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(54) **NUCLEIC ACID AND GENE SYNTHESIS**

(71) Applicant: **NunaBio Limited**, Newcastle upon Tyne (GB)

(72) Inventors: **Andrew Robert PIKE**, Newcastle upon Tyne (GB); **Eimer Mary TUITE**, Newcastle upon Tyne (GB); **Joseph Henry HEDLEY**, Morpeth (GB)

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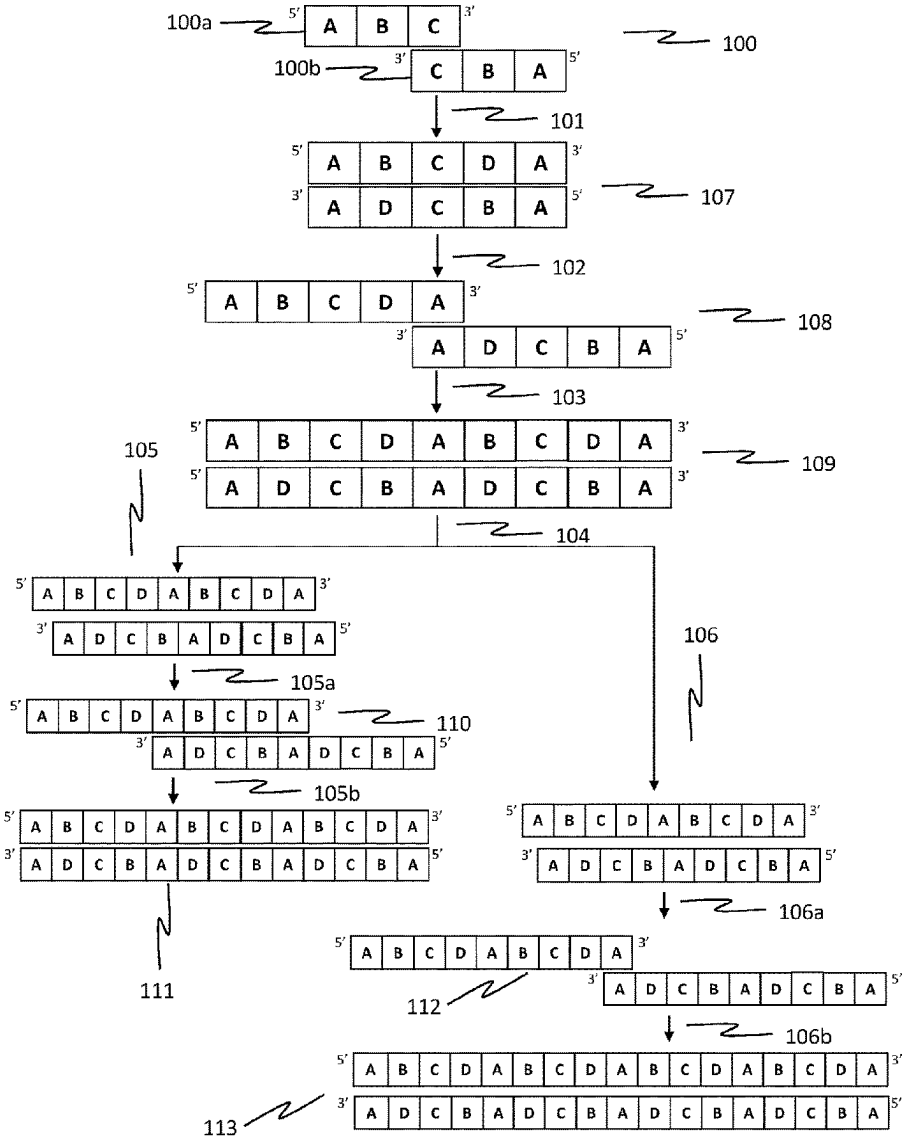
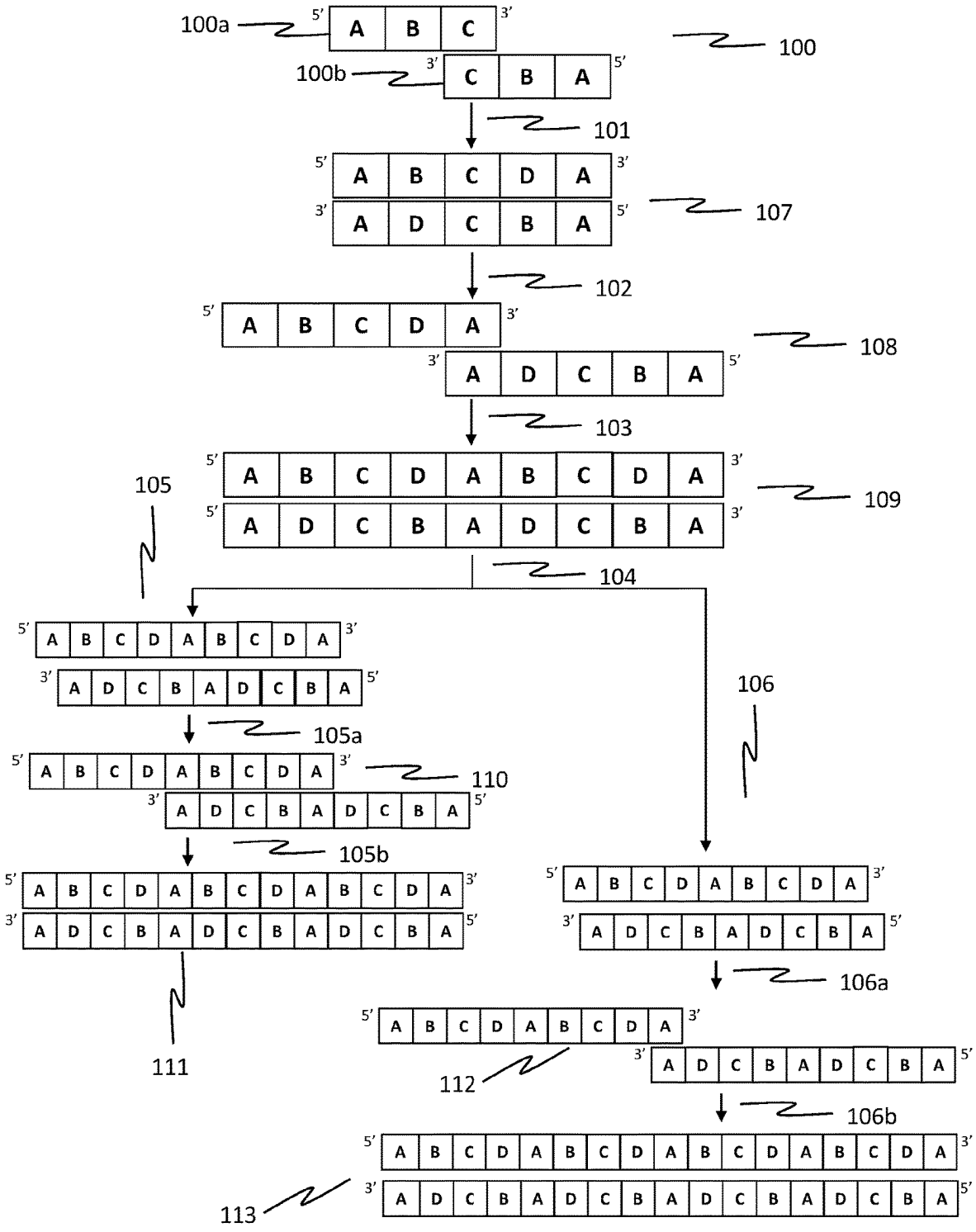
(57) **ABSTRACT**

The present invention relates to methods of synthesising nucleic acids, in particular to methods of extending an overlapping primer oligonucleotide to generate an oligonucleotide, polynucleotide, gene fragment or gene comprising repeating sequences. The present invention also relates to overlapping primer oligonucleotides for use in nucleic acid synthesis reactions.

Specification includes a Sequence Listing.




100a
100b
100
101
107
102
108
103
109
105
104
106
105a
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105b
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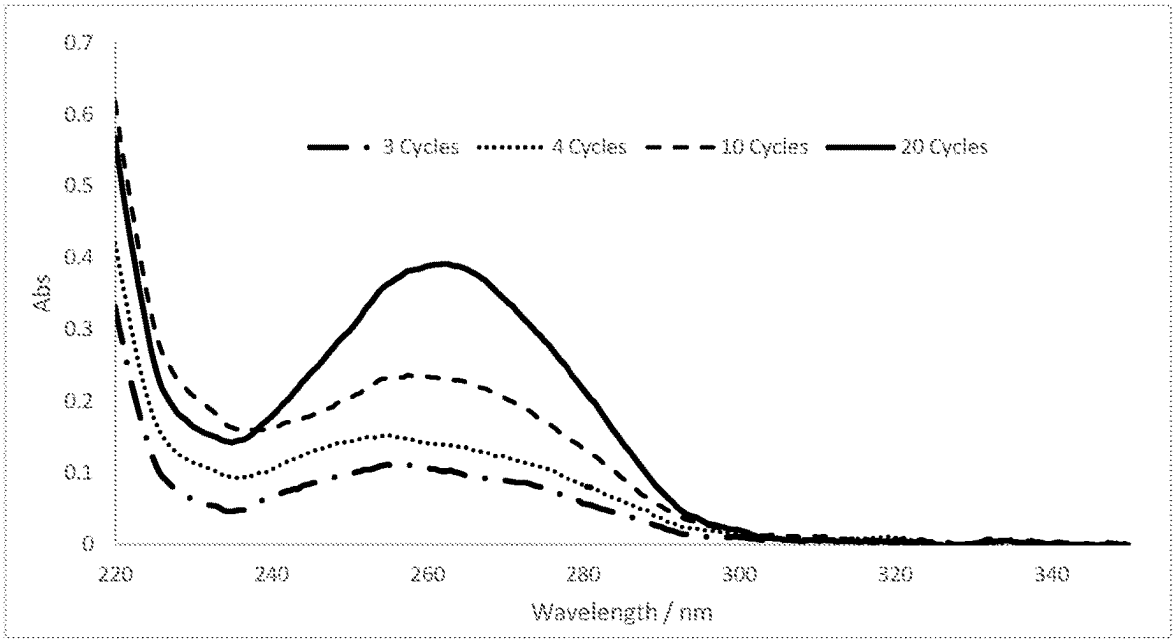
106a
112
106b
113

Fig 1


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Abs
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4 Cycles
10 Cycles
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240
260
280
300
320
340
Wavelength / nm

Fig 2

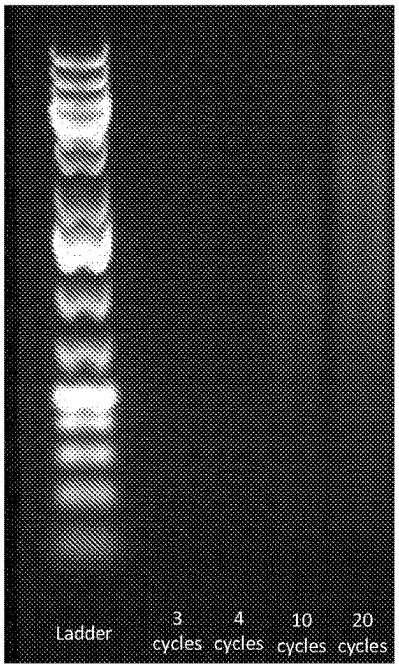

Ladder
3 cycles
4 cycles
10 cycles
20 cycles

Fig 3A

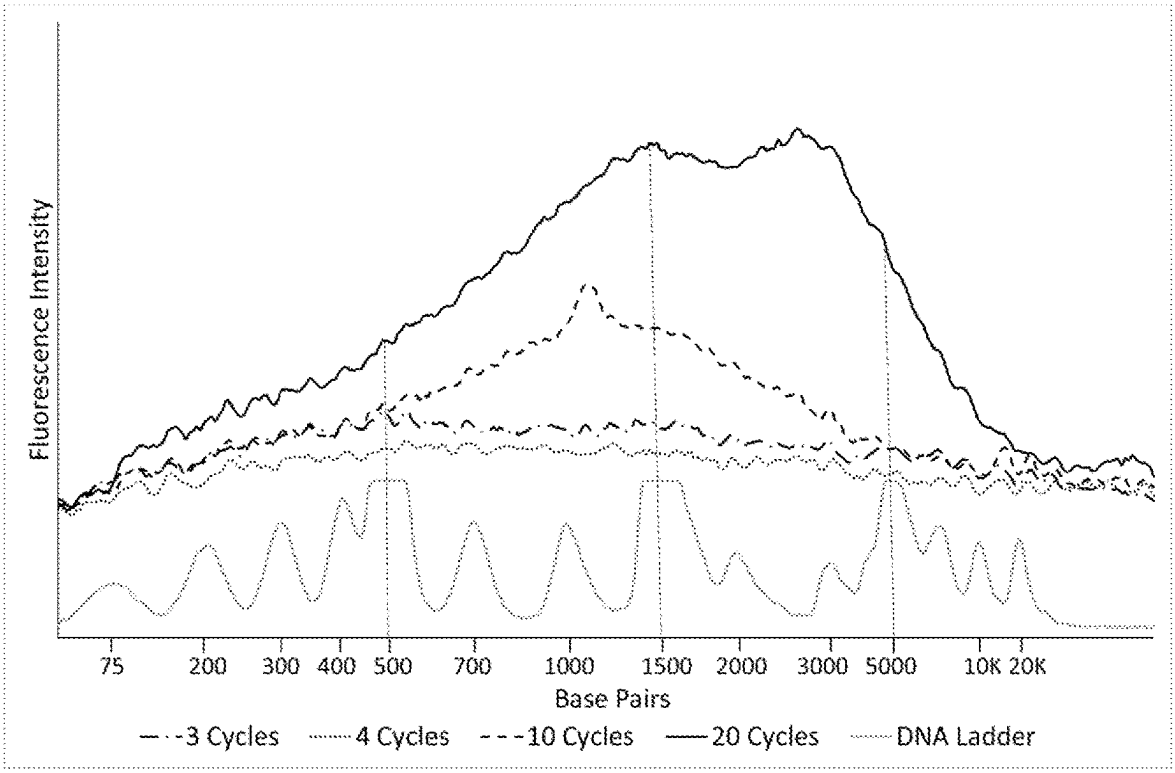
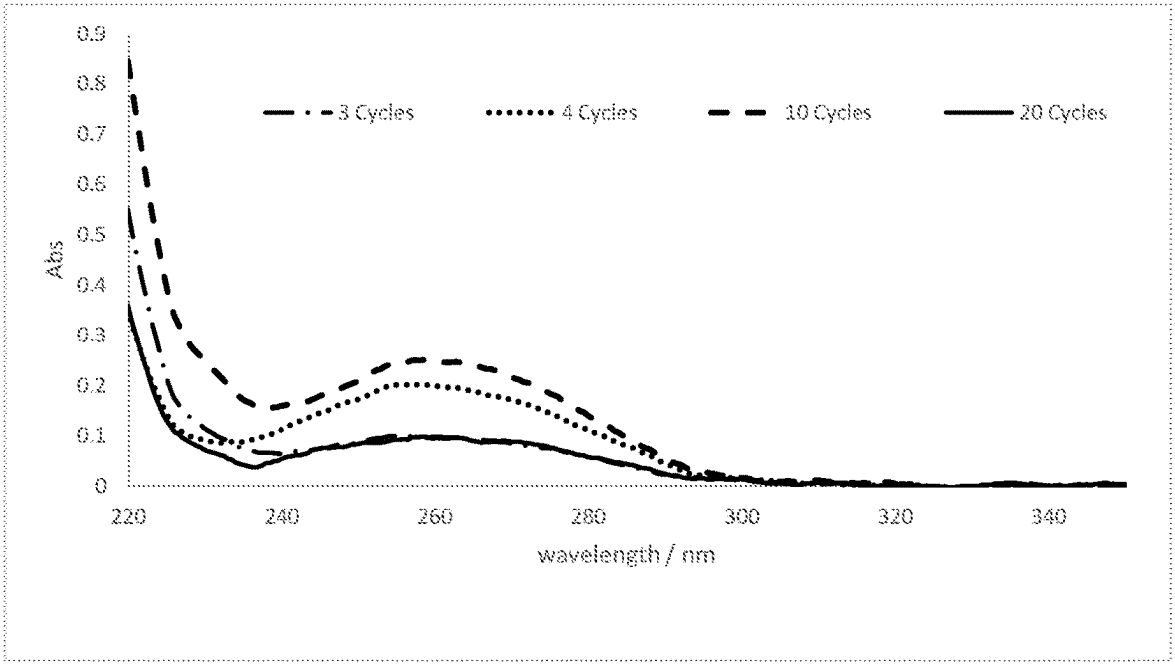

Fluorescence Intensity
75
200
300
400
500
700
1000
1500
2000
3000
5000
10K
20K
Base Pairs
3 Cycles
4 Cycles
10 Cycles
20 Cycles
DNA Ladder

Fig 3B


0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Abs
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10 Cycles
20 Cycles
220
240
260
280
300
320
340
wavelength / nm

Fig 4

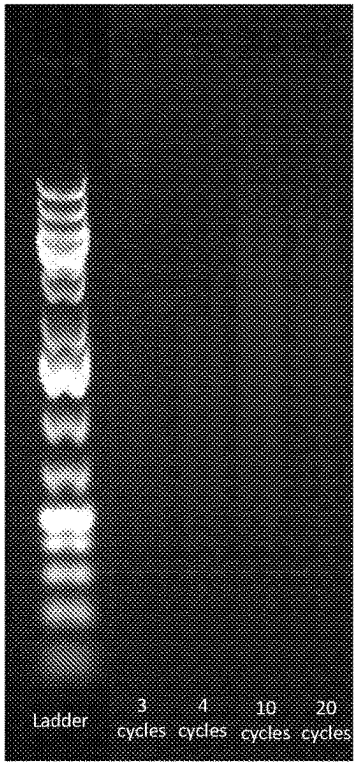

Ladder
3 cycles
4 cycles
10 cycles
20 cycles

Fig 5A

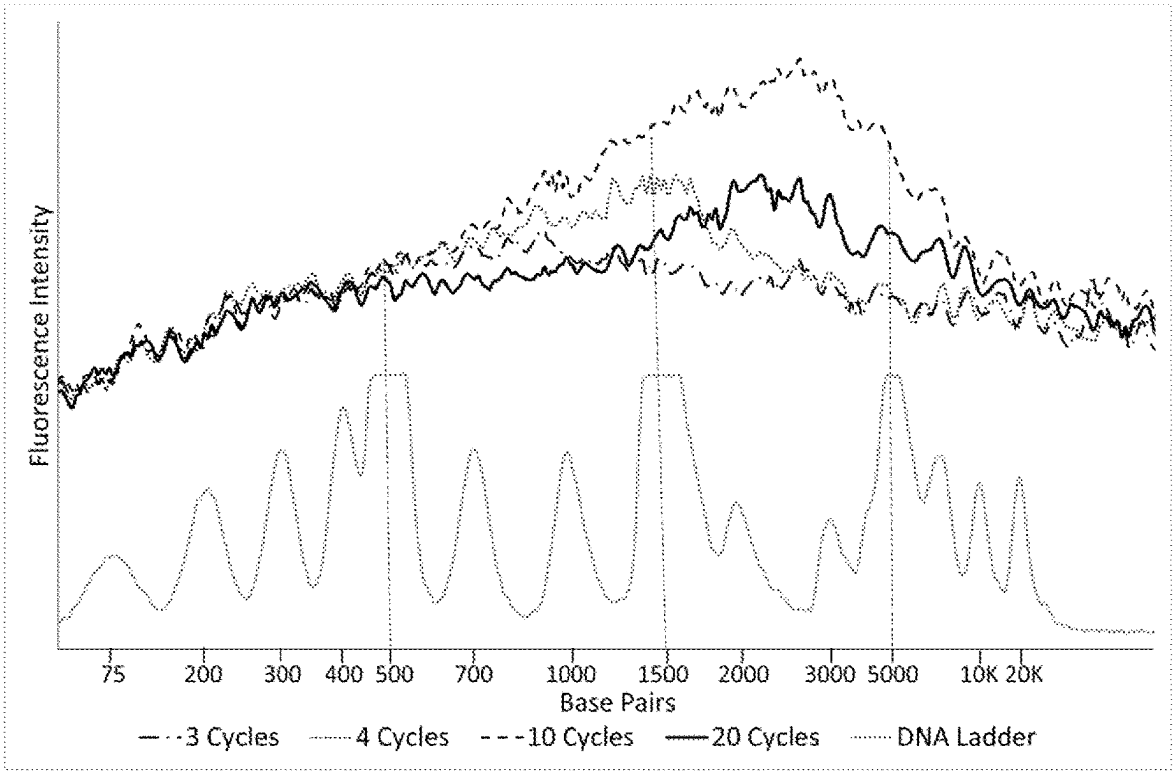
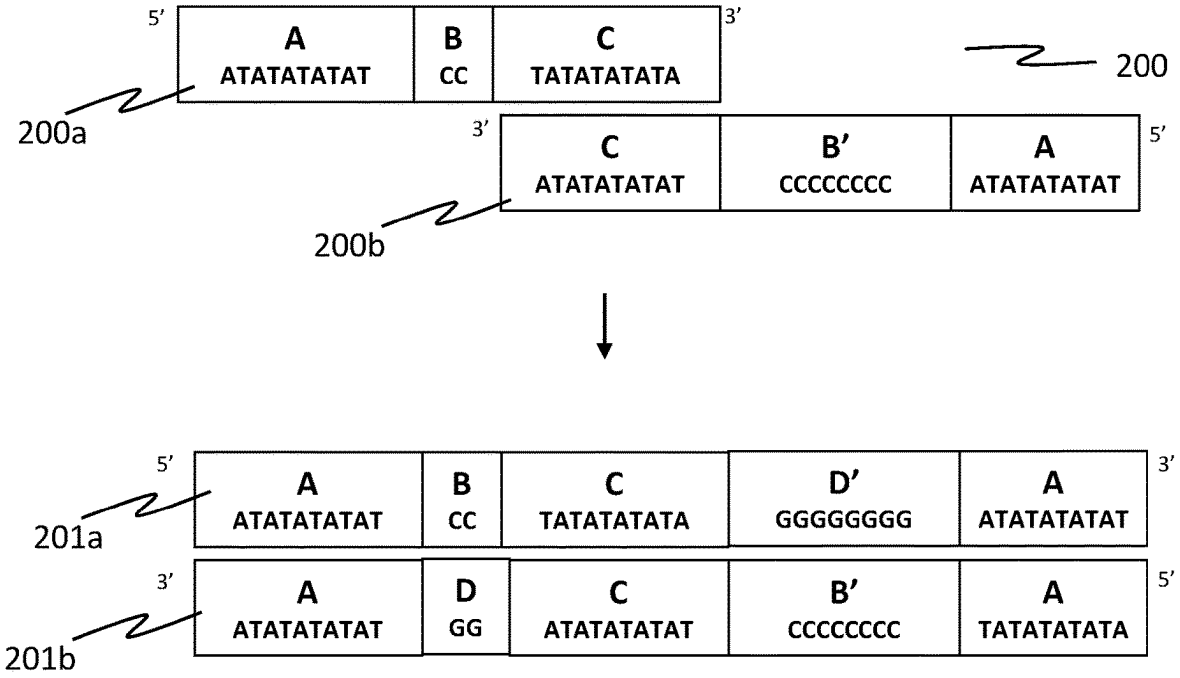

Fluorescence Intensity
75
200
300
400
500
700
1000
1500
2000
3000
5000
10K
20K
Base Pairs
3 Cycles
4 Cycles
10 Cycles
20 Cycles
DNA Ladder

Fig 5B


5'
A
ATATATATAT
B
CC
C
TATATATATA
3'
200
200a
3'
C
ATATATATAT
B'
CCCCCCCC
A
ATATATATAT
5'
200b
5'
A
ATATATATAT
B
CC
C
TATATATATA
D'
GGGGGGGG
A
ATATATATAT
3'
201a
3'
A
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D
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C
ATATATATAT
B'
CCCCCCCC
A
TATATATATA
5'
201b

Fig 6

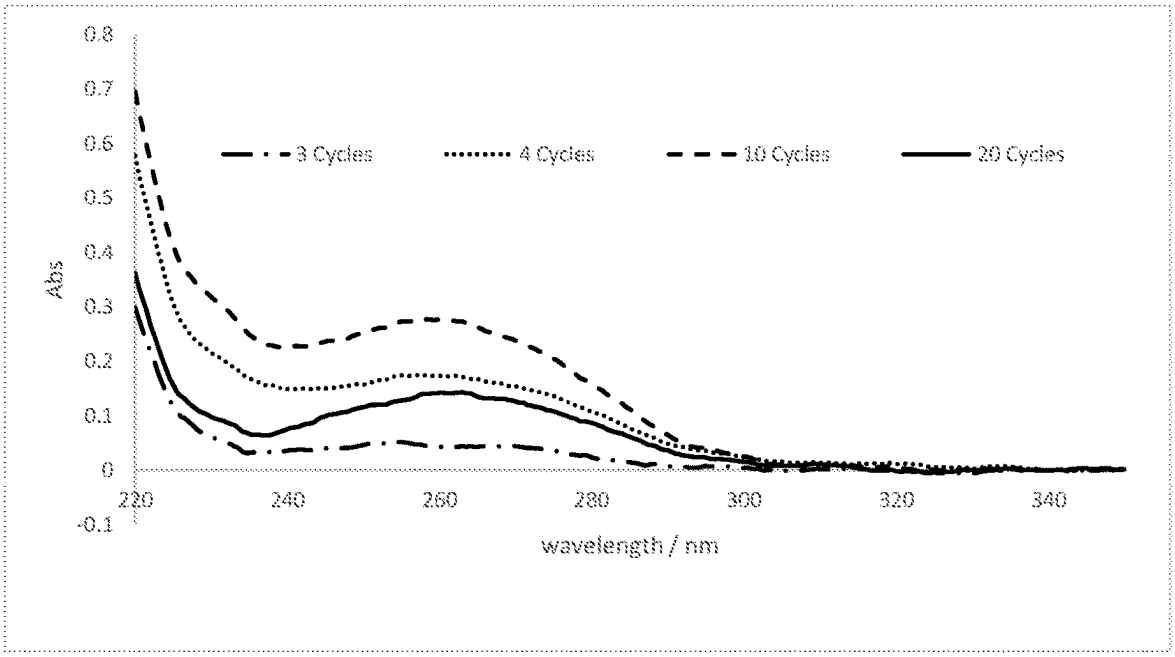
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
Abs
3 Cycles
4 Cycles
10 Cycles
20 Cycles
220
240
260
280
300
320
340
wavelength / nm

Fig 7

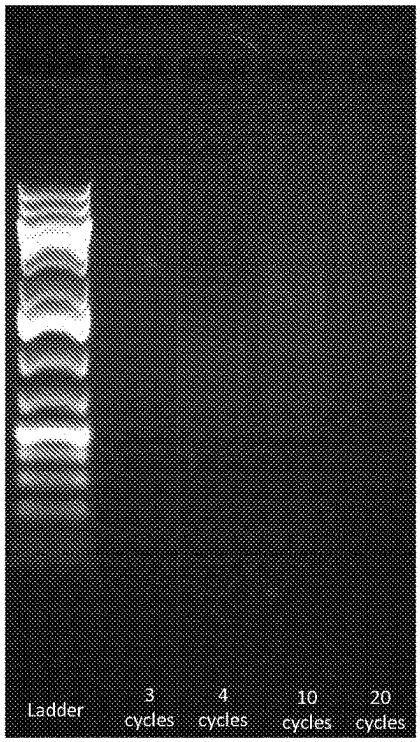
Ladder
3 cycles
4 cycles
10 cycles
20 cycles

Fig 8A

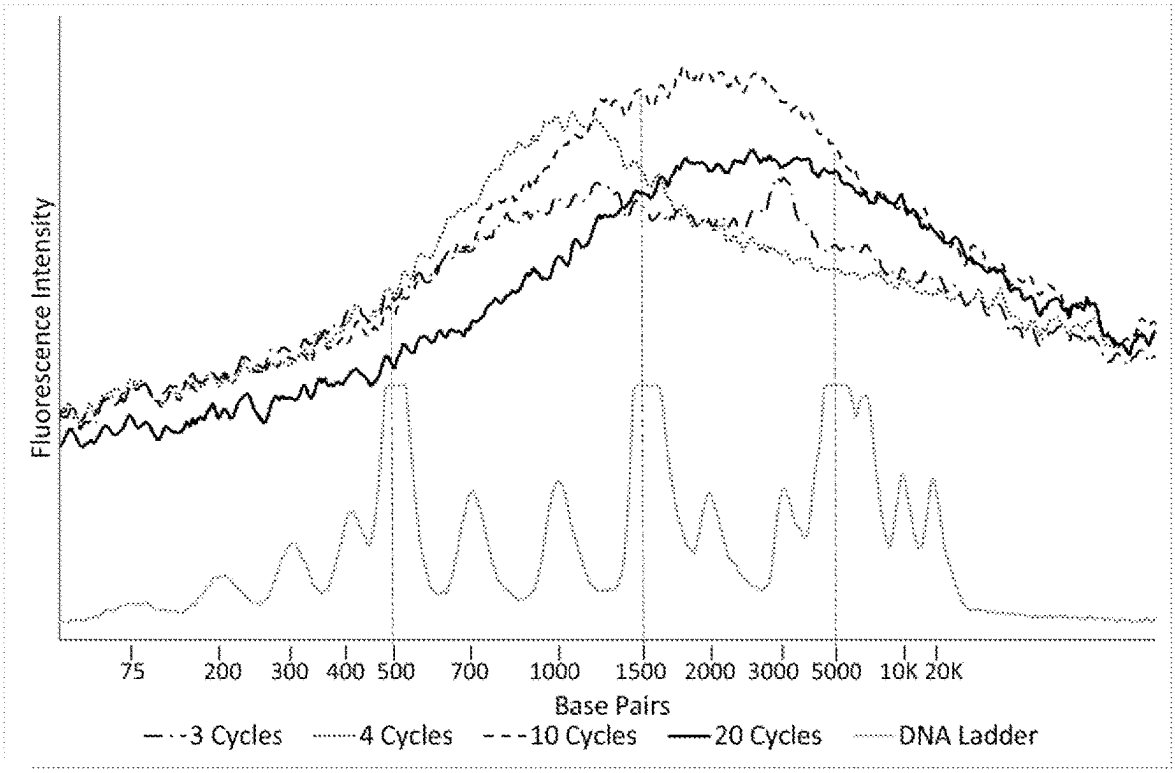
Fluorescence Intensity
75
200
300
400
500
700
1000
1500
2000
3000
5000
10K
20K
Base Pairs
3 Cycles
4 Cycles
10 Cycles
20 Cycles
DNA Ladder

Fig 8B

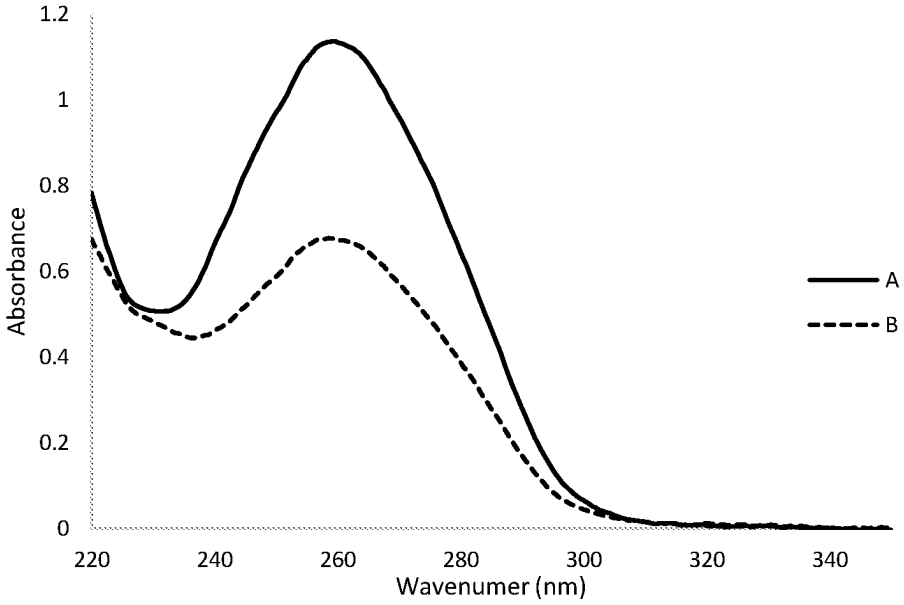
1.2
1
0.8
0.6
0.4
0.2
0
Absorbance
220
240
260
280
300
320
340
Wavenumer (nm)
A
B

Fig 9

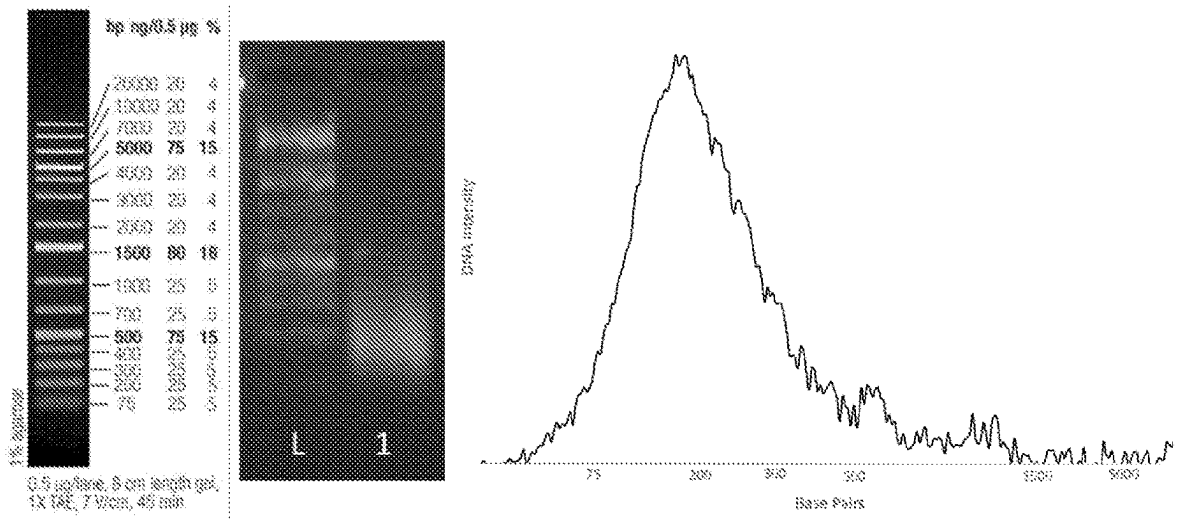

Fig 10A

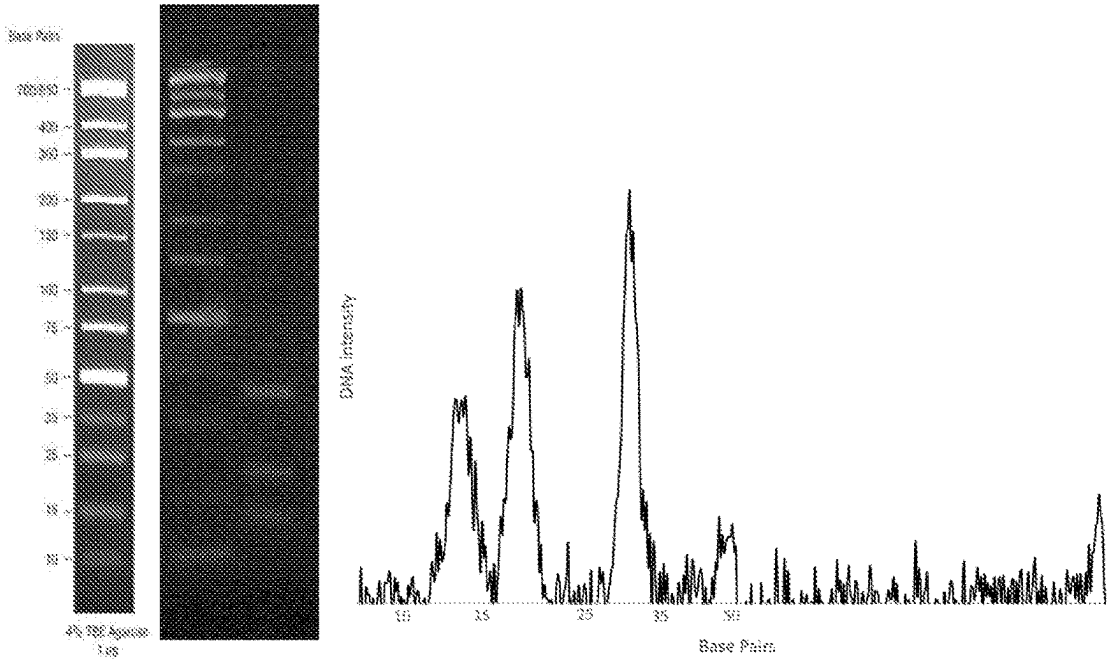

Fig 10B

NUCLEIC ACID AND GENE SYNTHESIS

TECHNICAL FIELD

[0001] The present invention relates to methods of synthesising nucleic acids. The present invention also relates to overlapping primer oligonucleotides for use in nucleic acid synthesis reactions.

BACKGROUND

[0002] Nucleic acid synthesis is the process whereby nucleotides are brought together to form nucleic acids such as deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Nucleic acids can be synthesised using a number of techniques such as phosphoramidite synthesis, the slippage reaction, loop-mediated amplification (LAMP), primer-template extension and thermal cycling amplification.

[0003] The synthesis of nucleic acids containing repeating sequences is challenging for the existing methods of nucleic acid synthesis, particularly in the synthesis of longer sequences (over 200 base pairs (bp)). Typically, the challenges associated with synthesising longer nucleic acid sequences are due to a reduction in yield and quality when producing longer sequences. One of the most commonly used methods of nucleic acid synthesis is column-based oligonucleotide synthesis which employs phosphoramidite synthesis chemistry to sequentially add bases to a growing oligonucleotide sequence on a solid support. This method utilises a four-step process of de-blocking (detritylation), coupling, capping and oxidation which is repeated to build up the oligonucleotide sequence. Once the synthesis is complete, the oligonucleotide is chemically cleaved from the solid support. This process is particularly amenable to automation and is therefore the basis of many commercial gene synthesiser systems. The major drawback with column-based oligonucleotide synthesis is that the yield and quality of the oligonucleotide products decrease with sequence length. This is due to spurious depurination, particularly on adenosines, during the synthesis. These spurious sites promote cleavage of the oligonucleotide backbone resulting in low yields. This reduction in yield and quality means that this method is not suitable for generation of oligonucleotides above 200 bases in length. Phosphoramidite synthesis is also inefficient in the production of sequences having a high GC content, hairpin structures and/or highly repetitive sequences. Alternatively, enzymatic techniques can be used to synthesise nucleic acids. Typically, these methods have fewer restrictions on sequence length when compared to phoshoramidite synthesis methods. Enzymatic techniques utilise a nucleic acid template or primer and polymerase enzyme to synthesise nucleic acid sequences using the polymerase chain reaction (PCR). One such example is polymerase cycling assembly (PCA). PCA uses PCR to extend short overlapping oligonucleotides into a double stranded sequence. Although enzymatic synthesis techniques are suitable for the generation of longer sequences, they are inefficient in the production of highly repetitive sequences and sequences with high GC content.

[0004] Long nucleic acid sequences comprising multiple repeat units have many uses in synthetic biology, diagnostics and therapeutics and therefore there is a need to identify a more efficient method for their production.

SUMMARY OF THE INVENTION

[0005] According to a first aspect of the present invention, there is provided a method for extending the length of a nucleic acid, the method comprising the steps of;

[0006] i) providing at least one overlapping primer oligonucleotide, said overlapping primer oligonucleotide comprising a first single-stranded oligonucleotide and a second single-stranded oligonucleotide, each of the first and second single-stranded oligonucleotides comprising at least a first, second and third sequence, said third sequence being located at the 3' end of each single-stranded oligonucleotide and said second sequence being located between the first and third sequences; wherein the first sequences and the third sequences of the first single-stranded oligonucleotide and the second single-stranded oligonucleotide are self-complementary, palindromic sequences;

wherein the third sequence of the first single-stranded oligonucleotide hybridises to the third sequence of the second single-stranded oligonucleotide to provide an overhanging region at the 5' end of at least one of the first or second single-stranded oligonucleotides;

[0007] (ii) enzymatically extending both the first and second single-stranded oligonucleotides to provide a duplex.

[0008] Advantageously, extension step (ii) incorporates a fourth sequence into the first single-stranded oligonucleotide, said fourth sequence being complementary to the second sequence on the second single-stranded oligonucleotide and wherein a fourth sequence is incorporated into the second single-stranded oligonucleotide, said fourth sequence being complementary to the second sequence on the first single-stranded oligonucleotide.

[0009] Advantageously, the present invention provides an improved method of synthesising nucleic acids. In particular, this method provides a more efficient means to produce longer sequences of nucleic acids which can be designed in accordance with the users requirements.

[0010] Furthermore, the method provides an improved means of synthesising long repeating sequences which are difficult to synthesise using existing methods due to low yields and high error rates.

[0011] Advantageously, having a 3' self-complementary sequence ensures the predictable incorporation of sequences into the oligonucleotides during extension permitting the user more control over the ordering of the sequences in the nucleotide product.

[0012] Typically, a fourth sequence is incorporated into the first and second single-stranded oligonucleotides during step (ii), said fourth sequence on the first single-stranded oligonucleotide being complementary to the second sequence on the second single-stranded oligonucleotide and said fourth sequence on the second single-stranded oligonucleotide being complementary to the second sequence on the first single-stranded oligonucleotide.

[0013] Optionally, the overlapping primer oligonucleotide is a DNA oligonucleotide.

[0014] Optionally, overlapping primer oligonucleotide is an RNA oligonucleotide.

[0015] Optionally, the second sequence on the first single-stranded oligonucleotide differs from the second sequence on the second single-stranded oligonucleotide.

[0016] Advantageously, when the second sequence differs between the first single-stranded oligonucleotide and the

second-single stranded oligonucleotide, a greater variety of sequences can be incorporated into the extending oligonucleotides due to complementary base pairing resulting in two distinct fourth sequences being incorporated into the nucleic acid sequence.

[0017] Preferably, the first and third sequences are each at least 6 bases in length and are preferably at least 8 bases in length.

[0018] Preferably, the second and fourth sequences are each at least 2 bases in length.

[0019] Optionally, at least one of the first and third sequences comprises at least one functional site.

[0020] Optionally the at least one functional site is in addition to the self-complementary, palindromic sequences (and could be considered separate sequences to the self-complementary, palindromic first and third sequences).

[0021] Optionally the at least one functional site is itself a self-complementary, palindromic sequence.

[0022] Optionally, the at least one functional site is a restriction enzyme site and/or a capping site.

[0023] Optionally, the at least one functional site is selected from the following: restriction enzyme site, capping site, chemically reactive modifications to the bases, e.g. alkynes, azides, halo-modifications, sequence specific drug binding site, enzyme inhibitor site.

[0024] In terms of a functional site that is a chemically reactive modification to the bases, the at least one functional site is selected from the following: alkyne-modifications, azide-modifications, halo-modifications.

[0025] In terms of a functional site that is more than one modified base, the at least one functional site is selected from the following: restriction enzyme site, capping site, sequence specific drug binding sites, enzyme inhibitor sites etc.

[0026] Advantageously, the inclusion of a restriction site in one of the sequences provides a means by which the nucleic acid sequence can be readily cut into shorter sequences. For example, sequences A and C may encode two desired oligonucleotides and sequences B and D may encode two restriction enzyme binding sites. In this example, once a long repeating sequence is generated in accordance with the method described herein, the relevant restriction enzymes can be used to readily digest the long repeating sequence into the desired oligonucleotide sequences.

[0027] Optionally, at least one of the first and third sequences comprise tandem repeats.

[0028] Optionally, both the first and third sequences comprise of tandem repeats.

[0029] Optionally, the tandem repeat is a di-nucleotide tandem repeat.

[0030] Advantageously, when the first and third sequences comprise di-nucleotide tandem repeats, controlled incorporation of modified bases into the second and fourth sequences is possible.

[0031] Optionally, at least one of the second and fourth sequences comprises at least one modification selected from the following: modified nucleotides, artificial bases, loop structures.

[0032] Advantageously, modified nucleotides can be readily incorporated into nucleic acid sequences using the method of the present invention. Modified nucleotides are used to alter the characteristic of nucleic acids which is particularly desirable in fields such as nanotechnology, biomedicine and diagnostics.

[0033] Optionally, the modified nucleotides are alkyne, azide or phosphorothioate modified nucleotides.

[0034] Optionally, the modified nucleotides may comprise: 5-Br-dUTP, 7-deaza-7-1-dATP, 6-S-dGTP,5-1-dCTP, 5-(octadiynyl)-dCTP, dye-labelled nucleotides, quencher-labelled nucleotides, intrinsically fluorescent nucleotides, α-phosphate modified nucleotides, y-[(6-aminohexyl)-imido]-ATPαS, α, βnon-hydrolyzable nucleotides, β-Phosphate modified nucleotides, β,γ non-hydrolyzable nucleotides, γ-Phosphate modified nucleotides, non-hydrolyzable di-nucleotides, non-hydrolyzable dye-labeled nucleotides, biotin labelled nucleotides, desthiobiotin labelled nucleotides, digoxigenin labelled nucleotides, DNP (dinitrophenol) labelled nucleotides, photo-labile groups labelled nucleotides, DBCO labelled nucleotides, TCO labelled nucleotides, vinyl labelled nucleotides, free amino group (—NH2) labelled nucleotides, redox dye labelled nucleotides, halogen atom labelled nucleotides, mercury labelled nucleotides, selenium labelled nucleotides, ferrocence labelled nucleotides, analogs and derivatives of cap, analogs and derivatives of puromycin, analogs and derivatives of coenzyme A (CoA), analogs and derivatives of NAD, analogs and derivatives of natural RNA nucleobases, cyclic-di-nucleotides, 3',5'-cyclic nucleotides, 2',3'-cyclic nucleotides, dinucleoside polyphosphates, 6-thio purines, 7-deaza purines, 7-methyl guanosines, substituted pyrimidines, 5-methyl cytidines and related, unmodified and modified ddNTPs 2'-Fluoro-2'-NTPs, 2'-O-Methyl-NTPs, LNA-NTPs, cleavable base labelled dNTP's, N1-modified purines, N6-modified purines, 6-modified purines, 8-oxo guanosines, 2'-deoxy uridines, 3'-deoxy nucleotides, nucleoside bisphosphates, ara-nucleotides, unmodified Purines, modified dNTPs, 3'-O-azidomethyl-dNTPs.

[0035] Optionally, the modified nucleotides comprise a linker which attaches the modification to the nucleotide.

[0036] Advantageously, using a linker to attach the modification to the base prevents the modification interfering with base recognition sites.

[0037] Optionally, the loop structure is a G quadruplex region or a C-motif or intercalated-motif (i-motif) DNA.

[0038] Preferably, the method further comprises the steps:

[0039] (iii) denaturing the duplex to provide a first and second single-stranded polynucleotide;

[0040] (iv) annealing the first and second single-stranded polynucleotides such that overhanging region is provided at the 5' end of at least one of the first or second single-stranded polynucleotides to permit polynucleotide duplex formation between the first and second single-stranded polynucleotides;

[0041] (v) enzymatically extending both the first and second single-stranded polynucleotides to provide a duplex.

[0042] Preferably, steps (iii) to (v) are repeated to increase the length of the nucleic acid sequence.

[0043] Optionally, the overlapping primer oligonucleotide is immobilised on a surface.

[0044] Preferably, the overlapping primer oligonucleotide is immobilised on a surface by the 5' end of one of the first or second single-stranded polynucleotides.

[0045] Preferably, the surface comprises glass, silica, gold, graphene, graphene oxide, epoxy, plastic, metal, gel matrix, template stripped metals or composites thereof.

[0046] Optionally, wherein the overlapping primer oligonucleotide is immobilised to the surface by covalent or non-covalent bonding.

[0047] Optionally, the overlapping primer oligonucleotide is immobilised to a chemically modified region of the surface.

[0048] Optionally, the overlapping primer oligonucleotide is immobilised to the surface by a linker.

[0049] Optionally, the linker comprises a silane linker molecule, a biotin-streptavidin complex, a thiol-Au linker, covalent Si—C bonds to silicon, covalent Si—O bonds to silicon, covalent Si—N bonds to silicon, a nanoparticle linker, or a dynamic covalent bond.

[0050] Preferably the step of providing at least one overlapping primer oligonucleotide comprises obtaining a first single-stranded oligonucleotide and second single-stranded oligonucleotide and hybridising the first and second single-stranded oligonucleotides under appropriate conditions.

[0051] According to a second aspect of the present invention, there is provided an overlapping primer oligonucleotide for extending the length of repeating sequences of nucleic acid, said overlapping primer oligonucleotide comprising two partially complementary single-stranded oligonucleotides, each single-stranded oligonucleotide comprising at least a first, second and third sequence wherein the third sequence is located at the 3' end of the first and second single-stranded oligonucleotide; the first sequences and the third sequences are palindromic, self-complementary sequences; and the second sequence is located in between the first and third sequences; wherein the third sequence of the first single-stranded oligonucleotide hybridises to the third sequence of the second single-stranded oligonucleotide to provide an overhanging region at the 5' end of at least one of the first or second single-stranded oligonucleotides.

[0052] Optionally, the second sequence on the first single-stranded oligonucleotide differs from the second sequence on the second single-stranded oligonucleotide.

[0053] Optionally, the second sequence on the first single-stranded oligonucleotide is not complementary to the second sequence on the second single-stranded oligonucleotide.

[0054] Preferably, the first and third sequences are each at least 6 bases in length and are preferably at least 8 bases in length.

[0055] Preferably, the second sequence is at least 2 bases in length.

[0056] Optionally, at least one of the first and third sequences comprises at least one functional site.

[0057] Optionally, the at least one functional site is selected from the following: restriction enzyme site, capping site.

[0058] Optionally, at least one of the first and third sequences comprise tandem repeats.

[0059] Optionally, both the first and third sequences comprise tandem repeats.

[0060] Optionally, the tandem repeat is a dinucleotide tandem repeat.

[0061] Optionally, the second sequence comprises at least one modification selected from the following: modified nucleotides, artificial bases, loop structures.

[0062] Optionally, the modified nucleotides are alkyne, azide or phosphorothioate modified nucleotides. Optionally, these may include: 5-Br-dUTP, 7-deaza-7-1-dATP, 6-S-dGTP,5-1-dCTP, 5-(octadiynyl)-dCTP.

[0063] Optionally, the modified nucleotides comprise a linker which attaches the modification to the nucleotide.

[0064] Optionally, the loop structure is a G quadruplex region.

[0065] Optionally, the overlapping primer oligonucleotide is a DNA primer oligonucleotide.

[0066] Optionally, overlapping primer oligonucleotide is an RNA primer oligonucleotide.

[0067] Various further features and aspects of the invention are defined in the claims.

[0068] Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this invention belongs.

[0069] A 'palindromic sequence' is a nucleic acid sequence in a double-stranded DNA or RNA molecule whereby reading in a certain direction (e.g. 5' to 3') on one strand is identical to the sequence in the same direction (e.g. 5' to 3') on the complementary strand.

[0070] A 'duplex' is a double stranded nucleic acid sequence comprising two complementary sequences annealed to one another. A "partial duplex" is a double stranded nucleic acid sequence wherein a section of one of the strands is complementary to the other strand and anneals to form a partial duplex, but the full lengths of the strands are not complementary, resulting in a single-stranded polynucleotide tail at least at one end of the partial duplex.

[0071] The terms 'hybridisation' 'binding' and "annealing' (or 'hybridise', 'bind' and 'anneal') in the context of nucleotide sequences, are used interchangeably herein. The ability of two nucleotide sequences to hybridize with each other is based on the degree of complementarity of the two nucleotide sequences, which in turn is based on the fraction of matched complementary nucleotide pairs. The more nucleotides in a given sequence that are complementary to another sequence, the more stringent the conditions can be for hybridization and the more specific the binding of the two sequences will be. Increased stringency is typically achieved by elevating the temperature, increasing the ratio of cosolvents, lowering the salt concentration, and other such methods well known in the field. The term "hybridisation conditions" refers to the reagents and reaction conditions (e.g. temperature, time etc) that are used for hybridisation. Typically, hybridisation conditions may be stringent or moderate. The hybridisation conditions used in the context of the methods described herein permit mismatched duplex formation and therefore may be either moderate or stringent. Preferably, the hybridisation between the unit sequence of the first single-stranded oligonucleotide and a second single-stranded oligonucleotide will form a stable duplex at 65° C. and below. It is preferred that a mismatched duplex may be formed at temperatures up to 65° C., for example between 55° C. and 65° C., optionally for a time period of between 1 to 30 seconds.

[0072] Moderate and stringent conditions are known to those skilled in the art and can be found in available references (e.g., Current Protocols in Molecular Biology, John Wiley & Sons, N.Y., 1989, 6.3.1-6.3.6). Aqueous and non-aqueous methods are described in that reference and either can be used. A preferred example of stringent hybridization conditions are hybridization in 6× sodium chloride/sodium citrate (SSC) at about 45° C., followed by one or more washes in 0.2×SSC, 0.1% (w/v) SDS at 50° C. Another example of stringent hybridization conditions are hybridiza-

tion in 6×SSC at about 45° C., followed by one or more washes in 0.2×SSC, 0.1% (w/v) SDS at 55° C. A further example of stringent hybridization conditions are hybridization in 6×SSC at about 45° C., followed by one or more washes in 0.2×SSC, 0.1% (w/v) SDS at 60° C. Preferably, stringent hybridization conditions are hybridization in 6×SSC at about 45° C., followed by one or more washes in 0.2×SSC, 0.1% (w/v) SDS at 65° C. Particularly preferred stringency conditions (and the conditions that should be used if the practitioner is uncertain about what conditions should be applied to determine if a molecule is within a hybridization limitation of the invention) are 0.5 molar sodium phosphate, 7% (w/v) SDS at 65° C., followed by one or more washes at 0.2×SSC, 1% (w/v) SDS at 65° C.

[0073] The terms 'extension' or 'elongation' in the context of nucleotide sequences, are used interchangeably herein. They refer to the extension of a 3'-end and/or a 5' end of a polynucleotide by the addition of nucleotides or bases. Chain extension relevant to the present invention is generally template dependent, that is, the appended nucleotides are determined by the sequence of a template nucleic acid to which the extending chain is hybridised.

[0074] The term 'enzymatic extension' refers to the extension of a nucleic acid which is catalysed by an enzyme, such as a polymerase enzyme. It is preferred that the polymerase enzyme can be template dependent, such as Deep Vent® polymerase.

[0075] The term "extension conditions" refers to the reagents and reaction conditions (e.g. temperature, time etc) that are used. It describes conditions for extension of the primer polynucleotide. In the present invention, contact between the mismatched duplex, polymerase and nucleotides is under extension conditions that permit polynucleotide extension in a 5' to 3' direction. Appropriate extension conditions are well known in the art. Preferably, extension is performed at a temperature of between about 65° C. and 75° C., optionally for a time period of between 30 to 120 seconds. Appropriate conditions may be found, for example, in Whitfield C J, Turley A T, Tuite E M, Connolly B A, Pike A R. Enzymatic Method for the Synthesis of Long DNA Sequences with Multiple Repeat Units. Angewandte Chemie International Edition 2015, 54(31), 8971-8974.

[0076] The term 'complementary' in the context of nucleotide sequences refers to when the sequence of one can bind to the sequence of the other in an anti-parallel sense wherein the 3'-end of one sequence binds to the 5'-end of the other sequence and each A, T(U), G, and C of one sequence is then aligned with a T(U), A, C, and G, respectively, of the other sequence.

[0077] The term 'amplification', as applied to nucleic acids refers to any method that results in the formation of one or more copies of a nucleic acid, where preferably the amplification is exponential. One such method for enzymatic amplification of specific sequences of DNA is known as the polymerase chain reaction (PCR), as described by Saiki et al, 1986, Science 230:1350-1354.

[0078] The terms 'nucleic acid' and 'polynucleotide' are interchangeable and refer to any nucleic acid, whether DNA, RNA, cDNA, DNA-RNA, peptide nucleic acid (PNA), a hybrid or any mixture of the same.

[0079] The terms 'nucleic acid', 'polynucleotide' and 'nucleotide' also specifically include nucleic acids composed of synthetic bases (i.e. bases other than the five biologically occurring bases —adenine, guanine, thymine, cytosine and uracil), modified bases or any combination of biologically occurring bases, synthetic bases and modified bases.

[0080] The term 'SS oligonucleotide' refers to 'single stranded oligonucleotide'.

[0081] The polynucleotides of the present invention can be from a human or non-human mammal, or any other organism, derived from any recombinant source, synthesized in vitro or by chemical synthesis.

BRIEF DESCRIPTION OF THE DRAWINGS

[0082] Embodiments of the present invention will now be described by way of example only with reference to the accompanying drawings where like parts are provided with corresponding reference numerals and in which:

[0083] FIG. **1** provides a schematic representation of the method according to the present invention.

[0084] FIG. **2** shows ultraviolet absorption of the PCR product after an increasing number of PCR cycles with the double-stranded primer oligonucleotide of example 1.

[0085] FIG. **3**A shows a visualisation of gel electrophoresis of the PCR products after 3, 4, 10 and 20 PCR cycles with the double-stranded primer oligonucleotide of example 1. FIG. **3**B shows the fluorescence intensity of the gel electrophoresis shown in FIG. **3**A.

[0086] FIG. **4** shows ultraviolet absorption of the PCR product after an increasing number of PCR cycles with the double-stranded primer oligonucleotide of example 2.

[0087] FIG. **5**A shows a visualisation of gel electrophoresis of the PCR products after 3, 4, 10 and 20 PCR cycles with the double-stranded primer oligonucleotide of example 2. FIG. **5**B shows the fluorescence intensity of the gel electrophoresis shown in FIG. **5**A.

[0088] FIG. **6** shows the double-stranded primer oligonucleotide of example 2 and the extended polynucleotide product.

[0089] FIG. **7** shows ultraviolet absorption of the PCR product after an increasing number of PCR cycles with the double-stranded primer oligonucleotide of example 3.

[0090] FIG. **8**A shows a visualisation of gel electrophoresis of the PCR products after 3, 4, 10 and 20 PCR cycles with the double-stranded primer oligonucleotide of example 3. FIG. **8**B shows the fluorescence intensity of the gel electrophoresis shown in FIG. **8**A.

[0091] FIG. **9** shows the UV/vis spectra of the extended DNA following purification.

[0092] FIG. **10** (A) shows a visualisation of gel electrophoresis of the extended DNA against the Gene Ruler 1kb plus DNA ladder, the fluorescence intensity of the gel electrophoresis was measured and analysed using Image-J software.

[0093] FIG. **10** (B) shows a visualisation of gel electrophoresis of the digested products against a low range ladder, the fluorescence intensity of the gel electrophoresis was measured and analysed using Image-J software, the results are shown.

DETAILED DESCRIPTION

[0094] FIG. **1** depicts a schematic representation of the method of the present invention. The starting overlapping primer oligonucleotide **100** comprises two single-stranded (SS) oligonucleotides **100***a*, **100***b*. Each of the SS oligonucleotides **100***a*, **100***b* comprises sequences A, B and C.

The first SS oligonucleotide **100***a* comprises sequences A, B and C in a 5' to 3' direction. The second SS oligonucleotide **100***b* comprises sequences C, B and A in a 3' to 5' direction.

[0095] In this embodiment, the A sequences are palindromic, self-complementary sequences and the C sequences are palindromic, self-complementary sequences and all sequences are identical in each of the SS oligonucleotides. Sequence B is complementary to a fourth sequence, sequence D, which will be incorporated into the SS oligonucleotides by complementary base pairing as described below. Sequences A and C are at least 6, preferably 8 bases in length. Sequences B and D are at least 2 bases in length.

[0096] It would be apparent to the skilled person that the nucleotide sequences of sequences B and D could differ between the first and second SS oligonucleotides provided that sequence B of the first SS oligonucleotide is complementary to sequence D of the second SS oligonucleotide and that sequence B of the second SS oligonucleotide is complementary to sequence D of the first SS oligonucleotide.

[0097] The overlapping primer oligonucleotide **100** is formed by the hybridisation of sequence C of the first SS oligonucleotide **100***a* with sequence C of the second SS oligonucleotide **100***b*. This hybridisation results in a partial duplex with an overhanging region on the 5' end of each of the SS oligonucleotide sequences **100***a*, **100***b*. The overhanging regions of each SS oligonucleotide comprise both sequence A and B.

[0098] The overlapping primer oligonucleotide **100** is extended 101 in the presence of a suitable polymerase and nucleotides under extension conditions. Any suitable polymerase can be used, for example Deep Vent DNA polymerase (New England Biolabs). Several well-known thermostable 5' to 3' polymerases are available and may be used in the methods described herein. It is preferred that a polymerase is thermostable and highly stable, such that its activity is substantially retained during prolonged incubation necessary for the extension reaction. The polymerase preferably has high processivity. It is preferred that a polymerase does not display non-specific nuclease activity. A polymerase preferably has good fidelity but can also accept a range of nucleotide analogues as both templates and substrates. A high-fidelity polymerase with highly efficient proof-reading activity is not particularly suitable. Preferably, a polymerase lacks 3'-95' exonuclease activity [3'>5' exo(-)], thereby possessing lower fidelity due to absence of proof-reading function. A person skilled in the art can determine whether a particular polymerase possesses required properties as defined above. Exemplary polymerases include, but are not limited to, Thermococcus gorgonarius family B polymerase (Tgo-Pol) enzyme variant, Z3 (Tgo-Pol Z3) exo(-) [Jozwiakowski et al., 2011 Chembiochem 12: 35-37], Deep Vent exo(-) (New England Biolabs), Vent exo(-) (New England Biolabs), Pfu exo(-) (Agilent Technologies), and Taq polymerase (many suppliers).

[0099] The extension of the overlapping primer oligonucleotides can be carried out using the PCR reaction. This results in the extension of the SS oligonucleotides **100***a*, **100***b* in the overhang regions to create a stable duplex **107**. In this step, a fourth sequence (D) is incorporated into the SS oligonucleotides **100***a*, **100***b*. Sequence D is the complementary sequence to sequence B.

[0100] In order to further extend the sequences, the stable duplex **107** is denatured **102** and reannealed under suitable conditions resulting in a second partial duplex **108**. The second partial duplex **108** is formed by the hybridisation of sequence A of the first SS oligonucleotide **100***a* with sequence A of the second SS oligonucleotide **100***b*. This hybridisation results in mismatched duplex with an overhanging region on the 5' end of each of the SS oligonucleotide sequences **100***a*, **100***b*. The overhanging regions on each SS oligonucleotide comprise sequences B, C and D once and sequence A twice.

[0101] In some embodiments the sequences are designed to have different melting temperatures (Tms) which allows the amount of mismatch to be controlled, however when Tms are similar then it is expected that 50% will form a mismatched duplex that can be extended.

[0102] The second partial duplex **108** is extended **103** in the presence of a suitable polymerase and nucleotides under extension conditions. This results in the extension of the SS oligonucleotides **100***a*, **100***b* in the overhanging regions to create a second stable duplex **109**. Further sequences A, B, C and D are incorporated into each of the SS oligonucleotides **100***a*, **100***b* such that sequences B, C and D appear in each SS oligonucleotide twice and sequence A appears three times.

[0103] To further extend the SS oligonucleotides **100***a*, **100***b* the second stable duplex **109** is denatured **104**. The further extension may occur under maximum overlap conditions **105** or minimum overlap conditions **106**. Under maximum overlap conditions **105**, the SS oligonucleotides anneal **105***a* such that there is a maximum amount of overlap between the first and second SS oligonucleotides **100***a*, **100***b*. In maximum overlap conditions **105**, a third partial duplex **110** is formed by the hybridisation of sequences A, B, C, D and A of the first SS oligonucleotide **100***a* with sequences A, D, C, B and A of the second SS oligonucleotide **100***b*. This hybridisation results in a third mismatched duplex **110** with an overhanging region on the 5' end of each of the SS oligonucleotide sequences **100***a*, **100***b*. The overhanging regions on each SS oligonucleotide comprise sequences A, B, C and D.

[0104] The reaction can be directed towards minimum overlap conditions by altering the temperatures of the denaturing and/or annealing steps. For example, increasing the denaturing and annealing temperatures can direct the reaction to minimum overlap conditions. It is also possible that, after a few cycles of extension reactions, increasing only the annealing temperature can direct the reaction to minimum overlap conditions. Directing the reaction to minimum overlap conditions is advantageous as it leads to products which are on average longer.

[0105] The third partial duplex **110** is extended **105***b* in the presence of a suitable polymerase and nucleotides under extension conditions. This results in the extension of the SS oligonucleotides **100***a*, **100***b* in the overhang regions to create a third stable duplex **111**. Further sequences A, B, C and D are incorporated into each of the SS oligonucleotides **100***a*, **100***b* such that each SS oligonucleotide contains sequences B, C and D three times and sequence A four times.

[0106] Under minimum overlap conditions **106**, the SS oligonucleotides anneal **106***a* such that there is a minimum amount of overlap between the first and second SS oligonucleotides **100***a*, **100***b*. In minimum overlap conditions **106**, a fourth partial duplex **112** is formed by the hybridisation of sequence A of the first SS oligonucleotide **100***a* with sequence A of the second SS oligonucleotide **100***b*. This hybridisation results in a fourth partial duplex **112** with

an overhanging region on the 5' end of each of the SS oligonucleotide sequences **100***a*, **100***b*. The overhanging regions on each SS oligonucleotide comprise sequences A, B, C and D twice.

[0107] The fourth partial duplex **112** is extended **106***b* in the presence of a suitable polymerase and nucleotides under extension conditions. This results in the extension of the SS oligonucleotides **100***a*, **100***b* in the overhanging regions to create a fourth stable duplex **113**. Two further of sequences A, B, C and D are incorporated into each of the SS oligonucleotides **100***a*, **100***b* such that each SS oligonucleotide now contains sequences B, C and D four times and sequence A 5 times.

[0108] The denaturing **104**, annealing **105***a*, **106***a* and extension steps **105***b*, **106***b* can be repeated any number of times to continue extending the sequences.

Example 1: $^{5'}$-$(AT)_5C_2(TA)_s$-$^{3'/3'}$-$(AT)_5C_2(TA)_5$-$^{5'}$

[0109] In one example of the present invention, the starting overlapping primer oligonucleotide comprised a first SS oligonucleotide and a second SS oligonucleotide. All custom oligonucleotides were purchased from Eurofins (Ebersberg, Germany). Each of the first and second SS oligonucleotides comprised identical sequences A, B and C. The nucleotide sequences of sequences A, B and C are shown in Table 1.

TABLE 1

Example 1 nucleotide sequences

Sequence name	Nucleotide sequence	
Sequence A	ATATATATAT	SEQ ID 1
Sequence B	CC	SEQ ID 2
Sequence C	TATATATATA	SEQ ID 3
Sequence D	GG	SEQ ID 4

[0110] The first SS oligonucleotide comprises the sequences in the following order: 5'-ABC-3' (SEQ ID 5) and the second SS oligonucleotide comprises the sequences in the following order 3'-CBA-5' (SEQ ID 6).

[0111] Sequences A and C are palindromic, self-complementary sequences and therefore the first and second SS oligonucleotides hybridise to form the overlapping primer oligonucleotide, which is a duplexwith 5' overhanging regions containing sequences A and B as shown in FIG. **1** (**100**). The overlapping primer oligonucleotide length is 22 base pairs. Sequence D is complementary to sequence B and will be incorporated into the oligonucleotides during the enzymatic extension. The nucleotide sequence of sequence D is shown in Table 1 above.

[0112] In the present example, sequences A and C comprise dinucleotide repeats of bases A and T. When sequences A and C are palindromic, self-complementary sequences and contain only 2 of the 4 DNA nucleotide bases (in this example A and T), modified bases can be incorporated into sequences B and D in a controlled manner. Advantageously, this allows the user greater control over where the modified bases incorporate and therefore greater control over the composition and characteristics of the extended polynucleotide product. The skilled person would understand that this is only one example of the present invention and in other examples, sequences A and C may contain sequences other than dinucleotide repeats. Likewise, it would be understood by the skilled person that in other examples sequences B and D may contain sequences other than mononucleotide repeats.

[0113] FIG. **2** shows ultraviolet absorption of the PCR product after an increasing number of PCR cycles. Increased absorption of UV light at a wavelength of 260 nm indicates increased DNA concentration. FIG. **2** shows that the concentration of DNA increases with an increased number of PCR cycles, demonstrating generation of new DNA with increasing PCR cycle numbers.

[0114] FIG. **3**A shows a visualisation of gel electrophoresis of the PCR products after 3, 4, 10 and 20 PCR cycles. The fluorescence intensity of the gel electrophoresis was measured and analysed using Image-J software, the results are shown in FIG. **3**B. As can be seen in FIG. **3**B, after 20 PCR cycles DNA products can be detected of between 1000 and 5000 base pairs in length.

Example 2: $^{5'}$-$(AT)_5C_4(TA)_5$-$^{3'/3'}$-$(AT)_5C_4(TA)_5$-$^{5'}$

[0115] In another example of the present invention, the starting overlapping primer oligonucleotide comprised a first and second SS oligonucleotide. Each of the first and second SS oligonucleotides comprised identical sequences A, B and C. The nucleotide sequences of sequences A, B and C are shown in Table 2.

TABLE 2

Example 2 nucleotide sequences

Sequence name	Nucleotide sequence	
Sequence A	ATATATATAT	SEQ ID 7
Sequence B	CCCC	SEQ ID 8
Sequence C	TATATATATA	SEQ ID 9
Sequence D	GGGG	SEQ ID 10

[0116] The first SS oligonucleotide comprises the sequences in the following order: $^{5'}$-ABC-$^{3'}$ (SEQ ID 11) and the second SS oligonucleotide comprises the sequences in the following order $^{3'}$-CBA-$^{5'}$ (SEQ ID 12).

[0117] Sequences A and C are palindromic, self-complementary sequences and therefore the first and second SS oligonucleotides hybridise to form a duplex with 5' overhang containing sequences A and B as shown in FIG. **1** (**100**). Sequence D is complementary to sequence B and will be incorporated into the oligonucleotides during the enzymatic extension. The nucleotide sequence of sequence D is shown in Table 2 above.

[0118] UV absorbance was measured as described in example 1. FIG. **4** shows the UV absorbance data. Increased absorption of UV light at a wavelength of 260 nm indicates increased DNA concentration. As can be seen in FIG. **4**, the DNA concentration in the PCR product increases with an increased number of PCR cycles, demonstrating generation of new DNA with increasing PCR cycle numbers.

[0119] FIG. **5**A shows a visualisation of gel electrophoresis of the PCR products after 3, 4, 10 and 20 PCR cycles. The fluorescence intensity of the gel electrophoresis was measured and analysed using Image-J software, the results are

shown in FIG. **5**B. As can be seen in FIG. **5**B, after 20 PCR cycles DNA products can be detected of between 1000 and 5000 base pairs in length.

Example 3: $^{5'}$-$(AT)_5C_2(TA)_5$-$^{3'/3'}$-$(AT)_5C_8(TA)_5$-$^{5'}$

[0120] In another example of the present invention, the starting overlapping primer oligonucleotide comprised a first and second SS oligonucleotide.

[0121] The first SS oligonucleotide comprised sequence A, sequence B and sequence C. The second SS oligonucleotide comprised sequence A, sequence B' and sequence C. The nucleotide sequences of sequences A, B, C and B' are shown in Table 3.

TABLE 3

Example 3 nucleotide sequences

Sequence name	Nucleotide sequence	
Sequence A	ATATATATAT	SEQ ID 13
Sequence B	CC	SEQ ID 14
Sequence C	TATATATATA	SEQ ID 15
Sequence D	GG	SEQ ID 16
Sequence B'	CCCCCCCC	SEQ ID 17
Sequence D'	GGGGGGGG	SEQ ID 18

[0122] The first SS oligonucleotide comprises the sequences in the following order: $^{5'}$-ABC-$^{3'}$ (SEQ ID 19) and the second SS oligonucleotide comprises the sequences in the following order $^{3'}$-CB'A-$^{5'}$ (SEQ ID 20).

[0123] As in the previous examples, the A sequences are palindromic, self-complementary sequences and the C sequences are palindromic, self-complementary sequences. As shown in FIG. **6**, a duplex **200** is formed by the hybridisation of sequence C in the first SS oligonucleotide **200***a* and second SS oligonucleotide **200***b*. The duplex **200** comprises a 5' overhang of sequences A and B on the first SS oligonucleotide **200***a* and sequences B' and A on the second SS oligonucleotide **200***b*.

[0124] During the enzymatic extension, complementary base pairing occurs to extend the first and second SS oligonucleotides at the 3' end. The first SS oligonucleotide is extended to include a sequence D' and A in an extended first SS oligonucleotide **201***a*. Sequence D' is complementary to sequence B'. The second SS oligonucleotide is extended to include sequence A and D in a second extended SS oligonucleotide **201***b*. Sequence D is complementary to sequence B. The nucleotide sequences of sequences D and D' can be found above in Table 3.

[0125] UV absorbance was measured as described in example 1. FIG. **7** shows the UV absorbance data. Increased absorption of UV light at a wavelength of 260 nm indicates increased DNA concentration. As can be seen in FIG. **7**, the DNA concentration in the PCR product increases with an increased number of PCR cycles, demonstrating generation of new DNA with increasing PCR cycle numbers.

[0126] FIG. **8**A shows a visualisation of gel electrophoresis of the PCR products after 3, 4, 10 and 20 PCR cycles. The fluorescence intensity of the gel electrophoresis was measured and analysed using Image-J software, the results are shown in FIG. **8**B. As can be seen in FIG. **8**B, after 20 PCR cycles DNA products can be detected of between 1000 and 5000 base pairs in length.

Materials and Methods

Overlapping Primer Oligonucleotide Preparation

[0127] The overlapping primer oligonucleotides (sequences shown in tables 1, 2 and 3) were prepared by adding the first and second SS oligonucleotides (10 μL of each SS oligonucleotide (100 μM)) to HEPES and potassium acetate DNA annealing buffer (480 μL, 1λ) in an Eppendorf. HEPES and potassium acetate buffer is made from 10 mM HEPES, 100 mM KCl and 1 mM EDTA. The solution was vortexed for a few seconds to ensure thorough mixing, and then heated to 95° C. for 10 minutes before being cooled slowly to room temperature (roughly 25° C.). When not in use, overlapping primer oligonucleotides were stored at −20° C. in the freezer.

DNA Extension

[0128] Nanopure-H_2O (60 μL) was added to 10 μL each of dATP, dTTP, dCTP, dGTP (all 100 mM) to provide a 100 μL dNTP mix of 10 mM concentration. Primer-specific dNTP mixtures were prepared for each of the above examples by varying the volume of dNTPs added, depending on the ratio of bases present in the overlapping primer oligonucleotide. For example, the overlapping primer oligonucleotide used in example 1 ($(AT)_5C_2(TA)_5/(AT)_5C_2(TA)_5$) has an A:T:C:G ratio of 5:5:1:1 and dNTPs were mixed at the same A:T:C:G ratio. For example, dATP (16.6 μL, 100 mM), dTTP (16.6 μL, 100 mM), dCTP (3.4 μL, 100 mM), and dGTP (3.4 μL, 100 mM) were added to nanopure-H_2O (60 μL) to make up the dNTP mix used in example 1.

Heat-Cool Cycles

[0129] The extension reaction mixture was prepared in a thin-walled 200 μL Eppendorf by adding nanopure-H_2O (36 μL) to ThermoPol buffer solution (5 μL, 10×), overlapping primer oligonucleotide (5 μL, 2 μM), primer-specific dNTP mix (2.5 μL, 10 mM), and $MgSO_4$ (1 μL, 100 mM). The mixture was then vortexed for a few seconds before adding DeepVent DNA polymerase enzyme (0.5 μL, 1U). At 75° C., one unit of DeepVent DNA polymerase enzyme can incorporate 10 nmol of dNTPs. To not destroy the polymerase enzyme, the mixture was mixed gently with a pipette tip instead of using a vortex. After 10 cycles, more primer-specific dNTP mix (2.5 μL, 10 mM) was added, along with polymerase enzyme (0.5 μL, 1U). The Eppendorf 15 was then placed back in the thermocycler on the same setting for a further 10 cycles (20 cycles in total). When not in use, reagents and products were stored at −20° C.

[0130] Each cycle consisted of:

[0131] 1) Denaturing at 95° C. for 30 seconds.

[0132] 2) Annealing at 55° C. for 30 seconds.

[0133] 3) Elongation at 72° C. for 120 seconds.

[0134] After 10 cycles, the thermocycler cooled the mixture to 4° C. and held the mixture at this temperature for as long as required.

[0135] Different numbers of cycles were performed in each of the above examples. For 20 cycles, the procedure outlined above was followed. For 10 cycles, the first half of the procedure outlined above was followed (i.e., experiment

was stopped after the first 10 cycles, thus not requiring the second addition of dNTP mix and polymerase enzyme).

Purification of Extended DNA Product

[0136] For purification, the Monarch® PCR and DNA Cleanup Kit was used. DNA binding buffer (100 μL) was added to the extended DNA solution and mixed with a pipette before transferring the whole sample to a thick-walled tube and column. The column was centrifuged at 13000 RPM for 1 minute, then the discarded buffer was disposed of in the aqueous waste. DNA wash buffer (200 μL) was added to the column, which was then centrifuged at 13000 RPM for 1 minute. This step was repeated, then all the wash solution was disposed of in the aqueous waste. The column was centrifuged again at 13000 RPM for 1 minute to ensure all excess liquid had been removed and the column was fully dry—any waste was disposed of in the aqueous waste. A 1.5 mL Eppendorf tube was heated to 65° C. in the heating block, into which the column (with DNA attached to the silica) was placed. This heating step was to increase yield. Elution buffer (20 μL) was added to the column in the heating block and was kept at 65° C. for 5 minutes. The sample was centrifuged for a final time at 13000 RPM for 1 minute. The purified extended DNA product was collected in the 1.5 mL Eppendorf tube. All binding/wash/elution buffers were stored at room temperature.

UV/Vis Spectroscopy

[0137] UV/Vis spectra, overlapping primer oligonucleotide concentrations, and purity ratios were recorded using a Thermo Scientific™ NanoDrop™ One Microvolume-UV/Vis Spectrophotometer allowing analysis of 1 μL samples, using surface tension to hold the small volume of sample in place between two pedestals. Most measurements of samples and blanks were recorded using the dsDNA setting. Blanks were obtained using Monarch® DNA Elution Buffer each time before performing measurements of each DNA sample. Between each measurement, a lint free wipe was used to clean the pedestals and limit any contamination between samples. An absorption band at 260 nm would confirm the presence of DNA.

Agarose Gel Electrophoresis

[0138] Gels were electrophoresed at 100V (400 mA) for 60 to 90 minutes. All gels were imaged using UviProMW1 software and a Uvitec Chemiluminescence fluorescence imaging box. All gels were analysed using ImageJ software, converting the data into graphs using Microsoft Excel.

Example 4: Inclusion of a Restriction Site

[0139] In another example of the present invention, a restriction site is included in the starting overlapping primer oligonucleotide. After extension the restriction site can be used to cut the nucleic acid to give two different length oligonucleotides. The nucleotide sequences of sequences A, B, C, D and E are shown in Table 4.

TABLE 4

Example 4 nucleotide sequences

Sequence name	Nucleotide sequence	
Sequence A	5'-TGGACTCTCTCA	SEQ ID 21
Sequence B	5'-GATATC	SEQ ID 22
Sequence C	5'-ATCGACT	SEQ ID 23
Sequence D	5'-AGTCGAT	SEQ ID 24
Sequence E	5'-TGAGAGAGTCCA	SEQ ID 25

In this example:

[0140] A is complementary to E;

[0141] C is complementary to D; and

[0142] B is a self-complementary restriction site which splits into two portions and adds 3 bases to each end of the oligomers after restriction digest.

[0143] After extension the product is long double-stranded 5'-[ABCB]$_n$-3'/3'-[EBDB]$_n$-5'

[0144] Restriction enzyme treatment yields two double stranded oligomers of 18 and 13 bases, derived from the A/E complementary sequence and the C/D complementary sequence respectively:

18 mer: (SEQ ID 26)

5'-ATCTGGACTCTCTCAGAT-3' (A)

3'-GATACCTGAGAGAGTCTA-5'(E)

13 mer: (SEQ ID 27)

5'-ATCATCGACTGAT-3' (C)

3'-GATTAGCTGACTA-3' (D)

[0145] The overlapping primer oligonucleotides (sequences shown below) were prepared by adding the first and second ss oligonucleotides (10 μL of each ss oligonucleotide (100 μM)) to HEPES and potassium acetate DNA annealing buffer (480 μL, 1λ) in an Eppendorf. HEPES and potassium acetate buffer is made from 10 mM HEPES, 100 mM KCl and 1 mM EDTA. The solution was vortexed for a few seconds to ensure thorough mixing, and then heated to 95° C. for 10 minutes before being cooled slowly to room temperature (roughly 25° C.). When not in use, overlapping primer oligonucleotides were stored at −20° C. in the freezer.

5'TGG ACT CTC TCAGAT ATCATC GAC T 3'

3'TAG CTGACT ATA GACCTG AGA GAG T 5'

[0146] Nanopure-H_2O (60 μL) was added to 10 μL each of dATP, dTTP, dCTP, dGTP (all 100 mM) to provide a 100 μL dNTP mix of 10 mM concentration.

[0147] The extension reaction mixture was prepared in a thin-walled 200 μL Eppendorf by adding nanopure-H_2O (36 μL) to ThermoPol buffer solution (5 μL, 10λ), overlapping primer oligonucleotide (5 μL, 2 μM), primer-specific dNTP mix (2.5 μL, 10 mM), and $MgSO_4$ (1 μL, 100 mM). The mixture was then vortexed for a few seconds before adding DeepVent DNA polymerase enzyme (0.5 μL, 1U). At 75° C.,

one unit of DeepVent (exo-) DNA polymerase enzyme can incorporate 10 nmol of dNTPs. To not destroy the polymerase enzyme, the mixture was mixed gently with a pipette tip instead of using a vortex. After 10 cycles, more primer-specific dNTP mix (2.5 μL, 10 mM) was added, along with polymerase enzyme (0.5 μL, 1U). The Eppendorf was then placed back in the thermocycler on the same setting for a further 10 cycles (20 cycles in total). When not in use, reagents and products were stored at −20° C.

[0148] Each cycle consisted of:

[0149] 1) Denaturing at 95° C. for 30 seconds.

[0150] 2) Annealing at 55° C. for 30 seconds.

[0151] 3) Elongation at 72° C. for 120 seconds.

[0152] After 20 cycles, the thermocycler cooled the mixture to 4° C. and held the mixture at this temperature for as long as required.

[0153] For purification, the Monarch® PCR and DNA Cleanup Kit was used. DNA binding buffer (100 μL) was added to the extended DNA solution and mixed with a pipette before transferring the whole sample to a thick-walled tube and column. The column was centrifuged at 13000 RPM for 1 minute, then the discarded buffer was disposed of in the aqueous waste. DNA wash buffer (200 μL) was added to the column, which was then centrifuged at 13000 RPM for 1 minute. This step was repeated, then all the wash solution was disposed of in the aqueous waste. The column was centrifuged again at 13000 RPM for 1 minute to ensure all excess liquid had been removed and the column was fully dry—any waste was disposed of in the aqueous waste. A 1.5 mL Eppendorf tube was heated to 60° C. in the heating block, into which the column (with DNA attached to the silica) was placed. This heating step was to increase yield. Elution buffer (20 μL) was added to the column in the heating block and was kept at 65° C. for 5 minutes. The sample was centrifuged for a final time at 13000 RPM for 1 minute. The purified extended DNA product was collected in the 1.5 mL Eppendorf tube. All binding/wash/elution buffers were stored at room temperature.

UV/Vis Spectroscopy

[0154] UV/Vis spectra, as shown in FIG. **9**, overlapping primer oligonucleotide concentrations, and purity ratios were recorded using a Thermo Scientific™ NanoDrop™ One Microvolume-UV/Vis Spectrophotometer allowing analysis of 1 μL samples, using surface tension to hold the small volume of sample in place between two pedestals. Most measurements of samples and blanks were recorded using the dsDNA setting. Blanks were obtained using Monarch® DNA Elution Buffer each time before performing measurements of each DNA sample. Between each measurement, a lint free wipe was used to clean the pedestals and limit any contamination between samples. An absorption band at 260 nm confirms the presence of DNA.

Agarose Gel Electrophoresis

[0155] Gels were electrophoresed at 100 V (400 mA) for 60 to 90 minutes. All gels were imaged using UviProMW1 software and a Uvitec Chemiluminescence fluorescence imaging box. All gels were analysed using ImageJ software, converting the data into graphs using Microsoft Excel.

Digestion

[0156] Reaction mixture was made up using the extended dsDNA product, rCutSmart Buffer and EcoRV HF in the concentrations and volumes in table 5. This reaction mixture was heated to 37° C. for 3 hours before purple loading dye was added to deactivate the enzyme.

TABLE 5

restriction enzyme digest reaction components

	Initial concentration	Volume (μL)	Final concentration
DNA	57 ng/μL	19	20 ng/μL
Water		25	
rCutSmart Buffer	10X	5	1X
EcoRV-HF		0.4	40 units

[0157] A 4% MetaPhor agarose gel was used to run the digestion product alongside a low range ladder and the gel electrophoresis was analysed using Image-J as shown in FIG. **10**.

[0158] FIG. **10** (A) shows a visualisation of gel electrophoresis of the extended DNA against the Gene Ruler 1kb plus DNA ladder, the fluorescence intensity of the gel electrophoresis was measured and analysed using Image-J software, the results show extension products of 75-300 base pairs.

[0159] FIG. **10** (B) shows a visualisation of gel electrophoresis of the digested products against a low range ladder, the fluorescence intensity of the gel electrophoresis was measured and analysed using Image-J software, the results are shown. As can be seen, after digestion, DNA bands of 31 bases (if only chopped at every other restriction site), 18 bases and 13 bases can be detected which correspond to the two products (A/E and C/D) as expected after digestion either side of the restriction sites.

[0160] All of the features disclosed in this specification (including any accompanying claims, abstract and drawings), and/or all of the steps of any method or process so disclosed, may be combined in any combination, except combinations where at least some of such features and/or steps are mutually exclusive. Each feature disclosed in this specification (including any accompanying claims, abstract and drawings) may be replaced by alternative features serving the same, equivalent or similar purpose, unless expressly stated otherwise. Thus, unless expressly stated otherwise, each feature disclosed is one example only of a generic series of equivalent or similar features. The invention is not restricted to the details of the foregoing embodiment(s). The invention extends to any novel one, or any novel combination, of the features disclosed in this specification (including any accompanying claims, abstract and drawings), or to any novel one, or any novel combination, of the steps of any method or process so disclosed.

[0161] With respect to the use of substantially any plural and/or singular terms herein, those having skill in the art can translate from the plural to the singular and/or from the singular to the plural as is appropriate to the context and/or application. The various singular/plural permutations may be expressly set forth herein for sake of clarity.

[0162] It will be understood by those within the art that, in general, terms used herein, and especially in the appended claims are generally intended as "open" terms (e.g., the term

"including" or "comprising" should be interpreted as "including but not limited to," the term "having" should be interpreted as "having at least," the term "includes" should be interpreted as "includes but is not limited to," etc.). It will be further understood by those within the art that if a specific number of an introduced claim recitation is intended, such an intent will be explicitly recited in the claim, and in the absence of such recitation no such intent is present. For example, as an aid to understanding, the following appended claims may contain usage of the introductory phrases "at least one" and "one or more" to introduce claim recitations. However, the use of such phrases should not be construed to imply that the introduction of a claim recitation by the indefinite articles "a" or "an" limits any particular claim containing such introduced claim recitation to embodiments containing only one such recitation, even when the same claim includes the introductory phrases "one or more" or "at least one" and indefinite articles such as "a" or "an" (e.g., "a" and/or "an" should be interpreted to mean "at least one" or "one or more"); the same holds true for the use of definite articles used to introduce claim recitations. In addition, even if a specific number of an introduced claim recitation is explicitly recited, those skilled in the art will recognize that such recitation should be interpreted to mean at least the recited number (e.g., the bare recitation of "two recitations," without other modifiers, means at least two recitations, or two or more recitations).

[0163] It will be appreciated that various embodiments of the present disclosure have been described herein for purposes of illustration, and that various modifications may be made without departing from the scope of the present disclosure. Accordingly, the various embodiments disclosed herein are not intended to be limiting, with the true scope being indicated by the following claims.

SEQUENCE LISTING

Sequence total quantity: 27

SEQ ID NO: 1 moltype = DNA length = 10
FEATURE Location/Qualifiers
source 1..10
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 1
atatatatat 10

SEQ ID NO: 2 moltype = length =
SEQUENCE: 2
000

SEQ ID NO: 3 moltype = DNA length = 10
FEATURE Location/Qualifiers
source 1..10
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 3
tatatatata 10

SEQ ID NO: 4 moltype = length =
SEQUENCE: 4
000

SEQ ID NO: 5 moltype = DNA length = 22
FEATURE Location/Qualifiers
source 1..22
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 5
atatatatat cctatatata ta 22

SEQ ID NO: 6 moltype = DNA length = 22
FEATURE Location/Qualifiers
source 1..22
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 6
tatatatata ccatatatat at 22

SEQ ID NO: 7 moltype = DNA length = 10
FEATURE Location/Qualifiers
source 1..10
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 7
atatatatat 10

SEQ ID NO: 8 moltype = length =
SEQUENCE: 8
000

-continued

```
SEQ ID NO: 9          moltype = DNA  length = 10
FEATURE               Location/Qualifiers
source                1..10
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 9
tatatatata                                                    10

SEQ ID NO: 10         moltype =   length =
SEQUENCE: 10
000

SEQ ID NO: 11         moltype = DNA  length = 24
FEATURE               Location/Qualifiers
source                1..24
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 11
atatatatat cccctatata tata                                    24

SEQ ID NO: 12         moltype = DNA  length = 24
FEATURE               Location/Qualifiers
source                1..24
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 12
tatatatata ccccatatat atat                                    24

SEQ ID NO: 13         moltype = DNA  length = 10
FEATURE               Location/Qualifiers
source                1..10
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 13
atatatatat                                                    10

SEQ ID NO: 14         moltype =   length =
SEQUENCE: 14
000

SEQ ID NO: 15         moltype = DNA  length = 10
FEATURE               Location/Qualifiers
source                1..10
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 15
tatatatata                                                    10

SEQ ID NO: 16         moltype =   length =
SEQUENCE: 16
000

SEQ ID NO: 17         moltype =   length =
SEQUENCE: 17
000

SEQ ID NO: 18         moltype =   length =
SEQUENCE: 18
000

SEQ ID NO: 19         moltype = DNA  length = 22
FEATURE               Location/Qualifiers
source                1..22
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 19
atatatatat cctatatata ta                                      22

SEQ ID NO: 20         moltype = DNA  length = 28
FEATURE               Location/Qualifiers
source                1..28
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 20
tatatatata cccccccat atatatat                                 28
```

-continued

```
SEQ ID NO: 21          moltype = DNA  length = 12
FEATURE                Location/Qualifiers
source                 1..12
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 21
tggactctct ca                                                  12

SEQ ID NO: 22          moltype =   length =
SEQUENCE: 22
000

SEQ ID NO: 23          moltype =   length =
SEQUENCE: 23
000

SEQ ID NO: 24          moltype =   length =
SEQUENCE: 24
000

SEQ ID NO: 25          moltype = DNA  length = 12
FEATURE                Location/Qualifiers
source                 1..12
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 25
tgagagagtc ca                                                  12

SEQ ID NO: 26          moltype = DNA  length = 18
FEATURE                Location/Qualifiers
source                 1..18
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 26
atctggactc tctcagat                                            18

SEQ ID NO: 27          moltype = DNA  length = 13
FEATURE                Location/Qualifiers
source                 1..13
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 27
atcatcgact gat                                                 13
```

1. A method for extending the length of a nucleic acid, the method comprising the steps of;

i) providing at least one overlapping primer oligonucleotide, said overlapping primer oligonucleotide comprising a first single-stranded oligonucleotide and a second single-stranded oligonucleotide, each of the first and second single-stranded oligonucleotides comprising at least a first, second and third sequence, said third sequence being located at the 3' end of each single-stranded oligonucleotide and said second sequence being located between the first and third sequences; wherein the first sequences and the third sequences of the first single-stranded oligonucleotide and the second single-stranded oligonucleotide are self-complementary, palindromic sequences;

wherein the third sequence of the first single-stranded oligonucleotide hybridises to the third sequence of the second single-stranded oligonucleotide to provide an overhanging region at the 5' end of at least one of the first or second single-stranded oligonucleotides;

(ii) enzymatically extending both the first and second single-stranded oligonucleotides to provide a duplex.

2. A method for extending the length of a nucleic acid according to claim **1**, wherein a fourth sequence is incorporated into the first and second single-stranded oligonucleotides during step (ii), said fourth sequence on the first single-stranded oligonucleotide being complementary to the second sequence on the second single-stranded oligonucleotide and said fourth sequence on the second single-stranded oligonucleotide being complementary to the second sequence on the first single-stranded oligonucleotide.

3. A method for extending the length of a nucleic acid according to any of claims **1** to **2**, wherein the overlapping primer oligonucleotide is a DNA oligonucleotide or an RNA oligonucleotide.

4. A method for extending the length of a nucleic acid according to any previous claim, wherein the second sequence on the first single-stranded oligonucleotide differs from the second sequence on the second single-stranded oligonucleotide.

5. A method for extending the length of a nucleic acid according to any previous claim, wherein the method further comprises the steps:

(iii) denaturing the duplex to provide a first and second single-stranded polynucleotide;

(iv) annealing the first and second single-stranded polynucleotides such that overhanging region is provided at the 5' end of at least one of the first or second single-

stranded polynucleotides to permit polynucleotide duplex formation between the first and second single-stranded polynucleotides;

(v) enzymatically extending both the first and second single-stranded polynucleotides to provide a duplex.

6. A method for extending the length of a nucleic acid according to claim **5**, wherein steps (iii) to (v) are repeated to increase the length of the nucleic acid sequence.

7. A method for extending the length of a nucleic acid according to any previous claim, wherein the overlapping primer oligonucleotide is immobilised on a surface, and, preferably, wherein the overlapping primer oligonucleotide is immobilised on a surface by the 5' end of one of the first or second single-stranded polynucleotides.

8. A method for extending the length of a nucleic acid according to claim **7**, wherein the surface comprises glass, silica, gold, graphene, graphene oxide, epoxy, plastic, metal, gel matrix, template stripped metals or composites thereof.

9. A method for extending the length of a nucleic acid according to any of claims **7** or **8**, wherein the overlapping primer oligonucleotide is immobilised to the surface by covalent or non-covalent bonding and/or wherein the overlapping primer oligonucleotide is immobilised to a chemically modified region of the surface.

10. A method for extending the length of a nucleic acid according to any of claims **7** to **9**, wherein the overlapping primer oligonucleotide is immobilised to the surface by a linker wherein, preferably, the linker comprises a silane linker molecule, a biotin-streptavidin complex, a thiol-Au linker, covalent Si—C bonds to silicon, covalent Si—O bonds to silicon, covalent Si—N bonds to silicon, a nanoparticle linker, or a dynamic covalent bond.

11. A method for extending the length of a nucleic acid according to any previous claim, wherein the step of providing at least one overlapping primer oligonucleotide comprises obtaining a first single-stranded oligonucleotide and second single-stranded oligonucleotide and hybridising the first and second single-stranded oligonucleotides under appropriate conditions.

12. An overlapping primer oligonucleotide for extending the length of repeating sequences of nucleic acid, said overlapping primer oligonucleotide comprising two partially complementary single-stranded oligonucleotides, each single-stranded oligonucleotide comprising at least a first, second and third sequence wherein the third sequence is located at the 3' end of the first and second single-stranded oligonucleotide; the first sequences and the third sequences are palindromic, self-complementary sequences; and the second sequence is located in between the first and third sequences; wherein the third sequence of the first single-stranded oligonucleotide hybridises to the third sequence of the second single-stranded oligonucleotide to provide an overhanging region at the 5' end of at least one of the first or second single-stranded oligonucleotides.

13. An overlapping primer oligonucleotide according to claim **12**, wherein the second sequence on the first single-stranded oligonucleotide differs from the second sequence on the second single-stranded oligonucleotide and/or wherein the second sequence on the first single-stranded oligonucleotide is not complementary to the second sequence on the second single-stranded oligonucleotide.

14. A method for extending the length of a nucleic acid according to any of claims **1** to **11** or an overlapping primer oligonucleotide according to any of claims **12** to **13**, wherein the first and third sequences are each at least 6 bases in length and are preferably at least 8 bases in length.

15. A method for extending the length of a nucleic acid according to any of claims **1** to **11** or **14**, or an overlapping primer oligonucleotide according to any of claims **12** to **14**, wherein the second sequence is at least 2 bases in length.

16. A method for extending the length of a nucleic acid according to any of claims **1** to **11**, **14** or **15** or an overlapping primer oligonucleotide according to any of claims **12** to **15**, wherein at least one of the first and third sequences comprises at least one functional site and wherein the at least one functional site may be selected from the following: restriction enzyme site, capping site, sequence specific drug binding site, enzyme inhibitor site.

17. A method for extending the length of a nucleic acid according to any of claims **1** to **11**, or **14** to **16**, or an overlapping primer oligonucleotide according to any of claims **12** to **17**, wherein at least one of the first and third sequences comprise tandem repeats.

18. A method for extending the length of a nucleic acid, or an overlapping primer oligonucleotide according to claim **17**, wherein both the first and third sequences comprise tandem repeats.

19. A method for extending the length of a nucleic acid, or an overlapping primer oligonucleotide according to any of claims **17** to **18**, wherein the tandem repeat is a dinucleotide tandem repeat.

20. A method for extending the length of a nucleic acid according to any of claims **1** to **11**, or **14** to **19**, or an overlapping primer oligonucleotide according to any of claims **12** to **19**, wherein the second sequence comprises at least one modification selected from the following: modified nucleotides, artificial bases, loop structures.

21. A method for extending the length of a nucleic acid, or an overlapping primer oligonucleotide according to claim **20**, wherein the modified nucleotides are alkyne, azide or phosphorothioate modified nucleotides. Optionally, these may include: 5-Br-dUTP, 7-deaza-7-1-dATP, 6-S-dGTP,5-I-dCTP, 5-(octadiynyl)-dCTP.

22. A method for extending the length of a nucleic acid, or an overlapping primer oligonucleotide according to any of claims **20** to **21**, wherein, the modified nucleotides comprise a linker which attaches the modification to the nucleotide.

23. A method for extending the length of a nucleic acid, or an overlapping primer oligonucleotide according to claim **20**, wherein the loop structure is a G quadruplex region or a C-motif or intercalated-motif DNA.

24. An overlapping primer oligonucleotide according to any of claims **12** to **23**, wherein the overlapping primer oligonucleotide is a DNA primer oligonucleotide or an RNA primer oligonucleotide.

* * * * *