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(54) **METHOD FOR TEMPLATE-FREE DE NOVO SYNTHESIS OF LONG-CHAIN NUCLEIC ACID, AND USE THEREOF**

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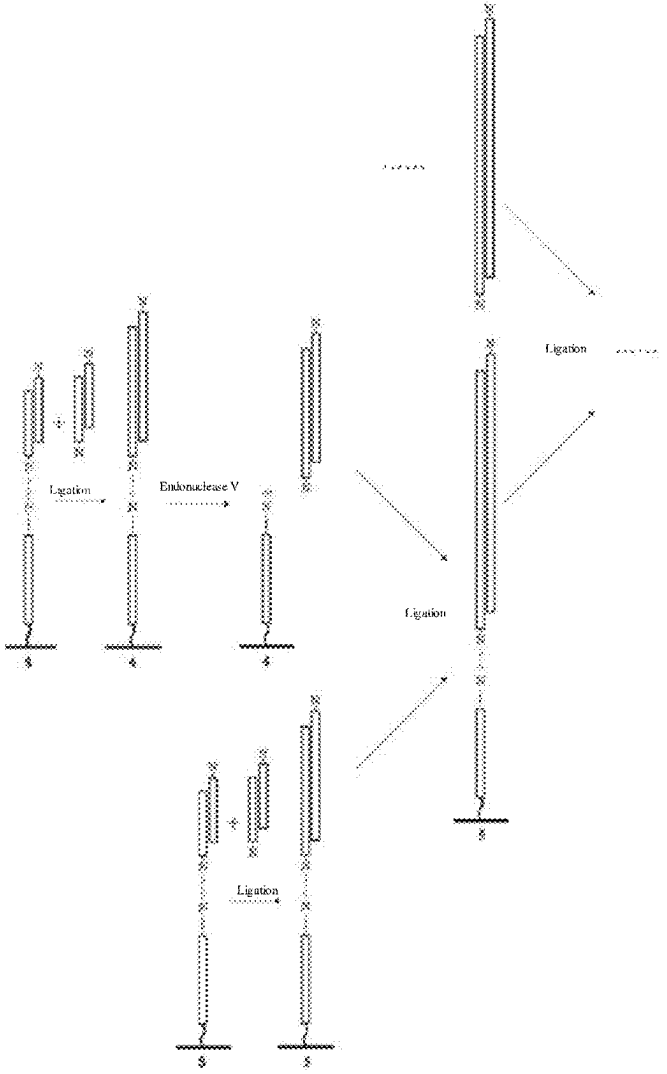
CPC ***C12P 19/34*** (2013.01)

(57) **ABSTRACT**

The present application relates to the fields of molecular biology and biotechnology, and in particular to a method for template-free de novo synthesis of a long-chain nucleic acid, including the following steps: S1, synthesis of double-stranded oligonucleotides; and S2, combination and ligation of the double-stranded oligonucleotides to obtain a target long-chain nucleic acid. The present application achieves continuous synthesis from single nucleotides to long-chain nucleic acids by means of the combination of S1 and S2, and has the advantages of no need for templates, high accuracy, low complexity and low cost.

Specification includes a Sequence Listing.



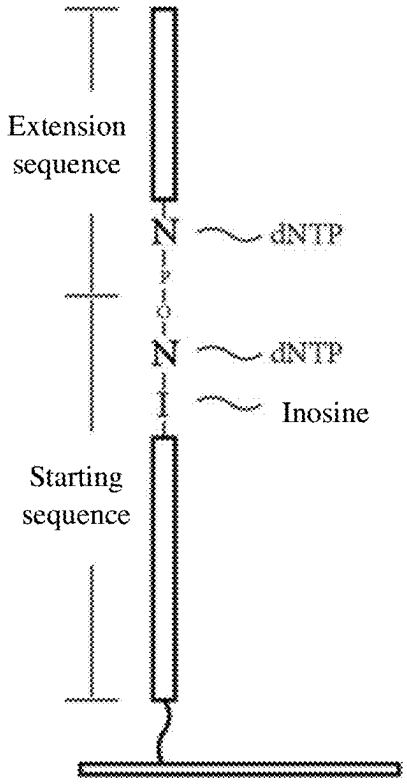

Extension sequence
dNTP
dNTP
Inosine
Starting sequence

FIG. 1

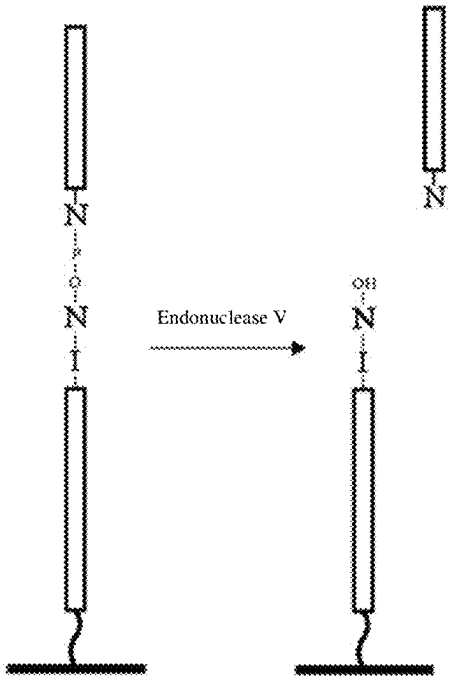

Endonuclease V
OH

FIG. 2

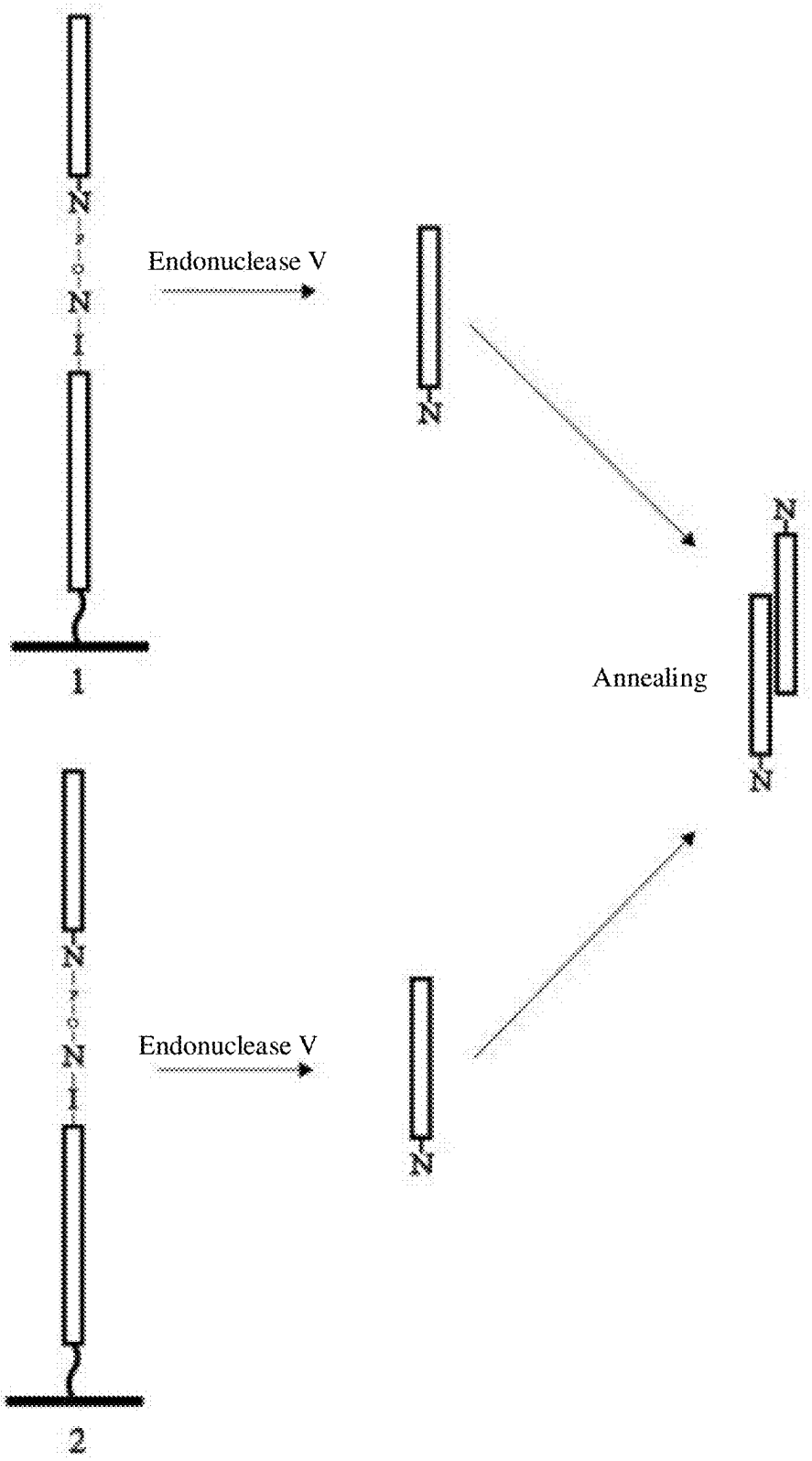


FIG. 3

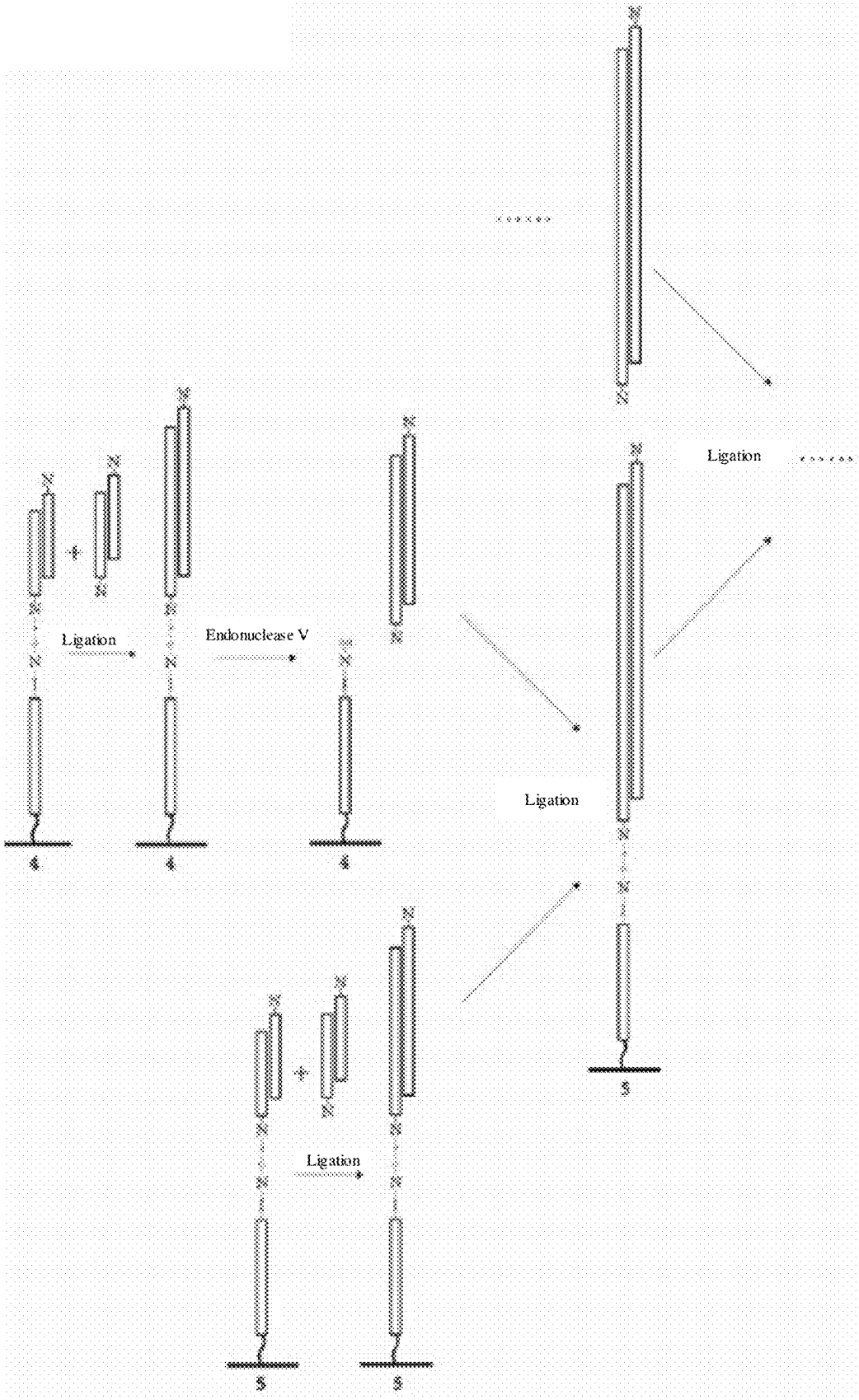


FIG. 4

Target long-chain nucleic acid sequence

atgaaaagga actacattct ggggctggac atcgggatta caagcgtggg gtatgggatt 60
attgactatg aaacaaggga cgtgatcgac gcaggcgtca gactgttcaa ggaggccaac 120
gtggaaaaca atgagggacg gagaagcaag aggggagcca ggcgcctgaa acgacggaga 180
aggcacagaa tccagagggt gaagaaactg ctgttcgatt acaacctgct gaccgaccat 240
tctgagctga gtggaattaa tccttatgaa gccagggtga aaggcctgag tcagaagctg 300
tcagaggaag agttttccgc agctctgctg cacctggcta agcgccgagg agtgcataac 360
gtcaatgagg tggaagagga caccggcaac gagctgtcta caaaggaaca gatctcacgc 420
aatagcaaag ctctggaaga gaagtatgtc gcagagctac agctggaacg gctgaagaaa 480
gatggcgagg tgagagggtc aattaatagg ttcaagacaa gcgactacgt caaagaagcc 540
aagcagctgc tgaaagtgca gaaggcttac caccagctgg atcagagctt catcgatact 600
tatatcgacc tgctggagac tcggagaacc tactatgagg gaccaggaga agggagcccc 660
ttcggatgga aagacatcaa ggaatggtac gagatgctga tgggacattg cacctatttt 720
ccagaagagc tgagaagcgt caagtacgct tataacgcag atctgtacaa cgccctgaat 780
gacctgaaca acctggtcat caccagggat gaaaacgaga aactggaata ctatgagaag 840
ttccagatca tcgaaaacgt gtttaagcag aagaaaaagc ctacactgaa acagattgct
aaggagatcc tggtcaacga agaggacatc aagggctacc gggtgacaag cactggaaaa 960
ccagagttca ccaatctgaa agtgtatcac gatattaagg acatcacagc acggaaagaa 1020
atcattgaga acgccgaact gctggatcag attgctaaga tcctgactat ctaccagagt 1080
tccgaggaca tccaggaaga gctgactaac ctgaacagcg agctgaccca ggaagagatc 1140
gaacagatta gtaatctgaa ggggtacacc ggaacacaca acctgtccct gaaagctatc 1200
aatctgattc tggatgagct gtggcataca aacgacaatc agattgcaat ctttaaccgg 1260
ctgaagctgg taccaaaaaa ggtggacctg agtcagcaga aagagatccc aaccacactg 1320
gtggacgatt tcattctgtc acccgtggtc aagcggagct tcatccagag catcaaagtg 1380
atcaacgcca tcatcaagaa gtacggcctg cccaatgata tcattatcga gctggctagg 1440
gagaagaaca gcaaggacgc acagaagatg atcaatgaga tgcagaaacg aaaccggcag 1500
accaatgaac gcattgaaga gattatccga actaccggga aagagaacgc aaagtacctg 1560
attgaaaaaa tcaagctgca cgatatgcag gagggaaagt gtctgtattc tctggaggcc 1620
atccccctgg aggacctgct gaacaatcca ttcaactacg aggtcgatca tattatcccc 1680
agaagcgtgt ccttcgacaa ttcctttaac aacaaggtgc tggtcaagca ggaagagaac 1740
tctaaaaagg gcaataggac tcctttccag tacctgtcta gttcagattc caagatctct 1800
tacgaaacct ttaaaaagca cattctgaat ctggccaaag gaaagggccg catcag 1856

FIG. 5

METHOD FOR TEMPLATE-FREE DE NOVO SYNTHESIS OF LONG-CHAIN NUCLEIC ACID, AND USE THEREOF

CROSS-REFERENCE TO RELATED APPLICATION

[0001] The present application is a continuation of PCT application serial no. PCT/CN2024/074510, filed on Jan. 29, 2024, which claims the priority benefits of China patent application No. 202310046681.4, filed on Jan. 31, 2023, and China patent application No. 202310408341.1, filed on Apr. 17, 2023. The entireties of PCT application serial no. PCT/CN2024/074510, China patent application No. 202310046681.4 and China patent application No. 202310408341.1 are hereby incorporated by reference herein and made a part of this specification.

REFERENCE TO AN ELECTRONIC SEQUENCE LISTING

[0002] The contents of the electronic sequence listing (SequenceListing.xml; Size: 117,731 bytes; and Date of Creation: May 1, 2025) is herein incorporated by reference.

TECHNICAL FIELD

[0003] The present application relates to the fields of molecular biology and biotechnology, in particular to a method for template-free de novo synthesis of a long-chain nucleic acid, and a use thereof.

BACKGROUND ART

[0004] At present, in the research and application of molecular biology, genetic engineering and synthetic biology, it is often necessary to synthesize large-fragment DNA sequences for detecting and studying their gene functions and other purposes. DNA synthesis methods mainly include a chemical synthesis method and an enzymatic assembly method.

[0005] The chemical synthesis method in related technologies is widely favored due to its mature technology and low cost, and allows for high-throughput production of DNAs or RNAs in chips in some embodiments. However, as the length of a synthetic template increases, errors accumulate gradually, a proportion of correct products sharply decreases, the cost of purification in the later stage rises, and the efficiency decreases. Therefore, an upper limit of sequences produced by this method in size is usually 200 bp.

[0006] The enzymatic assembly method in related technologies involves enzymatic ligation of oligonucleotides and polynucleotides of different sizes and sequences in a specific way to obtain larger molecules having desired target sequences. However, as the chain length increases or the number of ligated sequences increases, the accuracy and/or yield will decrease.

[0007] Moreover, a DNA fragment obtained by de novo synthesis has a limited length (below 600 bp). Longer genes or genomes (above 600 bp) need to be assembled through enzymatic/in-vivo assembly of oligonucleotide fragments, mainly in the form of LCR and PCA, but the synthesis efficiency and accuracy still cannot be guaranteed.

[0008] In summary, there is an urgent need to provide a method for template-free de novo synthesis of a long-chain nucleic acid with high accuracy, low complexity and low cost, and a use thereof.

SUMMARY

[0009] To solve the above technical problem, the present application provides a method for template-free de novo synthesis of a long-chain nucleic acid, and a use thereof. This method achieves a highly accurate and continuous synthesis from single nucleotides to the long-chain nucleic acid through a combination of a specific enzymatic method and a ligase assembly method, without a need for templates.

[0010] In a first aspect, the present application provides a method for template-free de novo synthesis of a long-chain nucleic acid, including the following steps:

[0011] S1, synthesis of double-stranded oligonucleotides; and

[0012] S2, combination and ligation of the double-stranded oligonucleotides to obtain a target long-chain nucleic acid, wherein

[0013] the double-stranded oligonucleotides in step S1 are combined and ligated according to a sequence of the target long-chain nucleic acid; sequence numbers of the double-stranded oligonucleotides to be used are marked as 1, 2, 3, 4 . . . . N−1, N;

[0014] wherein N is a power of 2 to a power of n, i.e., 2^n; and

[0015] a specific ligation method includes the following steps:

[0016] (1) moving free double-stranded oligonucleotides marked as N respectively to a region where the double-stranded oligonucleotide marked as N−1 sequence is located, for a first ligation reaction to obtain N/2 first composite sequences;

[0017] then, removing impurities by elution to remove a ligation system and unligated double-stranded oligonucleotide chains;

[0018] (2) then, treating first composite sequences marked as N/2 with endonuclease to obtain first free composite sequences, moving the first free composite sequences respectively to a region where the first composite sequence marked as N/2−1 is located, for a second ligation reaction to obtain $N/2^2$ second composite sequences, and then removing impurities by elution; and

[0019] (3) repeating the step (2) for several times until $N/2^n$, that is, one composite sequence is obtained, removing impurities by elution again after the reaction is completed, to remove the ligation system and unligated double-stranded oligonucleotide chains to obtain a fixed target long-chain nucleic acid.

[0020] Preferably, the double-stranded oligonucleotide marked as N−1 in step (1) is either a fixed nucleic acid chain or a free nucleic acid chain.

[0021] Preferably, the synthesis of double-stranded oligonucleotides in step S1 includes the following steps:

[0022] 1) firstly, fixing a starting single-stranded oligonucleotide at a reaction site by biotin;

[0023] 2) then, adding an amplification reaction system containing 3'-blocked dNTPs and a deblocking reaction system into the reaction site in sequence, and repeating said process for several times until fixed single-stranded oligonucleotides are obtained;

[0024] 3) subsequently, treating the fixed single-stranded oligonucleotides with endonuclease, such that an extension chain is separated from the starting single-stranded oligonucleotide to obtain free oligonucleotide chains by template-free synthesis; and

[0025] 4) finally, moving the free oligonucleotide chains to a region where complementary pairing chains are located, and performing a heating reaction to obtain fixed double-stranded oligonucleotides, the complementary pairing chains being the fixed single-stranded oligonucleotides in step 3).

[0026] Preferably, components and contents thereof of the amplification reaction system in step 2) are as follows:

[0027] the amplification reaction system contains 1.0-5.0 μM of TdT enzyme, 200 μM-500 μM of 3'-O-phosphate blocked dNTPs, 50-400 mM of a potassium cacodylate buffer, 20-50 mM of Tris, and 3-6 mM of $CoCl_2$; and

[0028] reaction conditions for the amplification reaction system applied in step 2) are as follows: a reaction volume is 0.1-50 μL, and incubation is carried out at 25-45° C. for 5-30 min.

[0029] Preferably, components and contents thereof of the deblocking reaction system in step 2) are as follows:

[0030] the deblocking reaction system contains 75-120 mM of Tris-HCl at pH 6.5, 8-15 mM of $MgCl_2$, 5-8 mM of 2-mercaptoethanol, and one unit of T4 polynucleotide kinase; and

[0031] reaction conditions for the deblocking reaction system applied in step 2) are as follows: a reaction volume is 0.1-50 μL, and incubation is carried out at 25-45° C. for 5-30 min.

[0032] Preferably, the step 3) includes the following steps:

[0033] treating the fixed single-stranded oligonucleotides with endonuclease first, such that the extension chain and the starting single-stranded oligonucleotide are free in lysis buffer for a lysis reaction; and after the lysis reaction is completed, transferring a lysis product and inactivating the endonuclease by heating, to obtain the free oligonucleotide chains by template-free synthesis.

[0034] Preferably, components and contents thereof of the lysis buffer, and lysis reaction conditions in step 3) are as follows:

[0035] the lysis buffer contains 40-60 mM of K—Ac, 15-25 mM of Tris-Ac, 8-15 mM of Mg—Ac, and 1-5 mM of DTT; and

[0036] the lysis reaction conditions are as follows: a reaction volume is 0.1-50 μL, and 1-100 U of endonuclease V is reacted at 25-45° C. for 15-60 min.

[0037] Preferably, the step 4) includes the following steps:

[0038] moving the free oligonucleotide chains by template-free synthesis to the region where the complementary pairing chains are located, maintaining the region at 95° C. for 3-5 min, and then cooling the region to 25° C. at a constant speed within 15 min to obtain the fixed double-stranded oligonucleotides, the complementary pairing chains being the fixed single-stranded oligonucleotides in step 3).

[0039] In a second aspect, the present application provides a use of the method for template-free de novo synthesis of the long-chain nucleic acid in a synthesis of a DNA fragment and a mutation of a DNA sequence.

[0040] Preferably, an application environment is any one of a centrifuge tube, a microfluidic device, a digital microfluidic device, a microarray or a biochip.

[0041] In summary, the present application has the following beneficial effects.

The present application achieves continuous synthesis from single nucleotides to the long-chain nucleic acid by means of a combination of S1 and S2, and the synthesis method has advantages of no need for templates, high efficiency, high accuracy, low complexity and low cost, which are reflected in the following aspects.

[0042] The high efficiency lies in that: the fixed single-stranded oligonucleotide synthesized by the enzymatic method in step S1 can be formed into a double-stranded oligonucleotide containing sticky ends required for ligation after simple separation and annealing in step S1, which may directly enter into the subsequent assembly reaction, and the assembly reaction proceeds exponentially.

[0043] The high accuracy lies in that:

[0044] the fixed single-stranded oligonucleotides in step S1 are synthesized by the enzymatic method instead of chemical methods, such that the whole synthesis reaction system is maintained in a relatively stable environment (all in a biological enzyme system), which is conducive to the unification of reaction conditions and eliminates the interference of chemical reagents and reactions on the system; and

[0045] in the synthesis of oligonucleotide chains and double-stranded oligonucleotides assembly and ligation reaction in step S2, an elution step is further inserted between each two extension reactions, and the elution step can minimize the influence of impurities in the reaction system on an extension effect during each elongation reaction, and further greatly reduce the number of erroneous products during short-chain assembly due to the properties of DNA ligase.

[0046] The low complexity and low cost lies in that: common reagents and instruments can be used for all steps in this method, unlike complex customized equipment (e.g., gene chips and automated pipette arms) or large raw material libraries (e.g., oligonucleotide libraries) or templates required in other patents.

BRIEF DESCRIPTION OF THE DRAWINGS

[0047] FIG. **1** is a schematic diagram of reactions in steps 1) and 2) for the synthesis of fixed single-stranded oligonucleotides in step S1.

[0048] FIG. **2** is a schematic diagram of a reaction in step 3) for a free oligonucleotide chain by template-free synthesis in step S1.

[0049] FIG. **3** is a schematic diagram of a reaction in step 4) for a free double-stranded oligonucleotide in step S1.

[0050] FIG. **4** is a schematic diagram of reactions in steps (1), (2) and (3) in step S2.

[0051] FIG. **5** is a schematic diagram of a sequence of a target long-chain nucleic acid to be synthesized.

DETAILED DESCRIPTION

[0052] The present application is further explained below through specific examples and in conjunction with the drawings, but is not limited thereto. Various reaction systems used in the present application were shown in the following table:

Types	Main components*
Amplification reaction system	2.0 μM of TdT enzyme, 400 μM of 3'-O-phosphate blocked dNTPs, 100 mM of a potassium cacodylate buffer (pH 7.2), 25 mM of Tris, and 5 mM of $CoCl_2$ (pH 7.2)
Deblocking reaction system	100 mM of Tris-HCl (pH 6.5), 10 mM of $MgCl_2$, 5 mM of 2-mercaptoethanol, and 5 U of T4 polynucleotide kinase
Lysis buffer	50 mM of K-Ac, 20 mM of Tris-HCl, 10 mM of Mg-Ac, 1 mM of DTT dissolved in pH 7.9

Note:

the materials, reagents and instruments used in the specific examples of the present application, unless otherwise specified can be obtained commercially.

[0053] Materials: PCR tubes used—an inner wall of the tube was modified to contain biotin-streptomycin; and a starting single-stranded oligonucleotide with a length of 12 bp-5' end of the starting single-stranded oligonucleotide was modified with streptavidin, a penultimate position at 3' end of the starting single-stranded oligonucleotide was hypoxanthine deoxyriboside (inosinate), and dNTPs-3' ends of the dNTPs were phosphorylated (3'-O-phosphate).

[0054] For sequences of the single-stranded oligonucleotides to be used, see SEQ ID NO 2 to SEQ ID NO 65 (a.1-64) and SEQ ID NO 66 to SEQ ID NO 129 (b.1-64), respectively:

positive strands (a.1-64): 5'-3'
ccatgaaaaggaactacattc 21

tggggctggacatcgggattacaagcgtg 29

gggtatgggattattgactatgaaacaag 29

ggacgtgatcgacgcaggcgtcagactgt 29

tcaaggaggccaacgtagaaaacaatgag 29

ggacggagaagcaagaggggagccaggcg 29

cctgaaacgacggagaaggcacagaatcc 29

agagggtgaagaaactgctgttcgattac 29

aacctgctgaccgaccattctgagctga 29

gtggaattaatccttatgaagccagggtga 29

aaggcctgagtcagaagctgtcagaggaa 29

gagttttccgcagctctgctgcacctggc 29

taagcgccgaggagtgcatatcgtcaatg 29

aggtggaagaggacaccggcaacgagctg 29

tctacaaaggaacagatctcacgcaatag 29

caaagctctggaagagaagtatgtcgcag 29

agctacagctggaacggctgaagaaagat 29

gccgaggtgagagggtcaattaataggtt 29

caagacaagcgactacgtcaaagaagcca 29

agcagctgctgaaagtgcagaaggcttac 29

caccagctcgatcagagcttcatcgata 29

cttatatcgacctgctggagactcggaga 29

-continued

acctactatgagggaccaggagaagggagc 29

cccttcggatggaaagacatcaaggaatg 29

gtacgagatgctgatgggacattgcacc 29

tattttccagaagagctgagaagcgtcaa 29

gtacgcttataacgcagatctgtacaacgc 29

cctgaatgacctgaacaacctggtcatca 29

ccagggatgaaaacgagaaactggaata 29

ctatgagaagttccagatcatcgaaaacg 29

tgtttaagcagaagaaaaagcctacactga 29

aacagattgctaaggagatcctggtcaac 29

gaagaggacatcaagggctaccgggtgac 29

aagcactcgaaaaccagagttcaccaat 29

ctgaaagtgtatcacgatattaaggacatc 29

acagcacggaaagaaatcattgagaacgc 29

cgaactgctggatcagattgctaagatc 29

ctgactatctaccagagttccgaggacatc 29

caggaagagctgactaacctgaacagcga 29

gctgacccaggaagagatcgaacagatt 29

agtaatctgaaggggtacaccggaacacac 29

aacctgtccctgaaagctatcaatctgat 29

tctggatgagctgtggcatacaaacgaca 29

atcagattgcaatctttaaccggctgaag 29

ctggtaccaaaaaaggtggacctgagtca 29

gcagaaagagatcccaaccacactggtgg 29

acgatttcattctgtcacccgtggtcaag 29

cggagcttcatccagagcatcaaagtgat 29

caacgccatcatcaagaagtacggcctg 29

cccaatgatatcattatcgagctggctag 29

ggagaagaacagcaaggacgcacagaagat 29

gatcaatgagatgcagaaacgaaaccggc 29

agaccaatgaacgcattgaagagattatc 29

cgaactaccgggaaagagaacgcaaagta 29

cctgattgaaaaaatcaagctgcacgata 29

tgcaggagggaaagtgtctgtattctctg 29

gaggccatccccctggaggacctgctgaa 29

caatccattcaactacgaggtcgatcata 29

ttatccccagaagcgtgtccttcgacaa 29

ttcctttaacaacaaggtgctggtcaagca 29

ggaagagaactctaaaaagggcaatagga 29

-continued

ctcctttccagtacctgtctagttcaga 29

ttccaagatctcttacgaaacctttaaaaa 29

gcacattctgaatctggccaaaggaaagg 29

reverse complementary strands (b.1-64): 5'-3'.

tccagccccagaatgtagttccttttcat 29

tcccataccccacgcttgtaatcccgatg 29

gatcacgtcccttgtttcatagtcaataa 29

gcctccttgaacagtctgacgcctgcgtc 29

ttctccgtccctcattgttttccacgttg 29

tcgtttcaggcgcctggctcccctcttgc 29

ttcaccctctcgattctgtgccttctccg 29

tcagcaggttgtaatcgaacagcagtttc 29

attaattccactcagctcagaatggtcgg 29

ctcaggcctttcaccctggcttcataagg 29

cggaaaactcttcctctgacagcttctga 29

tcggcgcttagccaggtgcagcagagctg 29

tcttccacctcattgacgttatgcactcc 29

cctttgtagacagctcgttgccggtgtcc 29

cagagctttgctattgcgtgagatctgtt 29

agctgtagctctgcgacatacttctcttc 29

tcacctcgccatctttcttcagccgttcc 29

gcttgtcttgaacctattaattgaccctc 29

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ccagctggtggtaagccttctgcactttc 29

gtcgatataagtatcgatgaagctctgat 29

tcatagtaggttctccgagtctccagcag 29

atccgaaggggctcccttctcctggtccc 29

catctcgtaccattccttgatgtctttcc 29

tctggaaaataggtgcaatgtcccatcag 29

tataagcgtacttgacgcttctcagctct 29

gtcattcagggcgttgtacagatctgcgt 29

tcatccctggtgatgaccaggttgttcag 29

acttctcatagtattccagtttctcgttt 29

ctgcttaaacacgttttcgatgatctgga 29

gcaatctgtttcagtgtaggcttttttctt 29

tgtcctcttcgttgaccaggatctcctta 29

tccagtgcttgtcacccggtagcccttga 29

tacactttcagattggtgaactctggttt 29

tccgtgctgtgatgtccttaatatcgtga 29

cagcagttcggcgttctcaatgatttctt 29

-continued

tagatagtcaggatcttagcaatctgatc 29

gctcttcctggatgtcctcggaactctcg 29

ctgggtcagctcgctgttcaggttagtca 29

ttcagattactaatctgttcgatctcttc 29

gggacaggttgtgtgttccggtgtacccc 29

ctcatccagaatcagattgatagctttca 29

gcaatctgattgtcgtttgtatgccacag 29

ttggtaccagcttcagccggttaaagatt 29

ctctttctgctgactcaggtccacctttt 29

atgaaatcgtccaccagtgtggttgggat 29

tgaagctccgcttgaccacgggtgacaga 29

gatggcgttgatcactttgatgctctgga 29

atatcattgggcaggccgtacttcttgat 29

tgttcttctccctagccagctcgataatg 29

ctcattgatcatcttctgtgcgtccttgc 29

tcattcgtctgccggtttcgtttctgcat 29

cggtagttcggataatctcttcaatgcgt 29

ttcaatcaggtactttgcgttctctttcc 29

ccctcctgcatatcgtgcagcttgatttt 29

ggatggcctccagagaatacagacacttt 29

gaatggattgttcagcaggtcctccaggg 29

ctggggataatatgatcgacctcgtagtt 29

tgttaaaggaattgtcgaaggacacgctt 29

gttctcttcctgcttgaccagcaccttgt 29

tggaaaggagtcctattgccctttttaga 29

agatcttcgaatctgaactagacaggtac 29

cagaatgtgctttttaaaggtttcgtaag 29

ctgatgcggccctttcctttggccagatt 29

Example 1

[0055] A method for template-free de novo synthesis of a long-chain nucleic acid was provided. A target long-chain nucleic acid to be synthesized had a length of 1856 bp, and a sequence as shown in FIG. **5**, i.e., SEQ ID NO 1. The specific steps were as follows.

[0056] S1, synthesis of double-stranded oligonucleotides

[0057] 1) Firstly, 10 nmol of starting single-stranded oligonucleotide (starting sequence) was dissolved in 50 μL of ddH_2O, then added to a biotin-modified PCR tube, the PCR tube was incubated for 30 min, such that the starting single-stranded oligonucleotide was fixed on a wall of the PCR tube; and the PCR tube was washed with 100 μL of ddH_2O twice, with five beats per blowing, to remove unfixed oligonucleotides.

[0058] 2) according to a sequence synthesis order of provided 128 oligonucleotide chains, the amplification reaction system containing 3'-O-phosphate blocked dNTPs was then added into 128 PCR tubes in sequence; each single nucleotide amplification reaction includes incubation with a reaction volume of 50 μL at 37° C. for 5 min for amplification; each PCR tube was then washed with 100 μL of ddH_2O twice, with five beats per blowing, to remove the amplification reaction system;

[0059] after each single nucleotide addition/amplification reaction, the deblocking reaction system was added to each washed PCR tube, and then incubated at 37° C. for 1 min for a deblocking reaction; After the deblocking reaction, each PCR tube was washed with 100 μL of ddH_2O twice, with five beats per blowing, to remove the deblocking reaction system; and

[0060] finally, repeating the above step 2) according to the sequence synthesis order of the oligonucleotide chains until the ligation and deprotection of the last nucleotide in the fixed single-stranded oligonucleotides were completed to obtain reverse complementary single-stranded oligonucleotides fixed on the wall of the PCR tube.

[0061] 3) The reverse complementary single-stranded oligonucleotides fixed on the wall of the PCR tube were then treated with endonuclease V, so that an extension chain and the starting single-stranded oligonucleotide were free in lysis buffer, and a lysis reaction was carried out with 100 U of endonuclease V with a reaction volume of 50 μL at 37° C. for 15 min; and

[0062] after the lysis reaction was completed, the buffer was transferred to a new tube, heated to 65° C. and maintained for 10 min to inactivate endonuclease V, thereby obtaining free oligonucleotide chains by template-free synthesis.

[0063] 4) Finally, the free oligonucleotide chains by template-free synthesis were respectively transferred to a tube where complementary pairing chains were located, the tube was maintained at 95° C. for 3 min, and then cooled to 25° C. at a constant speed within 15 min to obtain 64 double-stranded oligonucleotides for the synthesis of SEQ ID NO 1, wherein

[0064] the complementary pairing chains are positive-stranded oligonucleotides in step S1, the positive-stranded oligonucleotides in odd-numbered tubes were still fixed on the walls of the tubes, and the positive-stranded oligonucleotides in even-numbered tubes were in a free state.

[0065] S2, combination and ligation of the double-stranded oligonucleotides to obtain a target long-chain nucleic acid.

[0066] Free double-stranded oligonucleotides obtained in S1 were combined and ligated according to a sequence of the target long-chain nucleic acid; and the used PCR tubes for the double-stranded oligonucleotides were marked as 1, 2, 3, . . . , 63, 64; and a specific ligation method included the following steps:

[0067] (1) firstly, the double-stranded oligonucleotides marked as even numbers (set as N) were respectively moved to a tube where the double-stranded oligonucleotide marked as N−1 was located, and a ligation reaction was carried out by incubating with T4 ligase at room temperature for 60 min to obtain 32 tubes of extended composite sequences, which were renumbered as 1, 2, 3, . . . , 31, 32; each PCR tube was washed with 100 μL of ddH_2O twice, with five beats per blowing, to remove a ligation system and unligated double-stranded oligonucleotide chains;

[0068] (2) then, composite sequences marked as even numbers (set as N/2) were treated again with endonuclease V, such that the composite sequences marked as even numbers were free to obtain free composite sequences; the free composite sequences were then moved respectively to a tube where the composite sequence of N/2−1 was located; a ligation reaction was carried out by incubating with T4 ligase at room temperature for 60 min to obtain 16 tubes of extended composite sequences which were renumbered as 1, 2, 3, . . . , 15, 16; and similarly, each PCR tube was washed with 100 μL of ddH_2O twice, with five beats per blowing, to remove the ligation system and unligated double-stranded oligonucleotide chains;

[0069] (3) the operation in step (2) was repeated; after a total of 6 rounds of ligation (64 tubes-32 tubes-16 tubes-8 tubes-4 tubes-2 tubes-1 tube) were completed, the target long-chain nucleic acid, ultimately fixed to the wall of the PCR tube, can be obtained; and

[0070] similarly, the PCR tube was washed with 100 μL of ddH_2O twice, with five beats per blowing, to remove the ligation system and unligated double-stranded oligonucleotide chains.

[0071] Finally, the target long-chain nucleic acid fixed on the wall of each PCR tube was treated with endonuclease V and then free in buffer for subsequent detection. The specific detection step was as follows:

[0072] the free target long-chain nucleic acid was subjected to agarose gel electrophoresis, and then compared the band size of the target long-chain nucleic acid with that of the marker.

Comparative Example 1

[0073] A method for template-free de novo synthesis of a long-chain nucleic acid was provided, which was different from Example 1 in that the synthesis method to be used is a PCA (Polymerase Cycling Assembly) method.

[0074] In the PCA method, a pair of nucleic acid chains that were complementary end to end was used for PCR amplification to obtain full-length double-stranded nucleic acids. For each amplification, a pair of primers that were complementary to a first end of the existing sequence were required as an extension sequence. Due to the nature of the PCR, complementary regions of the oligonucleotide sequences required were longer (>20 bp), and thus the oligonucleotide sequences required were also longer (>40 bp). In this example, the oligonucleotide sequences used were 45 bp, with a complementary region of 22 bp.

[0075] The specific synthesis method was as follows:

[0076] 1) firstly, the oligonucleotide sequences including 1 positive strand and 80 reverse complementary amplification strands were synthesized;

[0077] 2) the positive strand and reverse complementary amplification strands (including the complementary regions) were added to a PCR reaction system (100 μL) at the same time, wherein a final concentration of each strand in the reaction system was 0.1 μM, and the PCR reaction system was commercially formulated; the PCR reaction conditions were as follows: denatur-

ation at 95° C. for 5 min, then annealing at 50° C. for 15 seconds, and extension at 72° C. for 120 seconds; annealing and extension reactions were repeated for 30 times; and

[0078] 3) after the reaction was completed, an amplification product was subjected to electrophoresis in agarose gel and band sizes of the amplification product were observed.

[0079] Experimental results: No target sequence-length fragment was observed in the agarose gel, indicating synthesis failure.

[0080] As can be seen from Example 1 and Comparative Example 1, the present application achieves continuous synthesis from single nucleotides to the long-chain nucleic acid by means of the combination of S1 and S2, and the synthesis method had the advantages of no need for templates, high efficiency, high accuracy, low complexity and low cost.

[0081] Obviously, Comparative Example 1 cannot possess the aforementioned advantages. The reasons were analyzed as follows.

[0082] The PCA method involved multiple DNA polymerization reactions, and a synthesis error rate of polymerase would affect the accuracy of the target nucleic acid chain; short chains may also mismatch during denaturation and annealing, and such mismatch would be ignored by DNA polymerase to increase the synthesis error rate. Since the PCA method was essentially a PCR reaction, accurate temperature control and repeated temperature changes were required during the reaction process. Such reaction conditions would also affect the efficiency of long-chain synthesis.

Comparative Example 2

[0083] A method for template-free de novo synthesis of a long-chain nucleic acid was provided, which was different from Example 1 in that the synthesis method to be used was a Gibson assembly method.

[0084] The Gibson assembly method used three enzymes—DNA exonuclease, DNA polymerase, and DNA ligase—as tools to ligate a plurality of blunt-ended double-stranded DNAs containing complementary regions into a single strand. The DNA exonuclease was used to generates sticky ends, the DNA polymerase was used to complement missing bases at the ends after complementary pairing (the DNA exonuclease can remove excessive bases), and the DNA ligase was used to ligate two strands.

[0085] The specific method was as follows:

[0086] 1) a total of 124 polynucleotide sequences, including 62 positive-stranded polynucleotide sequences and 62 negative-stranded polynucleotide sequences, were synthesized first and numbered from 1 to 62 in sequence; the positive-stranded and negative-stranded polynucleotide sequences with the same numbers were denatured and annealed at a final concentration of 20 μM in a 50 μL system to form complementary double-stranded nucleic acids; the sequence numbers of the products remained unchanged; and denaturation and annealing conditions were as follows: maintaining at 95° C. for 5 min, and cooling at 25° C. within 30 min;

[0087] 2) the above-mentioned annealed double-stranded nucleic acids were subjected to a Gibson assembly reaction, all double-stranded nucleic acids were added to a 100 μL reaction system to assemble at a final concentration of 0.2 μM, and the reaction was carried out using a commercial Gibson assembly kit; and

[0088] 3) after the reaction was completed, an amplification product was subjected to electrophoresis in agarose gel and band sizes of the amplification product were observed.

[0089] Experimental results: No target sequence-length fragment was observed in the agarose gel, indicating synthesis failure.

[0090] As can be seen from Example 1 and Comparative Example 2, the present application achieves continuous synthesis from single nucleotides to the long-chain nucleic acid by means of the combination of S1 and S2, and the synthesis method had the advantages of no need for templates, high efficiency, high accuracy, low complexity and low cost.

[0091] Obviously, Comparative Example 2 cannot possess the aforementioned advantages. The reasons were analyzed as follows.

[0092] Due to the uncontrollable nature of exonuclease, single-stranded nucleic acids that cannot be complemented by polymerases in time would be produced during the reaction, which affected the ligation efficiency, so the effect of this method depended on concentrations of the three enzymes and proportions of the buffer. In addition, when the ligated strand was too short, the DNA exonuclease would directly cut off the nucleic acid chains, so that the DNA polymerase failed to function. Therefore, this method was only suitable for the ligation of long-chain nucleic acids.

Comparative Example 3

[0093] A method for template-free de novo synthesis of a long-chain nucleic acid was provided, which was different from Example 1 in that the synthesis method to be used was an LCR (Ligation Chain Reaction) method.

[0094] In the LCR method, with the help of a single-strand bridging oligonucleotide, 5' and 3' ends of two nucleic acid chains were joined into a single chain by DNA ligase, wherein the bridging oligonucleotide was complementarily paired with 5' and 3' ends of chains to be ligated, respectively.

[0095] The specific method was as follows:

[0096] 1) 62 positive-stranded oligonucleotides and 61 bridging oligonucleotides were synthesized first, each bridging oligonucleotide corresponding to a complementary ligation reaction of two positive strands; all strands were added to a reaction system of 100 μL, wherein a final concentration of each strand was 0.1 μM; and the reaction conditions were as follows: maintaining at 95° C. for 5 min, and then cooling to 25° C. for DNA ligation for 30 min; a commercially available ligase was used;

[0097] 2) after the reaction was completed, 5 μL of amplification product was added to a PCR reaction system, and amplification primers were added at the same time, and a target sequence was obtained after 30 PCR cycles; and

[0098] 3) a PCR product was subjected to electrophoresis in agarose gel and band sizes of the PCR product were observed.

[0099] Experimental results: No target sequence-length fragment was observed in the agarose gel, indicating synthesis failure.

[0100] As can be seen from Example 1 and Comparative Example 3, the present application achieves continuous synthesis from single nucleotides to the long-chain nucleic acid by means of the combination of S1 and S2, and the synthesis method had the advantages of no need for templates, high efficiency, high accuracy, low complexity and low cost.

[0101] Obviously, Comparative Example 3 cannot possess the aforementioned advantages. The reasons were analyzed as follows.

[0102] The LCR method required the provision of double-stranded nucleic acids with sticky ends prior to ligation (the double-stranded nucleic acids in the present application were formed by annealing the synthesized oligonucleotides). Due to the specificity of the DNA ligase, the ligation reaction cannot occur when the sticky ends of the provided double-stranded nucleic acids were missing or mismatched and thus the ends were not aligned, thereby significantly reducing the number of false ligation products.

[0103] The specific examples are merely an explanation of the present application and not for limiting the present application. Those skilled in the art may make modifications, without creative contribution, to the examples as needed after reading this specification. Any of the modifications made within the scope of the claims of the present application shall be protected by the Patent Law.

SEQUENCE LISTING

Sequence total quantity: 129

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FEATURE Location/Qualifiers
source 1..1856
mol_type = other DNA
organism = synthetic construct

SEQUENCE: 1

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gtggaaaaca atgagggacg gagaagcaag aggggagcca ggcgcctgaa acgacggaga  180
aggcacagaa tccagagggt gaagaaactg ctgttcgatt acaacctgct gaccgaccat  240
tctgagctga gtggaattaa tccttatgaa gccagggtga aaggcctgag tcagaagctg  300
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aatagcaaag ctctggaaga gaagtatgtc gcagagctac agctggaacg gctgaagaaa  480
gatggcgagg tgagagggtc aattaatagg ttcaagacaa gcgactacgt caaagaagcc  540
aagcagctgc tgaaagtgca gaaggcttac caccagctgg atcagagctt catcgatact  600
tatatcgacc tgctggagac tcggagaacc tactatgagg gaccaggaga agggagcccc  660
ttcggatgga aagacatcaa ggaatggtac gagatgctga tgggacattg cacctatttt  720
ccagaagagc tgagaagcgt caagtacgct tataacgcag atctgtacaa cgccctgaat  780
gacctgaaca acctggtcat caccagggat gaaaacgaga aactggaata ctatgagaag  840
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aaggagatcc tggtcaacga agaggacatc aagggctacc gggtgacaag cactggaaaa  960
ccagagttca ccaatctgaa agtgtatcac gatattaagg acatcacagc acggaaagaa  1020
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tccgaggaca tccaggaaga gctgactaac ctgaacagcg agctgaccca ggaagagatc  1140
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accaatgaac gcattgaaga gattatccga actaccggga aagagaacgc aaagtacctg  1560
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tctaaaaagg gcaataggac tcctttccag tacctgtcta gttcagattc caagatctct  1800
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mol_type = other DNA
organism = synthetic construct

SEQUENCE: 2

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SEQ ID NO: 3 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct

SEQUENCE: 3

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SEQ ID NO: 4 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29

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source               1..29
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SEQUENCE: 5
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SEQ ID NO: 6         moltype = DNA  length = 29
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source               1..29
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                     organism = synthetic construct
SEQUENCE: 6
tcaaggaggc caacgtggaa aacaatgag                                    29

SEQ ID NO: 7         moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 7
ggacggagaa gcaagagggg agccaggcg                                    29

SEQ ID NO: 8         moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
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                     organism = synthetic construct
SEQUENCE: 8
cctgaaacga cggagaaggc acagaatcc                                    29

SEQ ID NO: 9         moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 9
agagggtgaa gaaactgctg ttcgattac                                    29

SEQ ID NO: 10        moltype = DNA  length = 28
FEATURE              Location/Qualifiers
source               1..28
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 10
aacctgctga ccgaccattc tgagctga                                     28

SEQ ID NO: 11        moltype = DNA  length = 30
FEATURE              Location/Qualifiers
source               1..30
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 11
gtggaattaa tccttatgaa gccagggtga                                   30

SEQ ID NO: 12        moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
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                     organism = synthetic construct
SEQUENCE: 12
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SEQ ID NO: 13        moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
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                     organism = synthetic construct
SEQUENCE: 13
gagttttccg cagctctgct gcacctggc                                    29
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FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 14
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SEQ ID NO: 15 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 15
aggtggaaga ggacaccggc aacgagctg 29

SEQ ID NO: 16 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 16
tctacaaagg aacagatctc acgcaatag 29

SEQ ID NO: 17 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 17
caaagctctg gaagagaagt atgtcgcag 29

SEQ ID NO: 18 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 18
agctacagct ggaacggctg aagaaagat 29

SEQ ID NO: 19 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 19
ggcgaggtga gagggtcaat taataggtt 29

SEQ ID NO: 20 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 20
caagacaagc gactacgtca aagaagcca 29

SEQ ID NO: 21 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 21
agcagctgct gaaagtgcag aaggcttac 29

SEQ ID NO: 22 moltype = DNA length = 28
FEATURE Location/Qualifiers
source 1..28
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 22
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SEQ ID NO: 23 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29

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SEQ ID NO: 25        moltype = DNA  length = 29
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SEQ ID NO: 26        moltype = DNA  length = 28
FEATURE              Location/Qualifiers
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SEQUENCE: 26
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SEQ ID NO: 27        moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
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SEQUENCE: 27
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SEQ ID NO: 28        moltype = DNA  length = 30
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FEATURE              Location/Qualifiers
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                     organism = synthetic construct
SEQUENCE: 29
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SEQ ID NO: 30        moltype = DNA  length = 28
FEATURE              Location/Qualifiers
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                     organism = synthetic construct
SEQUENCE: 30
ccagggatga aaacgagaaa ctggaata                                     28

SEQ ID NO: 31        moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
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SEQUENCE: 31
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SEQ ID NO: 32        moltype = DNA  length = 30
FEATURE              Location/Qualifiers
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                     organism = synthetic construct
SEQUENCE: 32
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source 1..29
mol_type = other DNA
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SEQUENCE: 33
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SEQ ID NO: 34 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 34
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SEQ ID NO: 35 moltype = DNA length = 28
FEATURE Location/Qualifiers
source 1..28
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 35
aagcactgga aaaccagagt tcaccaat 28

SEQ ID NO: 36 moltype = DNA length = 30
FEATURE Location/Qualifiers
source 1..30
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 36
ctgaaagtgt atcacgatat taaggacatc 30

SEQ ID NO: 37 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 37
acagcacgga aagaaatcat tgagaacgc 29

SEQ ID NO: 38 moltype = DNA length = 28
FEATURE Location/Qualifiers
source 1..28
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 38
cgaactgctg gatcagattg ctaagatc 28

SEQ ID NO: 39 moltype = DNA length = 30
FEATURE Location/Qualifiers
source 1..30
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 39
ctgactatct accagagttc cgaggacatc 30

SEQ ID NO: 40 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 40
caggaagagc tgactaacct gaacagcga 29

SEQ ID NO: 41 moltype = DNA length = 28
FEATURE Location/Qualifiers
source 1..28
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 41
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SEQ ID NO: 42 moltype = DNA length = 30
FEATURE Location/Qualifiers
source 1..30

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source               1..29
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                     organism = synthetic construct
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FEATURE              Location/Qualifiers
source               1..29
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                     organism = synthetic construct
SEQUENCE: 44
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FEATURE              Location/Qualifiers
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                     organism = synthetic construct
SEQUENCE: 45
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FEATURE              Location/Qualifiers
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                     organism = synthetic construct
SEQUENCE: 46
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SEQ ID NO: 47        moltype = DNA  length = 29
FEATURE              Location/Qualifiers
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SEQ ID NO: 48        moltype = DNA  length = 29
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SEQ ID NO: 49        moltype = DNA  length = 29
FEATURE              Location/Qualifiers
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                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 49
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SEQ ID NO: 50        moltype = DNA  length = 28
FEATURE              Location/Qualifiers
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                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 50
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SEQ ID NO: 51        moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 51
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mol_type = other DNA
organism = synthetic construct
SEQUENCE: 52
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SEQ ID NO: 53 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 53
gatcaatgag atgcagaaac gaaaccggc 29

SEQ ID NO: 54 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 54
agaccaatga acgcattgaa gagattatc 29

SEQ ID NO: 55 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 55
cgaactaccg ggaaagagaa cgcaaagta 29

SEQ ID NO: 56 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 56
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SEQ ID NO: 57 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 57
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SEQ ID NO: 58 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 58
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SEQ ID NO: 59 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 59
caatccattc aactacgagg tcgatcata 29

SEQ ID NO: 60 moltype = DNA length = 28
FEATURE Location/Qualifiers
source 1..28
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 60
ttatccccag aagcgtgtcc ttcgacaa 28

SEQ ID NO: 61 moltype = DNA length = 30
FEATURE Location/Qualifiers
source 1..30

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                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 61
ttcctttaac aacaaggtgc tggtcaagca                                    30

SEQ ID NO: 62        moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 62
ggaagagaac tctaaaaagg gcaatagga                                     29

SEQ ID NO: 63        moltype = DNA  length = 28
FEATURE              Location/Qualifiers
source               1..28
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 63
ctcctttcca gtacctgtct agttcaga                                      28

SEQ ID NO: 64        moltype = DNA  length = 30
FEATURE              Location/Qualifiers
source               1..30
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 64
ttccaagatc tcttacgaaa cctttaaaaa                                    30

SEQ ID NO: 65        moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 65
gcacattctg aatctggcca aaggaaagg                                     29

SEQ ID NO: 66        moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 66
tccagcccca gaatgtagtt ccttttcat                                     29

SEQ ID NO: 67        moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 67
tcccataccc cacgcttgta atcccgatg                                     29

SEQ ID NO: 68        moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 68
gatcacgtcc cttgtttcat agtcaataa                                     29

SEQ ID NO: 69        moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 69
gcctccttga acagtctgac gcctgcgtc                                     29

SEQ ID NO: 70        moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 70
ttctccgtcc ctcattgttt tccacgttg                                     29
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SEQ ID NO: 71 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 71
tcgtttcagg cgcctggctc ccctcttgc 29

SEQ ID NO: 72 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 72
ttcaccctct ggattctgtg ccttctccg 29

SEQ ID NO: 73 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 73
tcagcaggtt gtaatcgaac agcagtttc 29

SEQ ID NO: 74 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 74
attaattcca ctcagctcag aatggtcgg 29

SEQ ID NO: 75 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 75
ctcaggcctt tcaccctggc ttcataagg 29

SEQ ID NO: 76 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 76
cggaaaactc ttcctctgac agcttctga 29

SEQ ID NO: 77 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 77
tcggcgctta gccaggtgca gcagagctg 29

SEQ ID NO: 78 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 78
tcttccacct cattgacgtt atgcactcc 29

SEQ ID NO: 79 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 79
cctttgtaga cagctcgttg ccggtgtcc 29

SEQ ID NO: 80 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29

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                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 80
cagagctttg ctattgcgtg agatctgtt                                    29

SEQ ID NO: 81        moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 81
agctgtagct ctgcgacata cttctcttc                                    29

SEQ ID NO: 82        moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 82
tcacctcgcc atctttcttc agccgttcc                                    29

SEQ ID NO: 83        moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 83
gcttgtcttg aacctattaa ttgaccctc                                    29

SEQ ID NO: 84        moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 84
agcagctgct tggcttcttt gacgtagtc                                    29

SEQ ID NO: 85        moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 85
ccagctggtg gtaagccttc tgcactttc                                    29

SEQ ID NO: 86        moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 86
gtcgatataa gtatcgatga agctctgat                                    29

SEQ ID NO: 87        moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 87
tcatagtagg ttctccgagt ctccagcag                                    29

SEQ ID NO: 88        moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 88
atccgaaggg gctcccttct cctggtccc                                    29

SEQ ID NO: 89        moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 89
catctcgtac cattccttga tgtctttcc                                    29
```

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SEQ ID NO: 90 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 90
tctggaaaat aggtgcaatg tcccatcag 29

SEQ ID NO: 91 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 91
tataagcgta cttgacgctt ctcagctct 29

SEQ ID NO: 92 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 92
gtcattcagg gcgttgtaca gatctgcgt 29

SEQ ID NO: 93 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 93
tcatccctgg tgatgaccag gttgttcag 29

SEQ ID NO: 94 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 94
acttctcata gtattccagt ttctcgttt 29

SEQ ID NO: 95 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 95
ctgcttaaac acgttttcga tgatctgga 29

SEQ ID NO: 96 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 96
gcaatctgtt tcagtgtagg ctttttctt 29

SEQ ID NO: 97 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 97
tgtcctcttc gttgaccagg atctcctta 29

SEQ ID NO: 98 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 98
tccagtgctt gtcacccggt agcccttga 29

SEQ ID NO: 99 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29

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                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 99
tacactttca gattggtgaa ctctggttt                                    29

SEQ ID NO: 100       moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 100
tccgtgctgt gatgtcctta atatcgtga                                    29

SEQ ID NO: 101       moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 101
cagcagttcg gcgttctcaa tgatttctt                                    29

SEQ ID NO: 102       moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 102
tagatagtca ggatcttagc aatctgatc                                    29

SEQ ID NO: 103       moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 103
gctcttcctg gatgtcctcg gaactctgg                                    29

SEQ ID NO: 104       moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 104
ctgggtcagc tcgctgttca ggttagtca                                    29

SEQ ID NO: 105       moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 105
ttcagattac taatctgttc gatctcttc                                    29

SEQ ID NO: 106       moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 106
gggacaggtt gtgtgttccg gtgtacccc                                    29

SEQ ID NO: 107       moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 107
ctcatccaga atcagattga tagctttca                                    29

SEQ ID NO: 108       moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 108
gcaatctgat tgtcgtttgt atgccacag                                    29
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SEQ ID NO: 109 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 109
ttggtaccag cttcagccgg ttaaagatt 29

SEQ ID NO: 110 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 110
ctctttctgc tgactcaggt ccacctttt 29

SEQ ID NO: 111 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 111
atgaaatcgt ccaccagtgt ggttgggat 29

SEQ ID NO: 112 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 112
tgaagctccg cttgaccacg ggtgacaga 29

SEQ ID NO: 113 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 113
gatggcgttg atcactttga tgctctgga 29

SEQ ID NO: 114 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 114
atatcattgg gcaggccgta cttcttgat 29

SEQ ID NO: 115 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 115
tgttcttctc cctagccagc tcgataatg 29

SEQ ID NO: 116 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 116
ctcattgatc atcttctgtg cgtccttgc 29

SEQ ID NO: 117 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 117
tcattggtct gccggtttcg tttctgcat 29

SEQ ID NO: 118 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29

-continued

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                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 118
cggtagttcg gataatctct tcaatgcgt                                    29

SEQ ID NO: 119       moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 119
ttcaatcagg tactttgcgt tctctttcc                                    29

SEQ ID NO: 120       moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 120
ccctcctgca tatcgtgcag cttgatttt                                    29

SEQ ID NO: 121       moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 121
ggatggcctc cagagaatac agacacttt                                    29

SEQ ID NO: 122       moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 122
gaatggattg ttcagcaggt cctccaggg                                    29

SEQ ID NO: 123       moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 123
ctggggataa tatgatcgac ctcgtagtt                                    29

SEQ ID NO: 124       moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 124
tgttaaagga attgtcgaag gacacgctt                                    29

SEQ ID NO: 125       moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 125
gttctcttcc tgcttgacca gcaccttgt                                    29

SEQ ID NO: 126       moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 126
tggaaaggag tcctattgcc ctttttaga                                    29

SEQ ID NO: 127       moltype = DNA  length = 29
FEATURE              Location/Qualifiers
source               1..29
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 127
agatcttgga atctgaacta gacaggtac                                    29
```

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SEQ ID NO: 128 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 128
cagaatgtgc tttttaaagg tttcgtaag 29

SEQ ID NO: 129 moltype = DNA length = 29
FEATURE Location/Qualifiers
source 1..29
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 129
ctgatgcggc cctttccttt ggccagatt 29

What is claimed is:

1. A method for template-free de novo synthesis of a long-chain nucleic acid, comprising the following steps:

S1, synthesizing double-stranded oligonucleotides; and

S2, combining and ligating of the double-stranded oligonucleotides to obtain a target long-chain nucleic acid, wherein

the double-stranded oligonucleotides in the step S1 are combined and ligated according to a sequence of the target long-chain nucleic acid; sequence numbers of the double-stranded oligonucleotides to be used are marked as 1, 2, 3, 4 . . . N−1, N;

N is a power of 2 to a power of n; and

a specific ligation method comprises the following steps:

(1) moving free double-stranded oligonucleotides marked as N respectively to a region where a double-stranded oligonucleotide marked as N−1 is located, for a first ligation reaction to obtain N/2 first composite sequences;

then, removing impurities by elution to remove a ligation system and unligated double-stranded oligonucleotide chains;

(2) then, treating first composite sequences marked as N/2 with endonuclease to obtain first free composite sequences, moving the first free composite sequences respectively to a region where a first composite sequence marked as N/2−1 is located, for a second ligation reaction to obtain $N/2^2$ second composite sequences, and then removing the impurities by the elution; and

(3) repeating the step (2) for a plurality of times until $N/2^n$, that is, one composite sequence is obtained, removing the impurities by the elution again after the second ligation reaction is completed, to remove the ligation system and the unligated double-stranded oligonucleotide chains to obtain a fixed target long-chain nucleic acid.

2. The method for template-free de novo synthesis of the long-chain nucleic acid according to claim **1**, wherein the double-stranded oligonucleotide marked as N−1 in the step (1) is either a fixed nucleic acid chain or a free nucleic acid chain.

3. The method for template-free de novo synthesis of the long-chain nucleic acid according to claim **1**, wherein synthesizing the double-stranded oligonucleotides in the step S1 comprises the following steps:

a) firstly, fixing a starting single-stranded oligonucleotide at a reaction site by biotin;

b) then, adding an amplification reaction system containing 3'-blocked deoxynucleoside triphosphates (dNTPs) and a deblocking reaction system into the reaction site in sequence, and repeating the step b) for a plurality of times until fixed single-stranded oligonucleotides are obtained;

c) subsequently, treating the fixed single-stranded oligonucleotides with the endonuclease, such that an extension chain is separated from the starting single-stranded oligonucleotide to obtain free oligonucleotide chains for template-free synthesis; and

d) finally, moving the free oligonucleotide chains to a region where complementary pairing chains are located, and performing a heating reaction to obtain fixed double-stranded oligonucleotides, the complementary pairing chains being the fixed single-stranded oligonucleotides in the step c).

4. The method for template-free de novo synthesis of the long-chain nucleic acid according to claim **3**, wherein components and contents of the amplification reaction system in the step b) are as follows:

the amplification reaction system contains 1.0-5.0 μM of terminal deoxynucleotidyl transferase (TdT) enzyme, 200 μM-500 μM of 3'-O-phosphate blocked dNTPs, 50-400 mM of a potassium cacodylate buffer, 20-50 mM of Tris, and 3-6 mM of $CoCl_2$; and

reaction conditions for the amplification reaction system applied in the step b) are as follows: a reaction volume is 0.1-50 μL, and incubation is carried out at 25-45° C. for 5-30 min.

5. The method for template-free de novo synthesis of the long-chain nucleic acid according to claim **3**, wherein components and contents of the deblocking reaction system in the step b) are as follows:

the deblocking reaction system contains 75-120 mM of Tris-HCl at pH 6.5, 8-15 mM of $MgCl_2$, 5-8 mM of 2-mercaptoethanol, and one unit of T4 polynucleotide kinase; and

reaction conditions for the deblocking reaction system applied in the step b) are as follows: a reaction volume is 0.1-50 μL, and incubation is carried out at 25-45° C. for 5-30 min.

6. The method for template-free de novo synthesis of the long-chain nucleic acid according to claim **3**, wherein the step c) comprises the following steps:

treating the fixed single-stranded oligonucleotides with the endonuclease first, such that the extension chain and the starting single-stranded oligonucleotide are free in lysis buffer for a lysis reaction; and after the lysis reaction is completed, transferring a lysis product and inactivating the endonuclease by heating, to obtain the free oligonucleotide chains for the template-free synthesis.

7. The method for template-free de novo synthesis of the long-chain nucleic acid according to claim **6**, wherein components and contents of the lysis buffer, and lysis reaction conditions in the step c) are as follows:

the lysis buffer contains 40-60 mM of K—Ac, 15-25 mM of Tris-Ac, 8-15 mM of Mg—Ac, and 1-5 mM of dithiothreitol (DTT); and

the lysis reaction conditions are as follows: a reaction volume is 0.1-50 μL, and 1-100 U of endonuclease V is reacted at 25-45° C. for 15-60 min.

8. The method for template-free de novo synthesis of the long-chain nucleic acid according to claim **3**, wherein the step d) comprises the following steps:

moving the free oligonucleotide chains for the template-free synthesis to the region where the complementary pairing chains are located, maintaining the region at 95° C. for 3-5 min, and then cooling the region to 25° C. at a constant speed within 15 min to obtain the fixed double-stranded oligonucleotides, the complementary pairing chains being the fixed single-stranded oligonucleotides in the step c).

9. Use of the method for template-free de novo synthesis of the long-chain nucleic acid according to claim **1** in a synthesis of a deoxyribonucleic acid (DNA) fragment and a mutation of a DNA sequence.

10. Use of the method for template-free de novo synthesis of the long-chain nucleic acid according to claim **9**, wherein an application environment is any one of a centrifuge tube, a microfluidic device, a digital microfluidic device, a microarray or a biochip.

* * * * *