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

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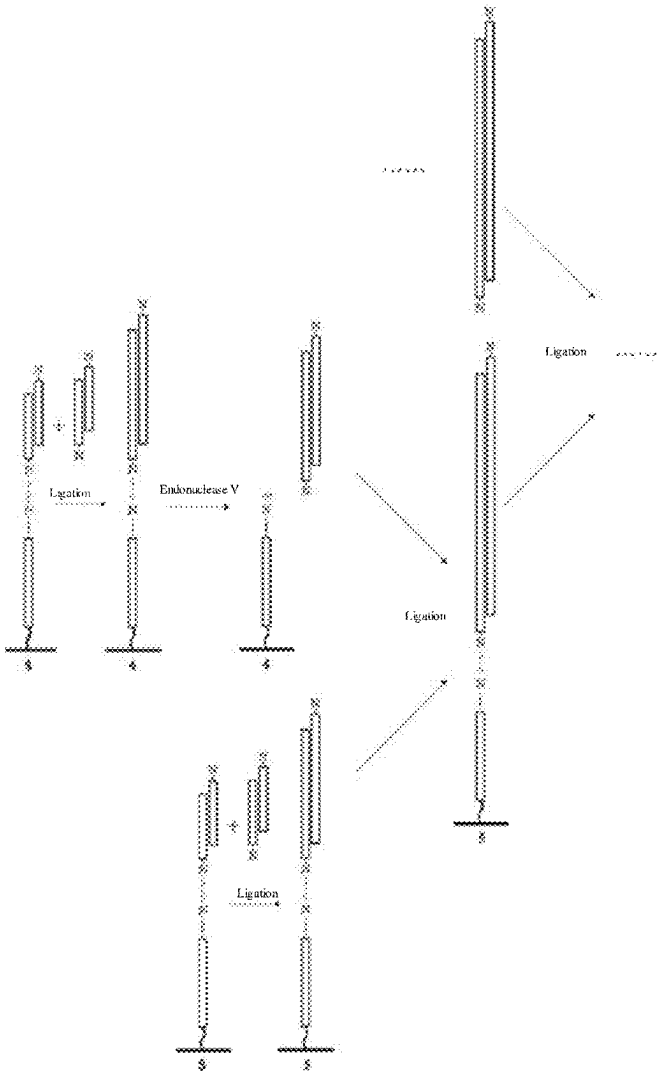
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(57) **ABSTRACT**

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.**



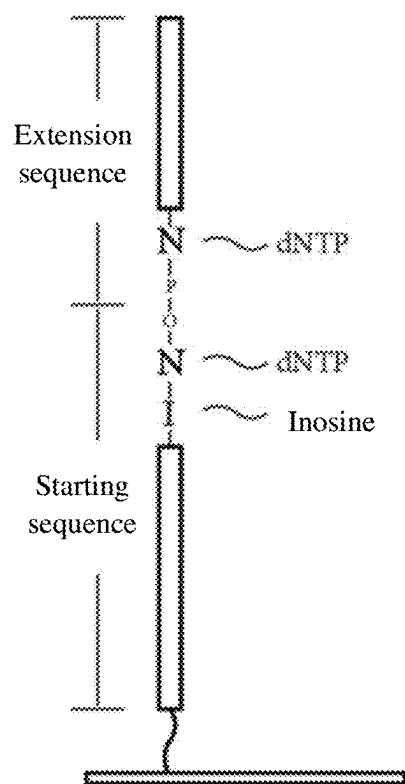


FIG. 1

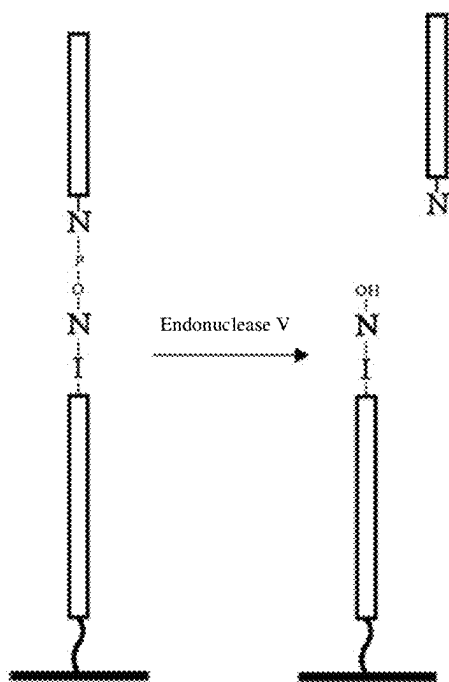


FIG. 2

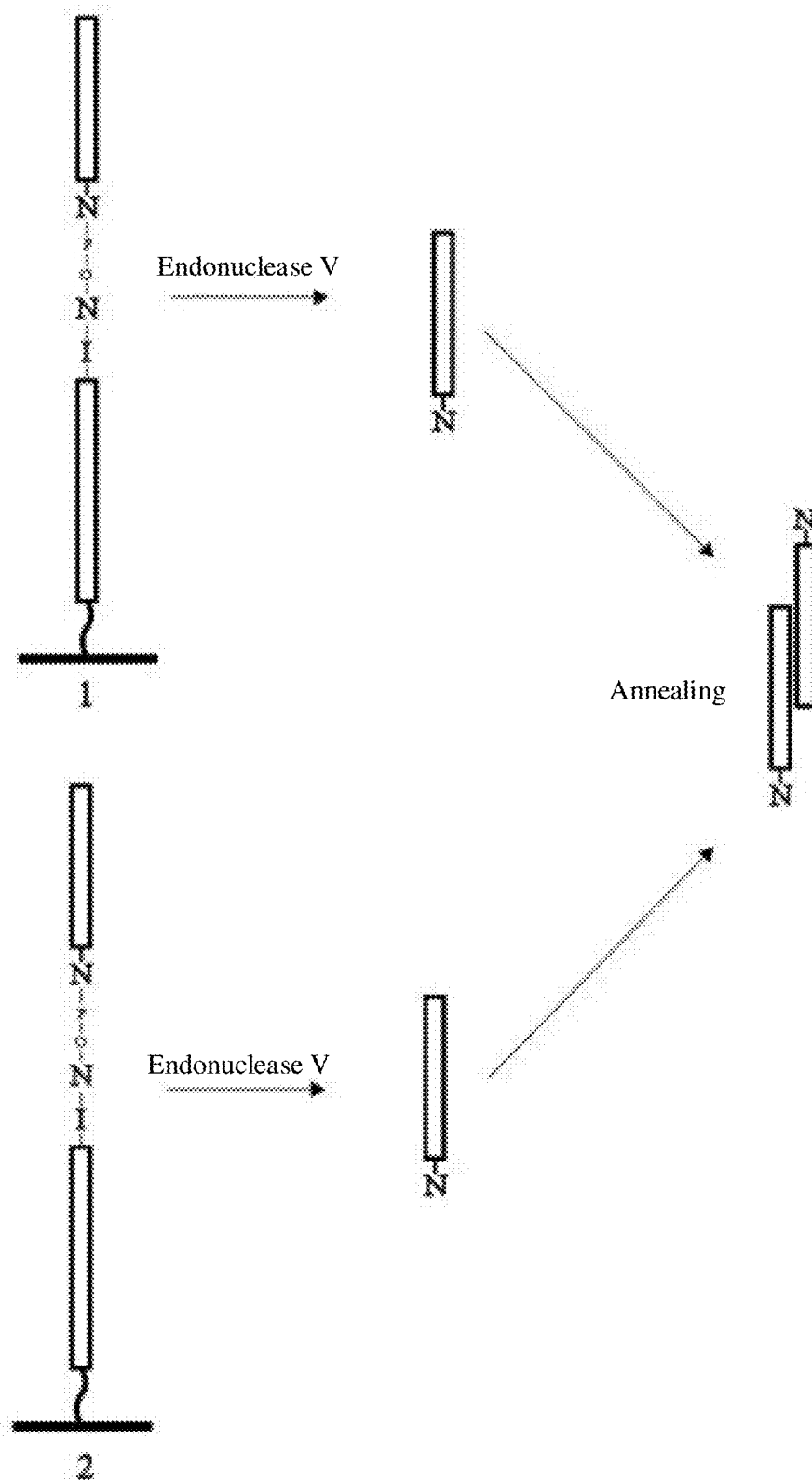


FIG. 3

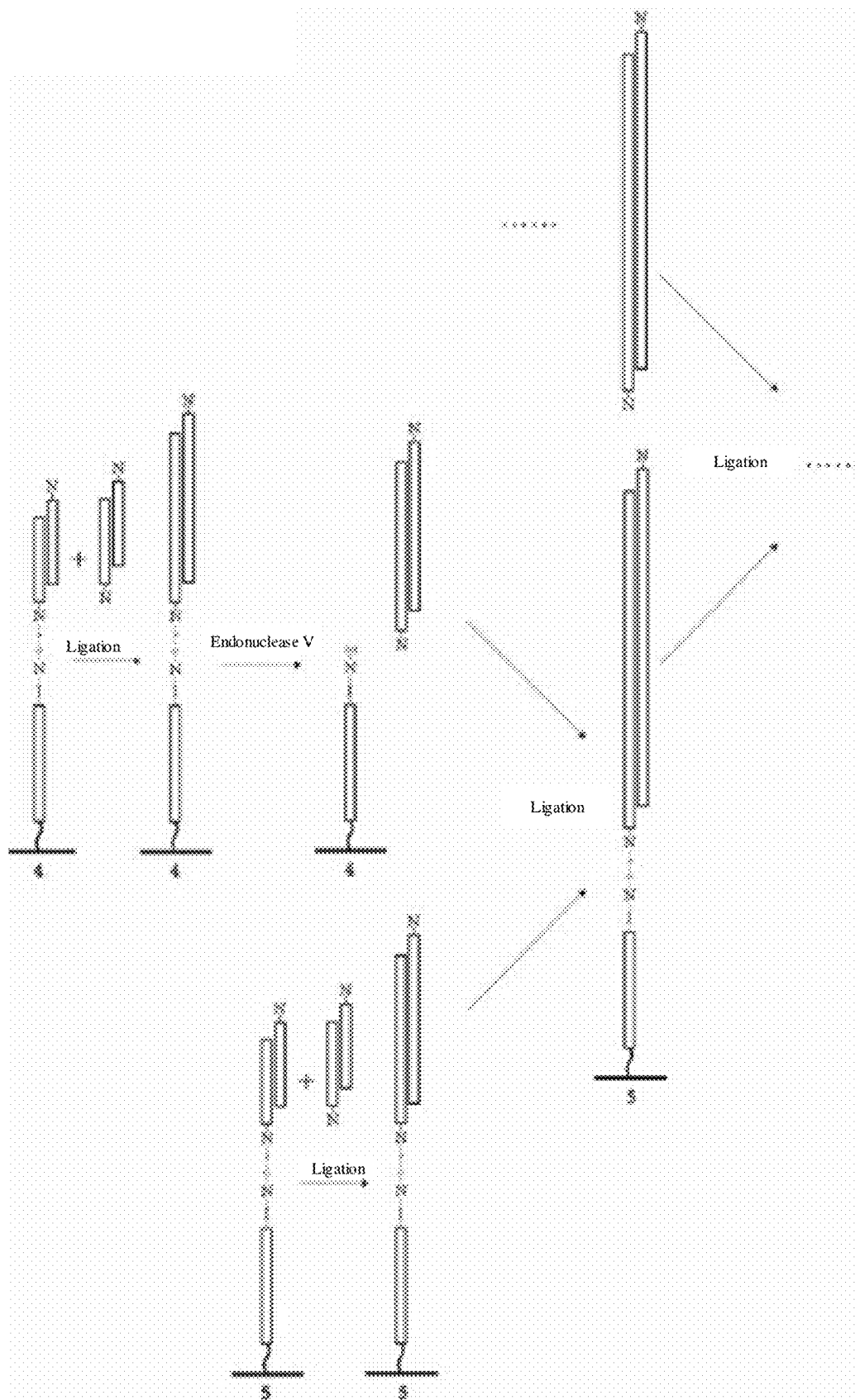


FIG. 4

## 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 tccagagggg gaagaaactg ctgttcgatt acaacctgct gaccgaccat 240  
tctgagctga gtggaattaa tccttatgaa gccagggtga aaggcctgag tcagaagctg 300  
tcagaggaag agttttccgc agctctgctg cacctggcta agcgccgagg agtgcataac 360  
gtcaatgagg tggaaagagga caccggcaac gagctgtcta caaaggaaca gatctacgc 420  
aatagcaaag ctctggaaga gaagtatgtc gcagagctac agctggaacg gctgaagaaa 480  
gatggcgagg tgagaggggc aattaatagg ttcaagacaa gcgactacgt caaagaagcc 540  
aagcagctgc tgaaagtgc gaaggcttac caccagctgg atcagagctt catcgatact 600  
tatatcgacc tgctggagac tcggagaacc tactatgagg gaccaggaga agggagcccc 660  
ttcggatgga aagacatcaa ggaatggtag gagatgctga tgggacattg cacctatatt 720  
ccagaagagc tgagaagcgt caagtacgt tataacgcag atctgtacaa cgccctgaat 780  
gacctgaaca acctggtcat caccagggat gaaaacgaga aactggaata ctatgagaag 840  
ttccagatca tcgaaaacgt gtttaagcag aagaaaaagc ctacactgaa acagattgct  
aaggagatcc tggtaacga agaggacatc aagggtacc gggtgacaag cactggaaaa 960  
ccagagtcca ccaatctgaa agtgtatcac gatattaagg acatcacagc acggaaagaa 1020  
atcattgaga acgccgaact gctggatcag attgctaaga tctgactat ctaccagagt 1080  
tccgaggaca tccaggaaga gctgactaac ctgaacagcg agctgacca ggaagagatc 1140  
gaacagatta gtaatctgaa ggggtacacc ggaacacaca acctgtccct gaaagctatc 1200  
aatctgattc tggatgagct gtggcatata aacgacaatc agattgcaat cttaaccgg 1260  
ctgaagctgg taccaaaaaa ggtggacctg agtcagcaga aagagatccc aaccacactg 1320  
gtggacgatt tcattctgtc acccgtggtc aagcggagct tcatccagag catcaaagt 1380  
atcaacgcca tcatcaagaa gtacggcctg ccaatgata 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 aggacctgt gaacaatcca ttcaactacg aggtcgatca tattatcccc 1680  
agaagcgtgt ccttcgacaa ttctttaac aacaaggtgc tggtaagca ggaagagaa 1740  
tctaaaaagg gcaataggac tccttcag tacctgtcta gtcagattc caagatctct 1800  
tacgaaacct ttaaaagca 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<sup>2</sup> second composite sequences, and then removing impurities by elution; and

[0019] (3) repeating the step (2) for several times until N/2<sup>n</sup>, 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  $\mu\text{M}$  of TdT enzyme, 200  $\mu\text{M}$ -500  $\mu\text{M}$  of 3'-O-phosphate blocked dNTPs, 50-400 mM of a potassium cacodylate buffer, 20-50 mM of Tris, and 3-6 mM of  $\text{CoCl}_2$ ; and

[0028] reaction conditions for the amplification reaction system applied in step 2) are as follows: a reaction volume is 0.1-50  $\mu\text{L}$ , 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  $\text{MgCl}_2$ , 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  $\mu\text{L}$ , 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  $\mu\text{L}$ , 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 $\mu$ M of TdT enzyme, 400 $\mu$ M of 3'-O-phosphate blocked dNTPs, 100 mM of a potassium cacodylate buffer (pH 7.2), 25 mM of Tris, and 5 mM of $\text{CoCl}_2$ (pH 7.2)
Deblocking reaction system	100 mM of Tris-HCl (pH 6.5), 10 mM of $\text{MgCl}_2$ , 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

tggggctggacatcggtattacaagcgtg 29  
gggtatgggattattgactatgaaacaag 29  
ggacgtgatcgacgcaggcgctcagactgt 29  
tcaaggaggccaacgtagaaaaaatgag 29  
ggacggagaagcaagaggggagccaggcg 29  
cctgaaacgacggagaaggcacagaatcc 29  
agaggggtgaagaaactgctgttcgattac 29  
aacctgctgaccgaccattctgagctga 29  
gtggaattaatccttatgaagccagggtga 29  
aaggcctgagtcagaagctgtcagaggaa 29  
gagttttccgcagctctgctgcacctggc 29  
taagcgcgaggagtgcatatcgctcaatg 29  
aggtggaagagacacgggcaacgagctg 29  
tctacaaaggaacagatctcacgcaatag 29  
caaagctctggaagagaagtatgtcgag 29  
agctacagctggaacggctgaagaagat 29  
gccgaggtgagaggggtcaattaataggtt 29  
caagacaagcgactacgtcaaagaagcca 29  
agcagctgctgaaagtgcagaaggcttac 29  
caccagctcgatcagagcttcacgata 29  
cttatatcgacctgctggagactcggaga 29

-continued

acctactatgagggaccaggagaaggagc 29  
cccttcggatggaaagacatcaaggaatg 29  
gtacgagatgctgatgggacattgcacc 29  
tattttccagaagagctgagaagcgtcaa 29  
gtacgcttataacgcagatctgtacaacgc 29  
cctgaatgacctgaacaacctggcatca 29  
ccagggatgaaaacgagaaactggaata 29  
ctatgagaagttccagatcatcgaaaacg 29  
tgtttaagcagaagaaaaagcctacactga 29  
aacagattgctaaggagatcctgggtcaac 29  
gaagaggacatcaaggggtaccgggtgac 29  
aagcactcgaaaaccagagttcaccaat 29  
ctgaaagtgtatcacgatatattaaggacatc 29  
acagcacggaaagaatcattgagaacgc 29  
cgaactgctggatcagattgctaagatc 29  
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cccaatgatatcattatcgagctggctag 29  
ggagaagaacagcaaggacgcacagaagat 29  
gatcaatgagatgcagaaacgaaaccggc 29  
agaccaatgaacgcattgaagagattatc 29  
cgaactaccgggaaagagaacgcaaagta 29  
cctgattgaaaaaatcaagctgcacgata 29  
tgcaggagggaaagtgtctgtattctctg 29  
gaggccatccccctggaggacctgctgaa 29  
caatccattcaactacgaggtcgatcata 29  
ttatccccagaagcgtgtccttcgacaa 29  
ttcctttaacaacaaggtgctgggtcaagca 29  
ggaagagaactctaaaaagggaatagga 29



-continued

ctcctttccagtagctgtctagttcaga 29

ttccaagatctcttacgaaccttttaaaa 29

gcacattctgaatctggccaaaggaaagg 29

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

tccagccccagaatgtagttccttttcat 29

tcccataccccacgcttgtaatcccgatg 29

gatcacgtcccttggttcatagtcaataa 29

gcctccttgaaacagtctgacgcctgcgtc 29

ttctccgtccctcattgttttccacgttg 29

tcgtttcaggcgctggctccctcttgc 29

ttcacctctcgtattctgtgccttctccg 29

tcagcaggttgtaatcgaaacagcagtttc 29

attaattccactcagctcagaatggtcgg 29

ctcaggcctttcacctggcctcataagg 29

cggaaaactcttccctctgacagcttctga 29

tcggcgcttagccaggtgcagcagagctg 29

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tcatagtaggttctccgagcttccagcag 29

atccgaaggggtcccttctctgtgtccc 29

catctcgtaccattccttgatgtctttcc 29

tctggaaaataagtgcaatgtcccatcag 29

tataagcgtacttgacgcttctcagctct 29

gtcattcagggcggtgtacagatctgcgt 29

tcacccctgggtgatgaccaggtgttcag 29

acttctcatagtagtccagtttctcggtt 29

ctgcttaaacacgcttttcgatgatctgga 29

gcaatctgtttcagtgtaggctttttctt 29

tgctctctcgttgaccaggatctcctta 29

tccagtgttgacacccggtagcccttga 29

tacactttcagattgggtgaactctgggtt 29

tccgtgctgtgatgtccttaatatcgtga 29

cagcagttcggcggttctcaatgatttctt 29

-continued

tagatagtcaggatcttagcaatctgato 29

gctcttctggatgtcctcggaactctcg 29

ctgggtcagctcgtgttcaggttagtca 29

ttcagattactaatctgttcgatctcttc 29

gggacaggttggtgttcgggtgtacccc 29

ctcatccagaatcagattgatagctttca 29

gcaatctgattgtcggttgatgccacag 29

ttggtaccagcttcagccggttaagatt 29

ctctttctgctgactcaggtccacctttt 29

atgaaatcgccaccagtgtggtgggat 29

tgaagctccgcttgaccacgggtgacaga 29

gatggcgttgatcactttgatgctctgga 29

atatcattgggcaggccgtacttcttgat 29

tggtcttctccctagccagctcgataatg 29

ctcattgatcatcttctgtgcgtccttgc 29

tcattcgtctgcgggttctggttctgcat 29

cggtagttcggataatctcttcaatgcgt 29

ttcaatcaggtactttgcgttctctttcc 29

ccctcctgcatactgtgcagcttgatttt 29

ggatggcctccagagaatacacagacttt 29

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ctggggataaatatgatcgacctcgtagtt 29

tggttaaagggaattgtcgaaggacacgctt 29

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tggaaaggagtcctattgccctttttaga 29

agatcttcgaatctgaactagacaggtac 29

cagaatgtgcttttaagggttctgtaag 29

ctgatgcggcccttctcttggccagatt 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  $\mu\text{L}$  of ddH<sub>2</sub>O, 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  $\mu\text{L}$  of ddH<sub>2</sub>O 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  $\mu$ L at 37° C. for 5 min for amplification; each PCR tube was then washed with 100  $\mu$ L of ddH<sub>2</sub>O 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  $\mu$ L of ddH<sub>2</sub>O 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  $\mu$ L 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  $\mu$ L of ddH<sub>2</sub>O 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  $\mu$ L of ddH<sub>2</sub>O 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  $\mu$ L of ddH<sub>2</sub>O 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  $\mu$ L) at the same time, wherein a final concentration of each strand in the reaction system was 0.1  $\mu$ M, 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 generate 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  $\mu$ M in a 50  $\mu$ L 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  $\mu$ L reaction system to assemble at

a final concentration of 0.2  $\mu$ M, 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  $\mu$ L, wherein a final concentration of each strand was 0.1  $\mu$ M; 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  $\mu$ L 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.

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#### SEQUENCE LISTING

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SEQUENCE: 54		
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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		
cctgattgaa aaaatcaagc tgcacgata		29
SEQ ID NO: 57	moltype = DNA length = 29	
FEATURE	Location/Qualifiers	
source	1..29	
	mol_type = other DNA	
	organism = synthetic construct	
SEQUENCE: 57		
tcaggaggag aaagtgtctg tattctctg		29
SEQ ID NO: 58	moltype = DNA length = 29	
FEATURE	Location/Qualifiers	
source	1..29	
	mol_type = other DNA	
	organism = synthetic construct	
SEQUENCE: 58		
gaggccatcc ccctggagga cctgctgaa		29
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 tggtaagca		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		
tcccatacc caccgttgta 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 acagtttgac 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 ctcatgttt 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 cgcttggtc cctcttgc		29
SEQ ID NO: 72	moltype = DNA length = 29	
FEATURE	Location/Qualifiers	
source	1..29	
	mol_type = other DNA	
	organism = synthetic construct	
SEQUENCE: 72		
ttcacctct 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 ctacagtcag 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		
gcttgctttg 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		
ccagctgggtg 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		
atccgaagg gctcccttct cctggctcc		29
SEQ ID NO: 89	moltype = DNA length = 29	
FEATURE	Location/Qualifiers	
source	1..29	
	mol_type = other DNA organism = synthetic construct	
SEQUENCE: 89		
catctcgtag cattccttga tgtcttttc		29

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 gcgttgtaga	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	gttggttcag	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		
gcaatctggt 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		
tccagtgcgt 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 atacgtga		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 tgattcttt		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 gatgtectcg 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		
ttgtaccag 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 cctagccacg 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 cgctcttgc		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	



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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		
tgtaaagga 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 tttttaagg 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

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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<sup>2</sup> 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<sup>n</sup>, 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<sub>2</sub>; 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<sub>2</sub>, 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  $\mu$ L, 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.

\* \* \* \* \*