rpm to remove residual PE buffer.

5. The spin column was then placed in a sterile 1.5 ml Eppendorf tube.

6. For DNA elution, 50 µl of Buffer EB QIAGEN (10mM Tris.CL, pH 8.5) was added to the centre of the column membrane and left to stand for 1min at room temperature.

7. The tube was then centrifuged at 13000 rpm for 1 minutes. Eluted DNA concentration was measured and stored at -20°C for subsequent use.

Measurement of purified DNA concentration was determined using the protocol below:

1. NanoDrop one (Thermo Scientific) was equilibrated by adding 2 µl of sterile distilled water (ELGA VEOLIA) onto the pedestal.

2. After equilibration, the water was gently wiped off using a lint-free lens cleaning tissue (Whatman).

3. NanoDrop one was blanked by adding 2 µl of Buffer EB QIAGEN (10mM Tris.CL, pH 8.5). Then step 2 was repeated after blanking.

4. DNA concentration was measured by adding 2 µl of the sample onto the pedestal and selecting the measure icon on the touch screen.

Cleavage of generated abasic site was carried out using the procedure below:

1. 2 µl (10-100ng/µl) DNA was added into a sterile 1.5ml Eppendorf tube.

2. 40 µl (0.2M) NaOH or 1.5 µl Endo VIII NEB (10units/µl) and 5 µl 10X Reaction Buffer NEB (10 mM Tris-HCl, 75 mM NaCl, 1 mM EDTA , pH 8 @ 25°C) was also added into the same tube and gently mixed by resuspension and centrifugation at 13000 rpm for 5 sec.

3. Resulting mixture was incubated at room temperature for 5 minutes for the 0.2 M NaOH treated sample while Endo VIII reaction mixture was incubated at 37°C for 1hr.

4. After incubation time had elapsed, the reaction mixture was purified using steps 1-7 of purification protocol as stated above.

Cleavage of generated abasic site using alternative basic chemical was carried out using the procedure below:

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 (100mM) which had been buffered at room temperature with acetic acid solution sigma (99.8%) to pH 7.4 was then added into the same tube.

2. 20 µl of sterile distilled water (ELGA VEOLIA) was added into the tube and gently mixed by resuspension and centrifugation at 13000 rpm for 5 sec.

3. The resulting mixture was incubated at 37°C for 20 minutes.

4. After incubation time had elapsed, the reaction mixture was purified using steps 1-7 of the purification protocol stated above.

Gel Electrophoresis and DNA Visualization:

1. 5 µl of DNA and TBE-Urea sample buffer (Novex) was added into a sterile 1.5 ml Eppendorf tube and heated to 95°C for 2 minutes using a heat thermoblock (Eppendorf).

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

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

4. Detection and visualization of DNA in the gel was carried out with ChemiDoc MP (BioRad) using Cy3 LEDS. Visualization and analysis was carried out on the Image lab 2.0 platform.

Results

Cleavage efficiency at a cleavage site comprising a universal nucleotide, in this particular case inosine, by hAAG DNA glycosylase was significantly increased from 10% in absence of helper strand to 50% in presence of helper strand (Figure 9b). hAAG and Endonuclease VIII cleave inosine with lower efficiency (10%) than hAAG and NaOH (50%). Chemical cleavage using 0.2M NaOH was shown to be preferable for cleavage of AP sites than Endonuclease VIII in the described system using nicked DNA (Figure 9c). 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 (Figures 9d-e).

Conclusion

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.

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.

Enzymatic cleavage using Endonuclease VIII as a substitute for NaOH was less efficient (10%) compared to NaOH (50%) in the presence of helper strand.

The inclusion of an alternative mild chemical base N,N'-dimethylethylenediamine led to high cleavage efficiency of AP sites, as efficient as for NaOH, and, together with addition of 10x hAAG enzyme, had a significant increase on cleaved DNA (see Figure 9e).

Step 3: Ligation

The third step describes ligation of polynucleotides with DNA ligase in the presence of a helper strand. A diagrammatic illustration is shown in Figure 10a.

Materials and Methods

Materials

1. Oligonucleotides were designed in-house and synthesized by Sigma Aldrich (see Figure 10d for sequences).

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

Methods

Ligation reaction on oligonucleotides was carried out using the procedure below:

1. A pipette (Gilson) was used to transfer 16µl sterile distilled water (ELGA VEOLIA) into a 1.5 ml Eppendorf tube.

2. 10µl of 2X Quick Ligation Reaction buffer NEB (132 mM Tris-HCl, 20mM $MgCl_2$, 2mM dithiothreitol, 2mM ATP, 15% Polyethylene glycol (PEG6000) and pH 7.6 at 25°C) was then added into the same Eppendorf tube.

3. 1µl each of oligonucleotides (Figure 10d); TAMRA or any fluorescently tagged phosphate strand (SEQ ID: No 18), primer with T (SEQ ID: No 19) and inosine strand (SEQ ID: No 20) and helper strand (SEQ ID: No 21), all at of 5 pmols, was added into the same tube.

4. 1µl of Quick T4 DNA Ligase NEB (400 units/µl) was added into the same tube.

5. Reaction mixture was then gently mixed by resuspension with a pipette, centrifuged at 13,000 rpm for 5seconds and incubated at room temperature for 20 minutes.

6. Typically after incubation time had elapsed, reaction was terminated with the addition of TBE-Urea sample Buffer (Novex).

7. Reaction mixture was purified using protocol outlined in purification steps 1-7 as described above.

Measurement of purified DNA concentration was determined using the protocol below:

1. NanoDrop one (Thermo Scientific) was equilibrated by adding 2 µl of sterile distilled water (ELGA VEOLIA) onto the pedestal.

2. After equilibration, the water was gently wiped off using a lint-free lens cleaning tissue (Whatman).

3. NanoDrop one was blanked by adding 2 µl of Buffer EB QIAGEN (10mM Tris.CL, pH 8.5). Then step 2 was repeated after blanking.

4. DNA concentration was measured by adding 2 µl of the sample onto the pedestal and selecting the measure icon on the touch screen.

5. Purified DNA was run on a polyacrylamide gel and visualized in accordance with the procedure in steps 5- 8 above. No change in conditions or reagents was introduced.

Results and Conclusion

In this specific example, and based on the specific reagents used, reduced ligation activity is observed in the absence of a helper strand (Figure 10b), whereas ligation proceeds with high efficiency in presence of a helper strand (Figure 10c) and the product is formed in high yield.

Example 3. Version 2 Chemistry with Helper Strand.

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.

Step1: Incorporation

Materials and Methods

Materials

The first step describes controlled addition of 3'-*O*-protected single nucleotide to oligonucleotide by enzymatic incorporation by DNA polymerase (Figure 11a).

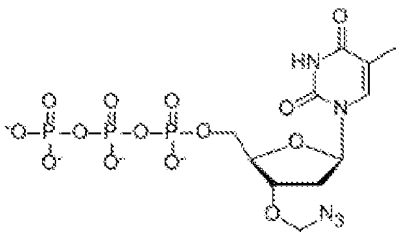
1. 3'-*O*-modified dNTPs were synthesized in-house according to the protocol described in PhD thesis Jian Wu: Molecular Engineering of Novel Nucleotide Analogues for DNA Sequencing by Synthesis. Columbia University, 2008. The protocol for synthesis is also described in patent: J. William Efcavitch, Juliesta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

2. Oligonucleotides were designed in house and obtained from Sigma-Aldrich (Figure 11j). The stock solutions are prepared in concentration of 100 μM.

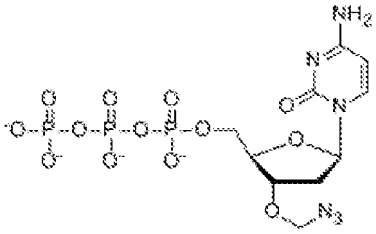
3. Therminator IX DNA polymerase was used that has been engineered by New England BioLabs with enhanced ability to incorporate 3-*O*-modified dNTPs.

3'-*O*-azidomethyl reversible terminators of all dNTPs were tested independently for incorporation:

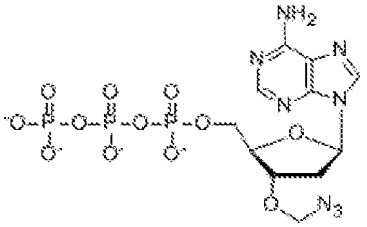
3'-*O*-azidomethyl-dTTP:



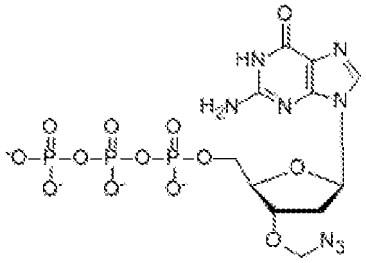
3'-*O*-azidomethyl-dCTP:



3'-*O*-azidomethyl-dATP:



3'-*O*-azidomethyl-dGTP:



Methods

1. 2 µl of 10x Thermopol® buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton® X-100, pH 8.8, New England BioLabs) was mixed with 12.25 µl of sterile deionized water (ELGA VEOLIA) in 1.5ml Eppendorf tube.

2. 0.5 µl of 10 µM primer (5 pmol, 1 equiv) (SEQ ID: No. 22, Figure 11j) and 0.75 µl of 10 µM template-A/G/T/C (6 pmol, 1.5equiv) (SEQ ID: Nos. 23 to 26, Figure 11j) and 1 µl of 10 µM helper strand-T/C/A/G (10 pmol, 2 equiv) (SEQ ID: Nos. 27 to 30, Figure 11j) were added to the reaction mixture.

3. 3'-*O*-modified-dTTP/dCTP/ dATP/dGTP (2 µl of 100 µM) and $MnCl_2$ (1 µl of 40 mM) were added.

4. 1.5 µl of Therminator IX DNA polymerase (15 U, New England BioLabs) was then added.

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

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

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

Gel Electrophoresis and DNA Visualization:

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

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

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

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

Results and Conclusions

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 presence of helper strand for ligation goes to 90% after 5 minutes. 10 % of primer remains unextended after 20 minutes at 37°C and 47°C.

Therminator IX DNA polymerase at 2mM Mn^{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

The second step describes a one-step cleavage of polynucleotides with Endonuclease V (Figure 12a).

Materials and Methods

Materials

1. Oligonucleotides utilized in Example 3 were designed in-house and synthesized by Sigma Aldrich (see table in Figure 12d for sequences).

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

Methods

Cleavage reaction on oligonucleotides was carried out using the procedure below:

1. A pipette (Gilson) was used to transfer 41 µl sterile distilled water (ELGA VEOLIA) into a 1.5 ml Eppendorf tube.

2. 5µl of 10X Reaction buffer® NEB (50 mM Potassium Acetate, 20 mM Tris-acetate, 10 mM Magnesium Acetate, 1 mM DTT, pH 7.9 @ 25°C) was then added into the same Eppendorf tube.

3. 1µl each of oligonucleotides (Figure 12d); Template (SEQ ID: No 31) or any fluorescently tagged long oligo strand, Primer with T (SEQ ID: No 32) and control (SEQ ID: No 33) and helper strand (SEQ ID: No 34), all at 5pmols, were added into the same tube.

4. 1µl of Human Endonuclease V (Endo V) NEB (10 units/µl) was added into the same tube.

5. Reaction mixture was then gently mixed by resuspension with a pipette, centrifuged at 13,000 rpm for 5 seconds and incubated at 37°C for 1hour.

6. Typically after incubation time had elapsed, reaction was terminated by enzymatic heat inactivation (i.e. 65°C for 20 minutes).

The sample mixture was purified using the protocol outlined below:

1. 500 µl of buffer PNI QIAGEN (5M guanidinium chloride) was added to the sample and mixed by gentle resuspension with a pipette.

2. The mixture was transferred into a QIAquick spin column (QIAGEN) and centrifuged for 1 min at 6000 rpm.

3. After centrifugation, flow-through was discarded and 750 µl of buffer PE QIAGEN (10mM Tris-HCl pH 7.5 and 80% ethanol) was added into the spin column and centrifuged for 1 min at 6000 rpm.

4. The flow-through was discarded and the spin column was centrifuged for an additional 1min at 13000 rpm to remove residual PE buffer.

5. The spin column was then placed in a sterile 1.5 ml Eppendorf tube.

6. For DNA elution, 50 µl of Buffer EB QIAGEN (10mM Tris.CL, pH 8.5) was added to the centre of the column membrane and left to stand for 1min at room temperature.

7. The tube was then centrifuged at 13000 rpm for 1 minutes. Eluted DNA concentration was measured and stored at -20°C for subsequent use.

Measurement of purified DNA concentration was determined using the protocol below:

1. NanoDrop one (Thermo Scientific) was equilibrated by adding 2 µl of sterile distilled water (ELGA VEOLIA) onto the pedestal.

2. After equilibration, the water was gently wiped off using a lint-free lens cleaning tissue (Whatman).

3. NanoDrop one was blanked by adding 2 µl of Buffer EB QIAGEN (10mM Tris.CL, pH 8.5). Then step 2 was repeated after blanking.

4. DNA concentration was measured by adding 2 µl of the sample onto the pedestal and selecting the measure icon on the touch screen.

Gel Electrophoresis and DNA Visualization:

1. 5 µl of DNA and TBE-Urea sample buffer (Novex) was added into a sterile 1.5ml Eppendorf tube and heated to 95°C for 2 minutes using a heat thermoblock (Eppendorf).

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

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

4. Detection and visualization of DNA in the gel was carried out with Chemidoc MP (BioRad) using Cy3 LEDS. Visualization and analysis was carried out on the Image lab 2.0 platform.

Results and Conclusions

Cleavage results from Example 3 showed that a significantly high yield of cleaved DNA could be obtained with Endonuclease V in the presence or absence of the helper strand (Figure 12c).

Step 3: Ligation

The third step describes ligation of polynucleotides with DNA ligase in the presence of a helper strand. A diagrammatic illustration is shown in Figure 13a.

Materials and Methods

Materials

1. Oligonucleotides utilized in Example 3 were designed in-house and synthesized by Sigma Aldrich (see table in Figure 13b for sequences).

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

Methods

Ligation reaction on oligonucleotides was carried out using the procedure below

1. A pipette (Gilson) was used to transfer 16 µl sterile distilled water (ELGA VEOLIA) into a 1.5ml Eppendorf tube.

2. 10 µl of 2X Quick Ligation Reaction buffer NEB (132 mM Tris-HCl, 20mM MgC_{l2}, 2mM dithiothreitol, 2mM ATP, 15% Polyethylene glycol (PEG6000) and pH 7.6 at 25°C) was then added into the same Eppendorf tube.

3. 1 µl each of oligonucleotides (Figure 13b); 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.

4. 1 µl of Quick T4 DNA Ligase NEB (400units/µl) was added into the same tube.

5. Reaction mixture was then gently mixed by resuspension with a pipette, centrifuged at 13,000 rpm for 5seconds and incubated at room temperature for 20 minutes.

6. Typically after incubation time had elapsed, reaction was terminated with the addition of TBE-Urea sample Buffer (Novex).

7. The reaction mixture was purified using protocol outlined in purification steps 1-7 as described above.

Measurement of purified DNA concentration was determined using the protocol below:

1. NanoDrop one (Thermo Scientific) was equilibrated by adding 2 µl of sterile distilled water (ELGA VEOLIA) onto the pedestal.

2. After equilibration, the water was gently wiped off using a lint-free lens cleaning tissue (Whatman).

3. NanoDrop one was blanked by adding 2 µl of Buffer EB QIAGEN (10mM Tris.CL, pH 8.5). Then step 2 was repeated after blanking.

4. DNA concentration was measured by adding 2 µl of the sample onto the pedestal and selecting the measure icon on the touch screen.

5. Purified DNA was run on a polyacrylamide gel and visualized in accordance with the procedure in steps 5- 8 described above. No change in conditions or reagents was introduced.

Gel Electrophoresis and DNA Visualization:

1. 5 µl of DNA and TBE-Urea sample buffer (Novex) was added into a sterile 1.5 ml Eppendorf tube and heated to 95°C for 2 minutes using a heat thermoblock (Eppendorf).

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

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

4. Detection and visualization of DNA in the gel was carried out with ChemiDoc MP (BioRad) using Cy3 LEDS. Visualization and analysis was carried out on the Image lab 2.0 platform.

Step 4: Deprotection

Deprotection step (Figure 14a) 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

1. Oligonucleotides utilized in Example 3 were designed in-house and synthesized by Sigma Aldrich (see Figure 14i for sequences).

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

3. Enzymes were purchased from New England BioLabs.

Methods

1. 2 µl of 10x Thermopol® buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 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.

2. 1 µl of 10 µM primer (10 pmol, 1 equiv) (SEQ ID: No 39, Figure 14i) and 1.5 µl of either 10 µM template-A/G/T/C (15 pmol, 1.5equiv) (SEQ ID: Nos: 40 to 43, Figure 14i) were added to the reaction mixture.

3. 3'-*O*-modified-dTTP/dCTP/dATP/dGTP (2 µl of 100 µM) and $MnCl_2$ (1 µl of 40mM) were added

4. 1.5 µl of Therminator IX DNA polymerase (15 U, New England BioLabs) was then added.

5. The reaction was incubated for 5 minutes at 37°C.

6. 4 μL of the sample was taken out and mixed with 0.5 ul of 5mM dNTP mix and let react for 10 minutes for control reaction.

7. 40 μL of the 500 mM TCEP in 1M TRIS buffer pH 7.4 was added to the reaction mixture and let react for 10 minutes at 37°C.

8. The reaction mixture was purified using QIAGEN Nucleotide removal kit eluting by 20 μL of 1x Thermopol® buffer.

9. 1 μL of 5mM dNTP mix and 1 μL of DeepVent (exo-) DNA polymerase were added to the purified reaction mixture and let react 10 minutes.

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

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

Results and Conclusion

50mM TCEP was not sufficient to cleave 3'-*O*-azidomethyl group with high efficiency on 0.2 μM DNA model (Figure 14h). In contrast, 300mM TCEP successfully cleaved 3'-*O*-azidomethyl group with 95% efficiency on 0.2 μM DNA model (Figure 14h).

Example 4. Version 2 Chemistry with Double Hairpin Model.

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.

Step1: Incorporation

The first step describes controlled addition of 3'-*O*-protected single nucleotide to oligonucleotide by enzymatic incorporation by DNA polymerase (Figure 15a).

Materials and Methods

Materials

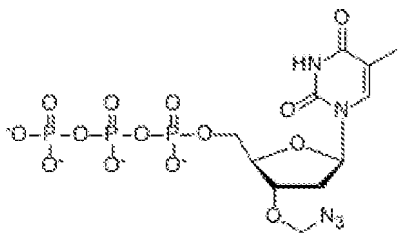
1. 3'-*O*-modified dNTPs were synthesized in-house according to the protocol described in PhD thesis Jian Wu: Molecular Engineering of Novel Nucleotide Analogues for DNA Sequencing by Synthesis. Columbia University, 2008. The protocol for synthesis is also described in patent: J. William Efcavitch, Juliesta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

2. Oligonucleotides were designed in house and obtained from Sigma-Aldrich (Figure 15c). The stock solutions were prepared in concentration of 100 μM.

3. Therminator IX DNA polymerase was used that has been engineered by New England BioLabs with enhanced ability to incorporate 3-*O*-modified dNTPs.

3'-*O*-azidomethyl-dTTP was tested for incorporation:

3'-*O*-azidomethyl-dTTP:



Method

1. 2 µl of 10x Thermopol® buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton® X-100, pH 8.8, New England BioLabs) was mixed with 10.25 µl of sterile deionized water (ELGA VEOLIA) in 1.5ml Eppendorf tube.

2. 0.5 µl of 10 µM hairpin oligonucleotide (5 pmol, 1 equiv) (SEQ ID: No 44, Figure 15c) was added to the reaction mixture.

3. 3'-*O*-modified-dTTP (2 µl of 100 µM) and $MnCl_2$ (1 µl of 40 mM) were added.

4. 1.5 µl of Therminator IX DNA polymerase (15 U, New England BioLabs) was then added.

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

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

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

Gel Electrophoresis and DNA Visualization:

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

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

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

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

Results

DNA polymerases incorporate 3'-*O*-modified-dTTPs opposite its naturally complementary base in a hairpin construct.

Step2: Cleavage

The second step describes a one-step cleavage of a hairpin model in this particular case with Endonuclease V (Figure 16a).

Materials and Methods

Materials

1. Oligonucleotides utilized in Example 4 were designed in-house and synthesized by Sigma Aldrich (see Figure 16c for sequences).

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

Methods

Cleavage reaction on hairpin oligonucleotides was carried out using the procedure below:

1. A pipette (Gilson) was used to transfer 43µl sterile distilled water (ELGA VEOLIA) into a 1.5ml Eppendorf tube.

2. 5µl of 10X Reaction buffer® NEB (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9 @ 25°C) was then added into the same Eppendorf tube.

3. 1µl of hairpin oligonucleotide (SEQ ID: No 45, Figure 16c) having an amount of 5pmols was added into the same tube.

4. 1µl of Human Endonuclease V (Endo V) NEB (30 units/µl) was added into the same tube.

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 1hour.

6. Typically after incubation time had elapsed, reaction was terminated by enzymatic heat inactivation (i.e. 65°C for 20 minutes).

The sample mixture was purified using the protocol outlined below:

1. 500 µl of buffer PNI QIAGEN (5M guanidinium chloride) was added to the sample and mixed by gentle resuspension with a pipette.

2. The mixture was transferred into a QIAquick spin column (QIAGEN) and centrifuged for 1min at 6000 rpm.

3. After centrifugation, flow-through was discarded and 750 µl of buffer PE QIAGEN (10mM Tris-HCl pH 7.5 and 80% ethanol) was added into the spin column and centrifuged for 1min at 6000 rpm.

4. The flow-through was discarded and the spin column was centrifuged for an additional 1min at 13000 rpm to remove residual PE buffer.

5. The spin column was then placed in a sterile 1.5ml Eppendorf tube.

6. For DNA elution, 50 µl of Buffer EB QIAGEN (10mM Tris.CL, pH 8.5) was added to the centre of the column membrane and left to stand for 1min at room temperature.

7. The tube was then centrifuged at 13000 rpm for 1 minute. Eluted DNA concentration was measured and stored at -20°C for subsequent use.

Measurement of purified DNA concentration was determined using the protocol below:

1. NanoDrop One (Thermo Scientific) was equilibrated by adding 2 µl of sterile distilled water (ELGA VEOLIA) onto the pedestal.

2. After equilibration, the water was gently wiped off using a lint-free lens cleaning tissue (Whatman).

3. NanoDrop One was blanked by adding 2 µl of Buffer EB QIAGEN (10mM Tris.CL, pH 8.5). Then step 2 was repeated after blanking.

4. DNA concentration was measured by adding 2 µl of the sample onto the pedestal and selecting the measure icon on the touch screen.

Gel Electrophoresis and DNA Visualization:

1. 5 µl of DNA and TBE-Urea sample buffer (Novex) was added into a sterile 1.5ml Eppendorf tube and heated to 95°C for 2 minutes using a heat ThermoMixer(Eppendorf).

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

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

4. Detection and visualization of DNA in the gel was carried out with ChemiDoc MP (BioRad) using Cy3 LEDS. Visualization and analysis was carried out on the Image lab 2.0 platform.

Results and Conclusion

Cleavage results from Example 4 showed that a significantly high yield of digested hairpin DNA was obtained with Endonuclease V at 37°C (Figure 16b).

Step 3: Ligation

The third step describes ligation of a hairpin model with DNA ligase. Diagrammatic illustration is shown in Figure 17a.

Materials and Methods

Materials

1. Oligonucleotides utilized in Example 4 were designed in-house and synthesized by Sigma Aldrich (see Figure 17c for sequences).

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

Method

Ligation reaction on oligonucleotides was carried out using the procedure below:

1. A pipette (Gilson) was used to transfer 1µl (5pmols) of TAMRA or any fluorescently tagged phosphate hairpin oligo (SEQ ID: 46) into a 1.5ml Eppendorf tube.

2. 15µl (100pmols) of inosine-containing hairpin construct (SEQ ID: 47) was then added into the same tube and gently mixed by resuspension with a pipette for 3 seconds.

3. 40µl of Blunt/TA DNA Ligase NEB (180 units/µl) was added into the same tube.

4. Reaction mixture was then gently mixed by resuspension with a pipette, centrifuged at 13,000 rpm for 5seconds and incubated at room temperature for 20 minutes.

5. Typically after incubation time had elapsed, reaction was terminated with the addition of TBE-Urea sample buffer (Novex).

6. The reaction mixture was purified using the protocol outlined in purification steps 1-7 above.

Measurement of purified DNA concentration was determined using the protocol below:

1. NanoDrop One (Thermo Scientific) was equilibrated by adding 2 µl of sterile distilled water (ELGA VEOLIA) onto the pedestal.

2. After equilibration, the water was gently wiped off using a lint-free lens cleaning tissue (Whatman).

3. NanoDrop One was blanked by adding 2 µl of Buffer EB QIAGEN (10mM Tris.CL, pH 8.5). Then step 2 was repeated after blanking.

4. DNA concentration was measured by adding 2 µl of the sample onto the pedestal and selecting the measure icon on the touch screen.

5. Purified DNA was run on a polyacrylamide gel and visualized in accordance with the procedure in steps 5- 8 as described above. No change in conditions or reagents was introduced.

Gel Electrophoresis and DNA Visualization.

1. 5 µl of DNA and TBE-Urea sample buffer (Novex) was added into a sterile 1.5 ml Eppendorf tube and heated to 95°C for 2 minutes using a heat ThermoMixer (Eppendorf).

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

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

4. Detection and visualization of DNA in the gel was carried out with ChemiDoc MP (BioRad) using Cy3 LEDS. Visualization and analysis was carried out on the Image lab 2.0 platform.

Results

Ligation of hairpin oligonucleotides with blunt/TA DNA ligase at room temperature (24°C) in the presence of a helper strand resulted high yield of ligated product. Ligated hairpin oligonucleotide after 1 minute showed a high yield of ligated DNA product with a ratio of ~ 85%. The ligated hairpin oligonucleotide after 2 minutes showed a high yield of ligated DNA with a ratio of ~ 85%. The ligated hairpin oligonucleotide after 3 minutes showed a high yield of ligated DNA product with a ratio of ~ 85%. The ligated hairpin oligonucleotide after 4 minutes showed a high yield of ligated DNA product with a ratio of ~ >85% (Figure 17b).

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

This Example describes the synthesis of polynucleotides using 4 steps on a double hairpin model: incorporation of 3'-*O*-modified dNTPs from the nick site; cleavage, ligation and

deprotection with the first step taking place opposite a naturally complementary nucleotide which is positioned in the support strand adjacent to a universal nucleotide, in this particular case inosine. One end of the hairpin serves as an attachment anchor.

The method starts by controlled addition of 3'-*O*-protected single nucleotide to oligonucleotide by enzymatic incorporation by DNA polymerase followed by inosine cleavage, ligation and deprotection (Figure 18a).

Materials and Methods

Materials

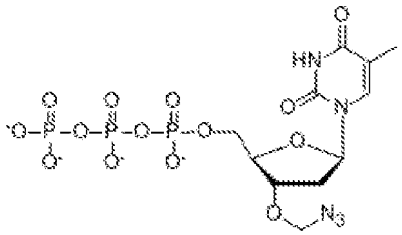
1. 3'-*O*-modified dNTPs were synthesized in-house according to the protocol described in PhD thesis Jian Wu: Molecular Engineering of Novel Nucleotide Analogues for DNA Sequencing by Synthesis. Columbia University, 2008. The protocol for synthesis is also described in patent: J. William Efcavitch, Juliesta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

2. Oligonucleotides were designed in house and obtained from Sigma-Aldrich (Figure 18c). The stock solutions are prepared in concentration of 100 μM.

3. Therminator IX DNA polymerase was used that has been engineered by New England BioLabs with enhanced ability to incorporate 3-*O*-modified dNTPs.

3'-*O*-azidomethyl-dTTP was tested for incorporation:

3'-*O*-azidomethyl-dTTP:



Method

1. 2 µl of 10x Thermopol® buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton® X-100, pH 8.8, New England BioLabs) was mixed with 12.5 µl of sterile deionized water (ELGA VEOLIA) in 1.5ml Eppendorf tube.

2. 2 µl of 10 µM double hairpin model oligonucleotide (20 pmol, 1 equiv) (SEQ ID: No 48, Figure 18c) were added to the reaction mixture.

3. 3'-*O*-modified-dTTP (2 µl of 100 µM) and $MnCl_2$ (1 µl of 40 mM) were added.

4. 1.5 µl of Therminator IX DNA polymerase (15 U, New England BioLabs) was then added.

5. The reaction was incubated for 10 minutes at 37°C.

6. The aliquot (5 µl) was taken out of the reaction mixture and 0.5 µl of natural dNTP mix was added and allowed to react for 10 minutes The reaction was analysed by gel electrophoresis.

7. The reaction mixture was purified using protocol outlined in purification steps 1-7.

8. The DNA sample was eluted by 20 µl of NEB reaction buffer® (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9 @ 25°C) into clean Eppendorf tube.

9. 1µl of Human Endonuclease V (Endo V) NEB (30 units/µl) was added into the same tube.

10. Reaction mixture was then gently mixed by resuspension with a pipette, centrifuged at 13,000 rpm for 5 seconds and incubated at 37°C for 1 hour.

11. After incubation time had elapsed, reaction was terminated by enzymatic heat inactivation (i.e. 65°C for 20 minutes).

12. The aliquot (5 µl) was taken out of the reaction mixture and analysed on polyacrylamide gel (15%) using TBE buffer and visualized by ChemiDoc MP imaging system (BioRad).

13. Reaction mixture was purified using the protocol outlined in purification steps 1-7 above.

14. The DNA sample was eluted by 20 µl of NEB Reaction buffer® (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9 @ 25°C) into clean Eppendorf tube.

15. 10 µl of 100 µM strand for ligation (1 nmol) (SEQ ID: No 49, Figure 18c) were added to the reaction mixture.

16. 40µl of Blunt/TA DNA Ligase NEB (180 units/µl) was added into the purified DNA sample.

17. Reaction mixture was then gently mixed by resuspension with a pipette, centrifuged at 13,000 rpm for 5 seconds and incubated at room temperature for 20 minutes.

18. 40 µL of the 500 mM TCEP in 1M TRIS buffer pH 7.4 was added to the reaction mixture and allowed to react for 10 minutes at 37°C.

19. The reaction mixture was purified using QIAGEN nucleotide removal kit eluting by 20 µL of 1x Thermopol® buffer.

Gel Electrophoresis and DNA Visualization:

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

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

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

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

Measurement of purified DNA concentration was determined using the protocol below:

1. NanoDrop One (Thermo Scientific) was equilibrated by adding 2 µl of sterile distilled water (ELGA VEOLIA) onto the pedestal.

2. After equilibration, the water was gently wiped off using a lint-free lens cleaning tissue (Whatman).

3. NanoDrop One was blanked by adding 2 µl of Buffer EB QIAGEN (10mM Tris.CL, pH 8.5). Then step 2 was repeated after blanking.

4. DNA concentration was measured by adding 2 µl of the sample onto the pedestal and selecting the measure icon on the touch screen.

5. Purified DNA was run on a polyacrylamide gel and visualized in accordance with the procedure in section 2 steps 5- 8. No change in conditions or reagents was introduced.

The sample mixture was purified after each step using the protocol outlined below:

1. 500 µl of buffer PNI QIAGEN (5M guanidinium chloride) was added to the sample and mixed by gentle resuspension with a pipette.

2. The mixture was transferred into a QIAquick spin column (QIAGEN) and centrifuged for 1 min at 6000 rpm.

3. After centrifugation, flow-through was discarded and 750 µl of buffer PE QIAGEN (10mM Tris-HCl pH 7.5 and 80% ethanol) was added into the spin column and centrifuged for 1 min at 6000 rpm.

4. The flow-through was discarded and the spin column was centrifuged for an additional 1min at 13000 rpm to remove residual PE buffer.

5. The spin column was then placed in a sterile 1.5 ml Eppendorf tube.

6. For DNA elution, 20 µl of appropriate buffer for the reaction was added to the centre of the column membrane and left to stand for 1min at room temperature.

7. The tube was then centrifuged at 13000 rpm for 1 minute. Eluted DNA concentration was measured and stored at -20°C for subsequent use.

Results

DNA polymerase incorporates 3'-*O*-modified-dTTPs opposite its naturally complementary base in a double hairpin construct (Figure 18b).

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

This Example describes the synthesis of polynucleotides using 4 steps on single-hairpin model: incorporation of 3'-*O*-modified dNTPs from nick site; cleavage, ligation and deprotection with the first step taking place opposite a naturally complementary base. The DNA synthesis uses a helper strand in the process.

The method starts by controlled addition of 3'-*O*-protected single nucleotide to oligonucleotide by enzymatic incorporation by DNA polymerase followed by inosine cleavage, ligation and deprotection (Figure 19a).

Materials and Methods

Materials

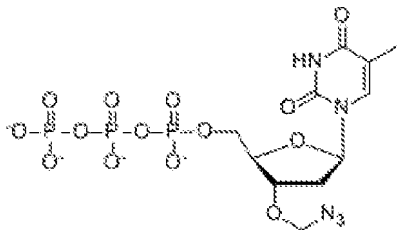
1. 3'-*O*-modified dNTPs were synthesized in-house according to the protocol described in PhD thesis Jian Wu: Molecular Engineering of Novel Nucleotide Analogues for DNA Sequencing by Synthesis. Columbia University, 2008. The protocol for synthesis is also described in patent: J. William Efcavitch, Juliesta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

2. Oligonucleotides were designed in house and obtained from Sigma Aldrich (Figure 19b). The stock solutions are prepared in concentration of 100 μM.

3. Therminator IX DNA polymerase was used that has been engineered by New England BioLabs with enhanced ability to incorporate 3-*O*-modified dNTPs.

3'-*O*-azidomethyl-dTTP was tested for incorporation:

3'-*O*-azidomethyl-dTTP:



Method

1. 2 µl of 10x Thermopol® buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton® X-100, pH 8.8, New England BioLabs) was mixed with 12.5 µl of sterile deionized water (ELGA VEOLIA) in 1.5ml Eppendorf tube.

2. 2 µl of 10 µM Single hairpin model oligonucleotide (20 pmol, 1 equiv) (SEQ ID: No 50, Figure 19b) and Helper strand (30 pmol, 1.5 equiv) (SEQ ID: No 51, Figure 19b) were added to the reaction mixture.

3. 3'-*O*-modified-dTTP (2 µl of 100 µM) and $MnCl_2$ (1 µl of 40 mM) were added

4. 1.5 µl of Therminator IX DNA polymerase (15 U, New England BioLabs) was then added.

5. The reaction was incubated for 10 minutes at 37°C.

6. The aliquot (5 µl) was taken out of the reaction mixture and 0.5 µl of natural dNTP mix was added and let react for 10 minutes The reaction was analysed by gel electrophoresis.

7. The reaction mixture was purified using the protocol outlined in purification steps 1-7 above.

8. The DNA sample was eluted by 20µl of NEB reaction buffer® (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9 @ 25°C) into clean Eppendorf tube.

9. 1µl of Human Endonuclease V (Endo V) NEB (30units/µl) was added into the same tube.

10. Reaction mixture was then gently mixed by resuspension with a pipette, centrifuged at 13,000 rpm for 5 seconds and incubated at 37°C for 1 hour.

11. After incubation time had elapsed, reaction was terminated by enzymatic heat inactivation (i.e. 65°C for 20 minutes).

12. The aliquot (5 µl) was taken out of the reaction mixture and analysed on polyacrylamide gel (15%) using TBE buffer and visualized by ChemiDoc MP imaging system (BioRad).

13. Reaction mixture was purified using the protocol outlined in purification steps 1-7 above.

14. The DNA sample was eluted by 20 µl of NEB reaction buffer® (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9 @ 25°C) into clean Eppendorf tube.

15. 10 µl of 100 µM strand for ligation (1 nmol) (SEQ ID: No 52, Figure 19b) and 10 µl of 100 µM helper strand for ligation (1 nmol) (SEQ ID: No 53, Figure 19b) were added to the reaction mixture.

16. 40µl of Blunt/TA DNA Ligase NEB (180 units/µl) was added into the same tube.

17. Reaction mixture was then gently mixed by resuspension with a pipette, centrifuged at 13,000rpm for 5seconds and incubated at room temperature for 20 minutes.

18. 40 µL of the 500 mM TCEP in 1M TRIS buffer pH 7.4 was added to the reaction mixture and let react for 10 minutes at 37°C.

19. The reaction mixture was purified using QIAGEN Nucleotide removal kit eluting by 20 µL of 1x NEB Thermopol® buffer.

20. Typically after incubation time had elapsed, reaction was terminated with the addition of TBE-Urea sample Buffer (Novex).

Gel Electrophoresis and DNA Visualization:

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

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

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

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

Measurement of purified DNA concentration was determined using the protocol below:

1. NanoDrop One (Thermo Scientific) was equilibrated by adding 2 µl of sterile distilled water (ELGA VEOLIA) onto the pedestal.

2. After equilibration, the water was gently wiped off using a lint-free lens cleaning tissue (Whatman).

3. NanoDrop One was blanked by adding 2 µl of Buffer EB QIAGEN (10mM Tris.CL, pH 8.5). Then step 2 was repeated after blanking.

4. DNA concentration was measured by adding 2 µl of the sample unto the pedestal and selecting the measure icon on the touch screen.

5. Purified DNA was run on a polyacrylamide gel and visualized in accordance with the procedure noted above in steps 5- 8. No change in conditions or reagents was introduced.

The sample mixture was purified after each step using the protocol outlined below:

1. 500 µl of buffer PNI QIAGEN (5M guanidinium chloride) was added to the sample and mixed by gentle resuspension with a pipette.

2. The mixture was transferred into a QIAquick spin column (QIAGEN) and centrifuged for 1min at 6000 rpm.

3. After centrifugation, flow-through was discarded and 750 µl of buffer PE QIAGEN (10mM Tris-HCl pH 7.5 and 80% ethanol) was added into the spin column and centrifuged for 1min at 6000 rpm.

4. The flow-through was discarded and the spin column was centrifuged for an additional 1min at 13000 rpm to remove residual PE buffer.

5. The spin column was then placed in a sterile 1.5ml Eppendorf tube.

6. For DNA elution, 20 µl of appropriate buffer for the reaction was added to the centre of the column membrane and left to stand for 1 minute at room temperature.

7. The tube was then centrifuged at 13000 rpm for 1 minute. Eluted DNA concentration was measured and stored at -20°C for subsequent use.

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

This Example describes the synthesis of polynucleotides using 4 steps on a double- hairpin construct model: incorporation of 3'-*O*-modified dNTPs from the nick site; cleavage, ligation and deprotection with the first step taking place opposite a universal nucleotide, in this particular case an inosine base.

The method starts by controlled addition of 3'-*O*-protected single nucleotide to oligonucleotide by enzymatic incorporation by DNA polymerase followed by inosine cleavage, ligation and deprotection (Figure 20a).

Materials and Methods

Materials

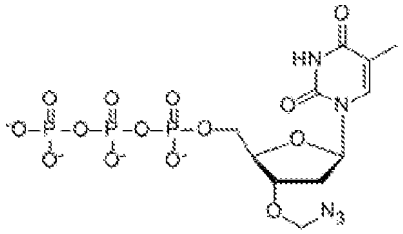
1. 3'-*O*-modified dNTPs were synthesized in-housed according to the protocol described in PhD thesis Jian Wu: Molecular Engineering of Novel Nucleotide Analogues for DNA Sequencing by Synthesis. Columbia University, 2008. The protocol for synthesis is also described in patent: J. William Efcavitch,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

2. Oligonucleotides were designed in house and obtained from Sigma-Aldrich (Figure 20b). The stock solutions are prepared in concentration of 100 µM.

3. Therminator IX DNA polymerase that has been engineered by New England BioLabs has enhanced ability to incorporate 3-*O*-modified dNTPs.

3'-*O*-azidomethyl-dTTP was tested for incorporation:

3'-*O*-azidomethyl-dTTP:



Method

1. 2 µl of 10x Thermopol® buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton® X-100, pH 8.8, New England BioLabs) was mixed with 12.5 µl of sterile deionized water (ELGA VEOLIA) in 1.5ml Eppendorf tube.

2. 2 µl of 10 µM double hairpin model oligonucleotide (20 pmol, 1 equiv) (SEQ ID: No 54, Figure 20b) were added to the reaction mixture.

3. 3'-*O*-modified-dTTP (2 µl of 100 µM) and $MnCl_2$ (1 µl of 40 mM) were added.

4. 1.5 µl of Therminator IX DNA polymerase (15 U, New England BioLabs) was then added.

5. The reaction was incubated for 10 minutes at 37°C.

6. The aliquot (5 µl) was taken out of the reaction mixture and 0.5 µl of natural dNTP mix was added and let react for 10 minutes. The reaction was analysed by gel electrophoresis.

7. The reaction mixture was purified using protocol outlined in purification steps 1-7.

8. The DNA sample was eluted by 20 µl of NEB Reaction buffer® (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9 @ 25°C) into clean Eppendorf tube.

9. 1µl of Human Endonuclease V (Endo V) NEB (30units/µl) was added into the same tube.

10. Reaction mixture was then gently mixed by resuspension with a pipette, centrifuged at 13,000 rpm for 5 seconds and incubated at 37°C for 1 hour.

11. After incubation time had elapsed, reaction was terminated by enzymatic heat inactivation (i.e. 65°C for 20 minutes).

12. The aliquot (5 µl) was taken out of the reaction mixture and analysed on polyacrylamide gel (15%) using TBE buffer and visualized by ChemiDoc MP imaging system (BioRad).

13. Reaction mixture was purified using the protocol outlined in purification steps 1-7 above.

14. The DNA sample was eluted by 20 µl of NEB Reaction buffer® (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9 @ 25°C) into clean Eppendorf tube.

15. 10 µl of 100 µM strand for ligation (1 nmol) (SEQ ID: No 55, Figure 20b), were added to the reaction mixture.

16. 40µl of Blunt/TA DNA Ligase NEB (180 units/µl) was added into the same tube.

17. Reaction mixture was then gently mixed by resuspension with a pipette, centrifuged at 13,000rpm for 5 seconds and incubated at room temperature for 20 minutes.

18. 40 µL of the 500 mM TCEP in 1M TRIS buffer pH 7.4 was added to the reaction mixture and let react for 10 minutes at 37°C.

19. The reaction mixture was purified using QIAGEN Nucleotide removal kit eluting by 20 µL of 1x NEB Thermopol® buffer.

20. Typically after incubation time had elapsed, reaction was terminated with the addition of TBE-Urea sample Buffer (Novex).

Gel Electrophoresis and DNA Visualization:

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

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

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

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

Measurement of purified DNA concentration was determined using the protocol below:

1. NanoDrop One (Thermo Scientific) was equilibrated by adding 2 µl of sterile distilled water (ELGA VEOLIA) onto the pedestal.

2. After equilibration, the water was gently wiped off using a lint-free lens cleaning tissue (Whatman).

3. NanoDrop One was blanked by adding 2 µl of Buffer EB QIAGEN (10mM Tris.CL, pH 8.5). Step 2 was then repeated after blanking.

4. DNA concentration was measured by adding 2 µl of the sample unto the pedestal and selecting the measure icon on the touch screen.

5. Purified DNA was run on a polyacrylamide gel and visualized in accordance with the procedure in section 2 steps 5- 8. No change in conditions or reagents was introduced.

The sample mixture was purified after each step using the protocol outlined below:

1. 500 µl of buffer PNI QIAGEN (5M guanidinium chloride) was added to the sample and mixed by gentle resuspension with a pipette.

2. The mixture was transferred into a QIAquick spin column (QIAGEN) and centrifuged for 1min at 6000 rpm.

3. After centrifugation, flow-through was discarded and 750 µl of buffer PE QIAGEN (10mM Tris-HCl pH 7.5 and 80% ethanol) was added into the spin column and centrifuged for 1min at 6000 rpm.

4. The flow-through was discarded and the spin column was centrifuged for an additional 1min at 13000 rpm to remove residual PE buffer.

5. The spin column was then placed in a sterile 1.5ml Eppendorf tube.

6. For DNA elution, 20 µl of appropriate buffer for the reaction was added to the centre of the column membrane and left to stand for 1min at room temperature.

7. The tube was then centrifuged at 13000 rpm for 1minutes. 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.

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.

The method starts by controlled addition of 3'-*O*-protected single nucleotide to 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 Figure 21a. Figure 21b shows a reaction schematic for the second cycle.

Materials and Methods

Materials

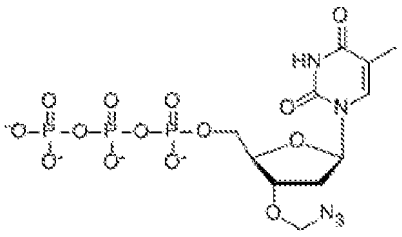
1. 3'-*O*-modified dNTPs were synthesized in-house according to the protocol described in PhD thesis Jian Wu: Molecular Engineering of Novel Nucleotide Analogues for DNA Sequencing by Synthesis. Columbia University, 2008. The protocol for synthesis is also described in patent: J. William Efcavitch, Juliesta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

2. Oligonucleotides were designed in house and obtained from Sigma-Aldrich (Figure 21d). The stock solutions are prepared in concentration of 100 μM.

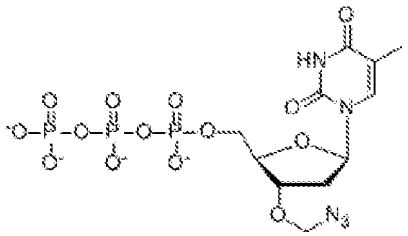
3. Therminator IX DNA polymerase that has been engineered by New England BioLabs has enhanced ability to incorporate 3'-*O*-modified dNTPs.

3'-*O*-azidomethyl-dTTP and 3'-O-azidomethyl-dCTP were used for incorporation:

3'-*O*-azidomethyl-dTTP:



3'-*O*-azidomethyl-dCTP:



Method

1st cycle:

1. 2 µl of 10x Thermopol® buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton® X-100, pH 8.8, New England BioLabs) was mixed with 12.5 µl of sterile deionized water (ELGA VEOLIA) in 1.5ml Eppendorf tube.

2. 2 µl of 10 µM double hairpin model oligonucleotide (20 pmol, 1 equiv) (SEQ ID: No 56, Figure 21d) were added to the reaction mixture.

3. 3'-*O*-modified-dTTP (2 µl of 100 µM) and $MnCl_2$ (1 µl of 40 mM) were added

4. 1.5 µl of Therminator IX DNA polymerase (15 U, New England BioLabs) was then added.

5. The reaction was incubated for 10 minutes at 37°C.

6. The aliquot (5 µl) was taken out of the reaction mixture and 0.5 µl of natural dNTP mix was added and allowed to react for 10 min. The reaction was analysed by gel electrophoresis.

7. 40 µL of the 500 mM TCEP in 1M TRIS buffer pH=7.4 was added to the reaction mixture and allowed to react for 10 minutes at 37°C.

8. The reaction mixture was purified using protocol outlined in purification steps 1-7.

9. The DNA sample was eluted by 20 µl of NEB Reaction buffer® (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9 @ 25°C) into a clean Eppendorf tube.

10. 1µl of Human Endonuclease V (Endo V) NEB (30units/µl) was added into the same tube.

11. Reaction mixture was then gently mixed by resuspension with a pipette, centrifuged at 13,000 rpm for 5 seconds and incubated at 37°C for 1 hour.

12. After incubation time had elapsed, the reaction was terminated by enzymatic heat inactivation (i.e. 65°C for 20mins).

13. The aliquot (5 µl) was taken out of the reaction mixture and analysed on polyacrylamide gel (15%) using TBE buffer and visualized by ChemiDoc MP imaging system (BioRad).

14. Reaction mixture was purified by QIAGEN Nucleotide Removal kit using protocol outlined in purification steps 1-7.

15. The DNA sample was eluted by 20 µl of NEB Reaction buffer® (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9 @ 25°C) into a clean Eppendorf tube.

16. 10 µl of 100 µM strand for ligation (1 nmol) (SEQ ID: No 57, Figure 21d), were added to the reaction mixture.

17. 40µl of Blunt/TA DNA Ligase NEB (180 units/µl) was added into the same tube.

18. Reaction mixture was then gently mixed by resuspension with a pipette, centrifuged at 13,000rpm for 5 seconds and incubated at room temperature for 20 mins.

19. Reaction mixture was purified by Streptavidin Magnetic Beads kit using protocol outlined in purification steps 1-5.

20. Unligated oligonucleotide was digested by Lambda Exonuclease.

21. Reaction mixture was purified by QIAGEN Nucleotide Removal kit using protocol outlined in purification steps 1-7.

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 clean Eppendorf tube.

2nd cycle:

23. 3'-*O*-modified-dCTP (2 µl of 100 µM) and $MnCl_2$ (1 µl of 40 mM) were added.

24. 1.5 µl of Therminator IX DNA polymerase (15 U, New England BioLabs) was then added.

25. The reaction was incubated for 10 minutes at 37°C.

26. The aliquot (5 µl) was taken out of the reaction mixture and 0.5 µl of natural dNTP mix was added and reacted for 10 min. The reaction was analysed by gel electrophoresis.

27. 40 µL of the 500 mM TCEP in 1M TRIS buffer pH=7.4 was added to the reaction mixture and reacted for 10 minutes at 37°C.

28. The reaction mixture was purified using protocol outlined in purification steps 1-7.

29. The DNA sample was eluted by 20 µl of NEB Reaction buffer® (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9 @ 25°C) into a clean Eppendorf tube.

30. 1µl of Human Endonuclease V (Endo V) NEB (30units/µl) was added into the same tube.

31. 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.

32. After incubation time had elapsed, the reaction was terminated by enzymatic heat inactivation (i.e. 65°C for 20mins).

33. The aliquot (5 µl) was taken out of the reaction mixture and analysed on polyacrylamide gel (15%) using TBE buffer and visualized by ChemiDoc MP imaging system (BioRad).

34. Reaction mixture was purified using protocol outlined in purification steps 1-7.

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.

36. 10 µl of 100 µM strand for ligation (1 nmol) (SEQ ID: No 58, Figure 21d), were added to the reaction mixture.

37. 40µl of Blunt/TA DNA Ligase NEB (180 units/µl) was added into the same tube.

38. Reaction mixture was then gently mixed by resuspension with a pipette, centrifuged at 13,000rpm for 5seconds and incubated at room temperature for 10mins.

39. After incubation time had elapsed, reaction was terminated with the addition of TBE-Urea sample Buffer (Novex).

Gel Electrophoresis and DNA Visualization:

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

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

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.

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

Measurement of purified DNA concentration was determined using the protocol below:

1. NanoDrop One (Thermo Scientific) was equilibrated by adding 2 µl of sterile distilled water (ELGA VEOLIA) onto the pedestal.

2. After equilibration, the water was gently wiped off using a lint-free lens cleaning tissue (Whatman).

3. NanoDrop One was blanked by adding 2 µl of Buffer EB QIAGEN (10mM Tris.CL, pH 8.5). Then step 2 was repeated after blanking.

4. DNA concentration was measured by adding 2 µl of the sample unto the pedestal and selecting the measure icon on the touch screen.

The sample mixture was purified by QIAGEN Nucleotide Removal kit using the protocol outlined below:

1. 500 µl of buffer PNI QIAGEN (5M guanidinium chloride) was added to the sample and mixed by gentle resuspension with a pipette.

2. The mixture was transferred into a QIAquick spin column (QIAGEN) and centrifuged for 1min at 6000 rpm.

3. After centrifugation, flow-through was discarded and 750 µl of buffer PE QIAGEN (10mM Tris-HCl pH 7.5 and 80% ethanol) was added into the spin column and centrifuged for 1min at 6000 rpm.

4. The flow-through was discarded and the spin column was centrifuged for an additional 1min at 13000 rpm to remove residual PE buffer.

5. The spin column was then placed in a sterile 1.5ml Eppendorf tube.

6. For DNA elution, 20 µl of appropriate buffer for the reaction was added to the centre of the column membrane and left to stand for 1min at room temperature.

7. The tube was then centrifuged at 13000 rpm for 1min.

After ligation step, the sample mixture was purified using Streptavidin Magnetic Beads via the protocol outlined below:

1. 100 µl of Streptavidin Magnetic Beads (New England BioLabs) were washed 3 times by 200 µl of binding buffer (20mM TRIS, 500 mM NaCl, pH = 7.4).

2. Reaction mixture after ligation step is mixed with 10 volumes of binding buffer (20mM TRIS, 500 mM NaCl, pH = 7.4) and incubated with Streptavidin Magnetic Beads for 15 minutes at 20 °C.

3. Streptavidin Magnetic Beads were washed 3 times by 200 µl of binding buffer (20mM TRIS, 500 mM NaCl, pH = 7.4).

4. Streptavidin Magnetic Beads were washed 3 times by deionized water.

5. The oligonucleotides were eluted by 40 µl of deionized water by heating to 95°C for 3 minutes.

The results shown in Figure 21c demonstrate the performance two complete synthesis cycles using an exemplary method of the invention.

It is to be understood that different applications of the disclosed methods and products may be tailored to the specific needs in the art. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments of the invention only, and is not intended to be limiting.

As used in this specification and the appended claims, the singular forms "a", "an", and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to "a ligation polynucleotide" includes two or more such polynucleotides, reference to "a scaffold polynucleotide" includes two or more such scaffold polynucleotides, and the like.

CLAIMS

1. An in vitro method for synthesising a double-stranded polynucleotide having a predefined sequence, the method comprising performing cycles of synthesis wherein in each cycle, a first strand is extended by the incorporation of a nucleotide of the predefined sequence and the second strand which is hybridized to the first strand is extended by the incorporation of a nucleotide thereby forming a nucleotide pair with the incorporated nucleotide of the first strand; wherein each cycle comprises extending the first strand by incorporating the nucleotide of the predefined sequence together with an attached reversible terminator group followed by extending the second strand; further wherein in each cycle the nucleotides are incorporated into a scaffold polynucleotide and wherein each cycle comprises:

(1) providing a scaffold polynucleotide;

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

(3) cleaving the scaffold polynucleotide at a cleavage site;

(4) ligating a ligation polynucleotide to the cleaved scaffold polynucleotide, the ligation polynucleotide comprising a partner nucleotide for the nucleotide of the predefined sequence, wherein upon ligation the nucleotide of the predefined sequence pairs with the partner nucleotide; and

(5) removing the reversible terminator group from the nucleotide of the predefined sequence after step (4) or removing the reversible terminator group from the nucleotide of predefined sequence after step (2) and before step (3), or after step (3) and before step (4).

2. A method according to claim 1, wherein step (1) comprises providing a scaffold polynucleotide comprising a synthesis strand and a support strand hybridized thereto, wherein the synthesis strand comprises a primer strand portion, and the support strand

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

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

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

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

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

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

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

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

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

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

(9) removing the reversible terminator group from the next nucleotide after step (8) and before step (10), or after step (6) and before step (7), or after step (7) and before step (8); and

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

4. A method according to claim 2 or claim 3, wherein the universal nucleotide occupies position n in: (a) the support strand of the scaffold polynucleotide in steps 1 and 6 and (b) the support strand of the ligation polynucleotide in steps 4 and 8, and wherein the support strand of the scaffold polynucleotide is cleaved between positions n and n-1 in steps 3 and 7; wherein position n is the nucleotide position in the support strand which is

opposite the position in the synthesis strand which will be occupied by the nucleotide of the predefined sequence upon its incorporation in the respective next incorporation step, and wherein position n-1 is the next nucleotide position in the support strand relative to the position occupied by the universal nucleotide in the direction distal to the helper strand.

5. A method according to claim 2 or claim 3, wherein the universal nucleotide occupies position n+1 in: (a) the support strand of the scaffold polynucleotide in steps 1 and 6 and (b) the support strand of the ligation polynucleotide in steps 4 and 8, and wherein the support strand of the scaffold polynucleotide is cleaved between positions n and n-1 in steps 3 and 7; wherein position n is the nucleotide position in the support strand which is opposite the position in the synthesis strand which will be occupied by the nucleotide of the predefined sequence upon its incorporation in the respective next incorporation step, wherein position n-1 is the next nucleotide position in the support strand relative to position n in the direction distal to the helper strand, and wherein position n+1 is the next nucleotide position in the support strand relative to position n in the direction proximal to the helper strand.

6. A method according to claim 2 or claim 3, wherein the universal nucleotide occupies position n in: (a) the support strand of the scaffold polynucleotide in steps 1 and 6 and (b) the support strand of the ligation polynucleotide in steps 4 and 8, and wherein the support strand of the scaffold polynucleotide is cleaved between positions n-1 and n-2 in steps 3 and 7; wherein position n is the nucleotide position in the support strand which is opposite the position in the synthesis strand which will be occupied by the nucleotide of the predefined sequence upon its incorporation in the respective next incorporation step, wherein position n-1 is the next nucleotide position in the support strand relative to the position occupied by the universal nucleotide in the direction distal to the helper strand, and wherein position n-2 is the next nucleotide position in the support strand relative to position n-1 in the direction distal to the helper strand.

7. A method according to claim 3 or claim 4, wherein:

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

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

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

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

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

ii. the universal nucleotide is positioned next to the terminal nucleotide of the support strand (position n);

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

8. A method according to claim 3 or claim 5, wherein:

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

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

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

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

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

ii. the universal nucleotide is positioned next to the penultimate nucleotide of the support strand (position n);

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

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

9. A method according to claim 3 or claim 6, wherein:

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

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

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

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

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

ii. the universal nucleotide is positioned next to the penultimate nucleotide of the support strand; and

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

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

11. A method according to any one of claims 1 to 4, claim 7 and claim 10, 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.

12. A method according to claim 11, wherein the first step is performed with a nucleotide-excising enzyme.

13. A method according to claim 12, wherein the nucleotide-excising enzyme is a 3-methyladenine DNA glycosylase enzyme.

14. A method according to claim 13, wherein the nucleotide-excising enzyme is human alkyladenine DNA glycosylase (hAAG).

15. A method according to any one of claims 11 to 14, wherein the second step is performed with a chemical which is a base.

16. A method according to claim 15, wherein the base is NaOH.

17. A method according to any one of claims 11 to 14, wherein the second step is performed with an organic chemical having abasic site cleavage activity.

18. A method according to claim 17, wherein the organic chemical is N,N'-dimethylethylenediamine.

19. A method according to any one of claims 11 to 14, wherein the second step is performed with an enzyme having abasic site lyase activity.

20. A method according to claim 19, wherein the enzyme is Endonuclease VIII.

21. A method according to any one of claims 1 to 10, wherein the cleavage step comprises cleaving the support strand with an enzyme.

22. A method according to claim 21, wherein the enzyme cleaves the support strand after the nucleotide which is next to the universal nucleotide, thereby creating the overhanging end in the synthesis strand comprising the first/next nucleotide.

23. A method according to claim 22, wherein the enzyme is Endonuclease V.

24. A method according to any one of the preceding claims, wherein both strands of the synthesized double-stranded polynucleotide are DNA strands.

25. A method according to any one of claims 2 to 24, wherein the synthesis strand and the support strand are DNA strands.

26. A method according to claim 24 or claim 25, wherein incorporated nucleotides are dNTPs.

27. A method according to claim 26 wherein incorporated nucleotides are dNTPs comprising a reversible terminator group.

28. A method according to claim 27, wherein one or more of the incorporated nucleotides comprising a reversible terminator group are 3'-*O*-allyl-dNTPs.

29. A method according to claim 27, wherein one or more of the incorporated nucleotides comprising a reversible terminator group are 3'-*O*-azidomethyl-dNTPs.

30. A method according to any one of claims 1 to 23, wherein a first strand of the synthesized double-stranded polynucleotide is a DNA strand and the second strand of the synthesized double-stranded polynucleotide is an RNA strand.

31. A method according to any one of claims 1 to 23 and claim 30, wherein the synthesis strand is an RNA strand and the support strand is a DNA strand.

32. A method according to claim 31, wherein incorporated nucleotides are NTPs.

33. A method according to claim 32, wherein incorporated nucleotides are NTPs comprising a reversible terminator group.

34. A method according to claim 33, wherein incorporated nucleotides comprising a reversible terminator group are 3'-*O*-allyl-NTPs.

35. A method according to claim 33, wherein incorporated nucleotides comprising a reversible terminator group are 3'-*O*-azidomethyl-NTPs.

36. A method according to any one of claims 1 to 29, wherein the polymerase is a DNA polymerase, preferably a modified DNA polymerase having an enhanced ability to incorporate a dNTP comprising a reversible terminator group compared to an unmodified polymerase.

37. A method according to claim 36, wherein the polymerase is a variant of the native DNA polymerase from *Thermococcus* species 9°N, preferably species 9°N-7.

38. A method according to any one of claims 1 to 23 and 30 to 35, wherein the polymerase is an RNA polymerase, optionally a modified RNA polymerase having an enhanced ability to incorporate an NTP comprising a reversible terminator group compared to an unmodified polymerase.

39. A method according to any one of claims 1 to 38, wherein the step of removing the reversible terminator group from the first/next nucleotide is performed with tris(carboxyethyl)phosphine (TCEP).

40. A method according to any one of claims 1 to 39, wherein the step of ligating a double-stranded ligation polynucleotide to the cleaved scaffold polynucleotide is performed with a ligase enzyme.

41. A method according to claim 40, wherein the ligase enzyme is a T4 DNA ligase.

42. A method according to any one of claims 1 to 41, wherein in step (1) the helper strand and the portion of the support strand hybridized thereto are connected by a hairpin loop.

43. A method according to any one of claims 1 to 41, wherein in step (1) the synthesis strand comprising the primer strand portion and the portion of the support strand hybridized thereto are connected by a hairpin loop.

44. A method according to any one of claims 1 to 41, wherein in step (1):

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

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

45. A method according to any one of claims 1 to 44, wherein at least one of the ligation polynucleotides is provided as a single molecule comprising a hairpin loop connecting the support strand and the helper strand at the end opposite the overhanging end.

46. A method according to any one of claims 1 to 45, wherein the ligation polynucleotides of each synthesis cycle are provided as single molecules each comprising a hairpin loop connecting the support strand and the helper strand at the end opposite the overhanging end.

47. A method according to any one of claims 1 to 46, wherein in step (1) the synthesis strand comprising the primer strand portion and the portion of the support strand hybridized thereto are tethered to a common surface.

48. A method according to claim 47 wherein the primer strand portion and the portion of the support strand hybridized thereto each comprise a cleavable linker, wherein the linkers may be cleaved to detach the double-stranded polynucleotide from the surface following synthesis.

49. A method according to any one of claims 1 to 46, wherein in step (1) the primer strand portion of the synthesis strand and the portion of the support strand hybridized thereto are connected by a hairpin loop, and wherein the hairpin loop is tethered to a surface.

50. A method according to claim 49 wherein the hairpin loop is 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.

51. A method according to claim 48 or claim 50, wherein the cleavable linker is a UV cleavable linker.

52. A method according to any one of claims 47 to 51, wherein the surface is a microparticle.

53. A method according to any one of claims 47 to 51, wherein the surface is a planar surface.

54. A method according to any one of claims 1 to 53, wherein the step of removing the reversible terminator group from a nucleotide of the predefined sequence is performed before the cleavage step, or before the ligation step.

55. A method according to any one of the preceding claims, wherein synthesis cycles are performed in droplets within a microfluidic system.

56. A method according to claim 55, wherein the microfluidic system is an electrowetting system.

57. A method according to claim 55, wherein the microfluidic system is an electrowetting-on-dielectric system (EWOD).

58. A method according to any one of the preceding claims, wherein following synthesis the strands of the double-stranded polynucleotides are separated to provide a single-stranded polynucleotide having a predefined sequence.

59. A method of assembling a polynucleotide having a predefined sequence, the method comprising performing the method of any one of the preceding claims to synthesize a first polynucleotide having a predefined sequence and one or more additional polynucleotides having a predefined sequence and joining together the first and one or more additional polynucleotides.

60. A method according to claim 59 wherein the first nucleotide and the one or more additional polynucleotides are double-stranded.

61. A method according to claim 59 wherein the first nucleotide and the one or more additional polynucleotides are single-stranded.

62. A method according to any one of claims 59 to 61, wherein the first polynucleotide and the one or more additional polynucleotides are cleaved to create compatible termini and joined together by ligation.

63. A method according to claim 62, wherein the first polynucleotide and the one or more additional polynucleotides are cleaved by a restriction enzyme at a cleavage site.

64. A method according to any one of claims 57 to 63, wherein the synthesis and/or assembly steps are performed in droplets within a microfluidic system.

65. A method according to claim 64 wherein the assembly steps comprise providing a first droplet comprising a first synthesized polynucleotide having a predefined sequence and a second droplet comprising an additional one or more synthesized polynucleotides having a predefined sequence, wherein the droplets are brought in contact with each other and wherein the synthesized polynucleotides are joined together thereby assembling a polynucleotide comprising the first and additional one or more polynucleotides.

66. A method according to claim 65 wherein the synthesis steps are 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 polynucleotide in accordance with the steps of the synthesis cycles.

67. A method according to claim 66, wherein following delivery of a droplet and prior to the delivery of a next droplet, a washing step is carried out to remove excess reaction reagents.

68. A method according to claim 66 and 67, wherein the microfluidic system is an electrowetting system.

69. A method according to claim 68, wherein the microfluidic system is an electrowetting-on-dielectric system (EWOD).

70. A method according to any one of claims 66 to 69, wherein synthesis and assembly steps are performed within the same system.

71. A polynucleotide synthesis system for carrying out the method according to any one of claims 1 to 64 and claims 66 to 70, 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 synthesized double-stranded polynucleotide from the scaffold polynucleotide.

72. A system according to claim 71 further comprising means for providing the reaction reagents in droplets and means for delivering the droplets to the scaffold polynucleotide in accordance with the synthesis cycles.

73. A kit for use with the system of claims 71 or 72 and for carrying out the method according to any one of claims 1 to 64, the kit comprising volumes of reaction reagents corresponding to the steps of the synthesis cycles.

74. A method of making a polynucleotide microarray, wherein the microarray comprises a plurality of reaction areas, each area comprising one or more polynucleotides having a predefined sequence, the method comprising:

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

b) performing cycles of synthesis according to the method of any one of claims 1 to 57 at each reaction area, thereby synthesising at each area one or more double-stranded polynucleotides having a predefined sequence.

75. A method according to claim 74, wherein following synthesis the strands of the double-stranded polynucleotides are separated to provide a microarray wherein each area comprises one or more single-stranded polynucleotides having a predefined sequence.