

MULTI-AMPLIFICATION METHOD

TECHNICAL FIELD

The present application relates to the field of biotechnology, and related to a method for multiplex amplification.

5 BACKGROUND

Multiplex polymerase chain reaction (MPCR) is a technology to simultaneously amplify multiple targets in a single PCR reaction and detect the amplified products with certain detection manners, so as to realize the detection of multiple targets. MPCR has been studied in depth as its characteristics of high efficiency, high throughput and low cost. It can not only significantly
10 improve the detection efficiency, but also reduce the detection cost. MPCR has been applied in many fields, including gene mutation and deletion, genotyping and quantification, genetic testing, companion diagnostics and so on. At present, MPCR can be divided into liquid-phase MPCR, microfluidic-based MPCR and solid-phase carrier multiplex PCR.

Liquid-phase MPCR employs specific primers of multiple targets to amplify the multiple
15 targets. This method introduces multiple amplification primers in a single reaction. As the number of molecules to be detected increase,, the number of nucleotide chains in the tube will also double. Dimers or even multimers are largely formed among nucleotide chains, which will lead to the emergence of non-specific amplification, where the amplification efficiency of targets reduce, resulting in low sensitivity and specificity , or even dominance of non-specific
20 amplification, causing the failure of target amplification. At the same time, this kind of design method will also lead to the problem of inconsistent amplification efficiency for each target molecule. At present, multiplex ligation-dependent probe amplification (MLPA), a detection strategy based on hairpin structure (PCT/US2002/037238) and a detection strategy of two-round amplification (PCT patent WO 96/41012) have been reported. In MLPA, specific ligation
25 primers and universal amplification primers are designed based on the terminal information of different targets . However, this technology not only needs a ligation reaction to start the reaction, but also is interfered by ligase specificity, difficulty in preparing ligation primers, number and concentration of primers, etc. PCT/US2002/037238 adopts an amplification mode based on long primers. Although the 5'-end information can be detected in principle, this solution however
30 requires an enzyme or chemical cleaving step and complicated pre-amplification and purification process after the first amplification step, which not only reduces the sensitivity of the reaction,

but also increases the complexity of operation. PCT patent WO 96/41012 discloses a detection strategy of two rounds of amplification. However, this solution not only cannot detect the 5'-end information of multiple specific target sequences, but also has the problems of a large number of primers and competition in amplification.

5 MPCR based on a microfluidic chip pre-loads different primer pairs in micropores of the chip, which confines each amplification to its own independent space. Proprietary detection instruments are used due to the unique amplification method. The technical complexity of this technology makes it difficult to implement.

10 MPCR based on a solid-phase carrier mainly comprises multiple detection technologies based on a "liquid chip". The liquid chip uses coded microspheres as a matrix for a multiplex detection. Although the liquid chip has the characteristics of multi-flux and high efficiency in detection, it does not solve the problem of mismatch between numerous primers and templates in the multiplex amplification, because it needs to perform liquid-phase multiplex amplification before using coded microspheres for hybridization identification.

15 In summary, MPCR has a broad application prospect, but at present, the existing technology is faced with the problems of decreased amplification efficiency, inconsistent amplification efficiency among different templates, and complicated operation steps. Therefore, it is urgent to develop a simple, convenient, high efficient technology for single reaction and equivalent multiplex nucleic acid amplification.

20 SUMMARY

25 In order to overcome the defects of traditional MPCR technology, such as reduced amplification efficiency, inconsistent amplification efficiency between templates, complicated operation steps and the like, the present application provides a novel method of multiplex amplification. The method includes the following two steps: Step 1, a capture oligonucleotide
30 binds to a target molecule, and specific linear amplification is initiated to obtain an intermediate sequence, which can be initiated by a universal primer for exponential amplification. Step 2, the multiplex intermediate sequence obtained by the linear amplification of the target molecule in Step 1 is subjected to multiplex exponential amplification initiated by a universal primer. Due to the special molecular sequence design, the capture oligonucleotide provided in the present application can not only ensure the specific linear amplification of the target molecule, but also ensure that the linear amplification product can be self-folded and self-extended to form a

universal sequence which can be initiated by the universal primer for exponential amplification and a specific sequence corresponding to multiplex target molecules. The linear amplification process is initiated with requiring only a low concentration (1/20-1/40 of the concentration of a common multiplex PCR primer) of a single specific capture oligonucleotide. Therefore, not only the specificity of amplification is ensured, but also the concentration and the number of primers required in the MPCR reaction are effectively reduced, and mutual interference between the primers is avoided. The multiplex amplification initiated by a universal primer not only significantly improves the amplification efficiency and ensures the sensitivity of the reaction, but also realizes the equivalent amplification of many different target molecules. The combination of the above two steps enables the present application to effectively solve the key problems existing in the traditional MPCR amplification technology.

To achieve the object, the present application adopts the technical solutions below.

In a first aspect, the present application provides a capture oligonucleotide for nucleic acid amplification, wherein the capture oligonucleotide includes a first universal sequence, a folding sequence and a binding capture sequence in sequence from 5'-end to 3'-end;

wherein, the folding sequence is at least partially the same as a 5'-end sequence of a target molecule; and

the binding capture sequence is complementary to a non-5'-end sequence of the target molecule.

In the present application, the capture oligonucleotide mainly includes three regions: a first universal sequence, a folding sequence and a binding capture sequence. The capture oligonucleotide firstly captures a target molecule having defined sequence information at the 5'-end via the binding capture sequence. Then, an extension reaction is performed with the target molecule as a template. A strand complementary to the target molecule is added to the 3'-end of the capture oligonucleotide to obtain an extended capture oligonucleotide. Due to the presence of the folding sequence and the extended 3'-end sequence that are at least partial complementary base pairing to each other, the extended capture oligonucleotide initiates intramolecular self-folding and further extends by the polymerase. A strand complementary to the first universal sequence is added at the 3'-end to obtain a product having a complete hairpin structure. PCR amplification detection can be performed using a universal primer(s) and/or a target molecule-specific primer(s).

In some embodiments of the present application, “a 5’-end sequence of a target molecule” refers to a contiguous sequence fragment of nucleotides from position 1 to position 4, nucleotides from position 1 to position 5, nucleotides from position 1 to position 6, nucleotides from position 1 to position 7, nucleotides from position 1 to position 8, nucleotides from position 1 to position 9, nucleotides from position 1 to position 10, nucleotides from position 1 to position 11, nucleotides from position 1 to position 12, nucleotides from position 1 to position 13, nucleotides from position 1 to position 14, nucleotides from position 1 to position 15, nucleotides from position 1 to position 16, nucleotides from position 1 to position 17, nucleotides from position 1 to position 18, nucleotides from position 1 to position 19, nucleotides from position 1 to position 20, or nucleotides from position 1 to greater position at the 5’-end of the target molecule.

In some embodiments of the present application, “the folding sequence is at least partially the same as a 5’-end sequence of a target molecule” means that the folding sequence is the same as or partially the same as the 5’-end sequence of the target molecule.

For example, in some embodiments, the folding sequence is the same as the 5’-end sequence of the target molecule. In such an embodiment, in the extended capture oligonucleotide obtained by extension with the target molecule as a template, the folding sequence is completely complementary to the extended 3’-end sequence and initiates intramolecular self-folding.

For example, in other embodiments, the folding sequence is partially the same as the 5’-end sequence of the target molecule. In such an embodiment, in the extended capture oligonucleotide obtained by extension with the target molecule as a template, the folding sequence is partially complementary to the extended 3’-end sequence and initiates intramolecular self-folding.

In some embodiments of the present application, “a non-5’-end sequence of a target molecule” refers to a sequence fragment that is 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or more nucleotides away from the first nucleotide at the 5’-end of the target molecule.

In the present application, the first universal sequence is an artificially designed sequence, which is independent of the sequence of the target molecule. Therefore, when detecting different target molecules, it is only necessary to design the folding sequence and the binding capture sequence of the capture oligonucleotide according to the target molecule, while keeping the first universal sequence unchanged.

Preferably, the capture oligonucleotide further includes a second universal sequence.

Preferably, the second universal sequence is located at the 5'-end of the binding capture sequence.

5 In the present application, the second universal sequence is arranged between the folding sequence and the binding capture sequence. After the capture oligonucleotide is extended and self-folded and a product having a hairpin structure is obtained, which can be detected via PCR amplification, by using universal primers which are the same as or partially the same as the first universal sequence and the second universal sequence respectively as an upstream primer and a downstream primer, so that the effect of equivalent amplification of multiplex target molecules
10 can be realized.

Preferably, the capture oligonucleotide further includes a nucleic acid extension blocking site, which is located at the 3'-end of the folding sequence for blocking the binding capture sequence and the folding sequence.

15 Preferably, the nucleic acid extension blocking site is located at the 5'-end of the second universal sequence for blocking the second universal sequence and the folding sequence.

In the present application, the nucleic acid extension blocking site is arranged at the 3'-end of the folding sequence, or further preferably between the second universal sequence and the folding sequence. The nucleic acid extension blocking site not only does not affect the self-folding of the extended capture oligonucleotide, but also allows the subsequent
20 self-extending by using itself as a template and adding a strand complementary to the first universal sequence at the 3'-end which is not belonged to the target molecule thus forming a product having a complete hairpin structure. The product having a complete hairpin structure can act as a template for PCR exponential amplification with a universal primer(s) and/or a target molecule-specific primer(s), thus effectively ensuring specificity and accuracy of amplification.

25 Preferably, the nucleic acid extension blocking site is modified with a substance that can block the extension of DNA (deoxyribonucleic acid) polymerase. The substance can terminate the extension at the nucleic acid extension blocking site when the product having a complete hairpin structure undergoes PCR amplification with a universal primer and/or a target molecule specific primer, thereby producing a linearized amplification template for subsequent exponential
30 amplification, and improving the efficiency of amplification.

Preferably, the substance that can block the extension of DNA polymerase is any one or a combination of at least two of Spacer, a thio group or a uracil base.

Preferably, the folding sequence is modified with a nucleic acid analogue.

5 In some embodiments of the present application, the Spacer refers to polyethylene glycol, preferably polyethylene glycol 18.

10 It is worth mentioning that due to limitations of the existing oligonucleotide synthesis technology, by-products with incomplete sequence fragments may be produced during the synthesis of the capture oligonucleotides. However, according to the present application, since the folding region sequence of the capture oligonucleotide is derived from the target molecule, the presence of the above by-products may initiate non-specific amplification. Therefore, in the present application, in order to further improve specificity of the detection system, the folding sequence of the capture oligonucleotide is modified with a nucleic acid analogue, and extension of synthesized by-products of the capture oligonucleotides is inhibited while ensuring the normal complementary base pairing function of the folding sequence, thus non-specific amplification of the original gene fragment or genome is blocked, therefore specificity of the detection system is improved.

15 Preferably, the nucleic acid analogue includes a peptide nucleic acid(s), a locked nucleic acid(s), a transposed base(s), 2'-O,4'-C-methylene bridge RNA (2'-O,4'-C-methylene bridge ribonucleic acid), 2'-O-methyl RNA or 2'-fluoro RNA.

20 In the present application, the transposed base is a kind of base with an opposite direction to a normal base. The 3'-end of the transposed base forms a 3-3-phosphodiester bond with the 3'-end of an upstream base and forms a 5-5-phosphodiester bond with the 5'-end of a downstream base, which can inhibit the extension of a DNA polymerase and the degradation of an exonuclease during the extension.

25 In a second aspect, the present application provides a kit for nucleic acid amplification, which includes the capture oligonucleotide described in the first aspect.

Preferably, the kit further includes a pretreatment reagent of a target nucleic acid, which is used for producing a target molecule having a defined sequence information at the 5'-end.

Preferably, the pretreatment reagent includes any one or a combination of at least two of a

nuclease for site-specific cleaving, a nucleic acid extension blocker or a specific primer.

Preferably, the specific primer has a modified group at the 5'-end.

Preferably, the modified group includes a phosphate group and/or a thio group.

Preferably, the kit further includes a universal primer.

- 5 Preferably, the universal primer has a nucleic acid sequence that is the same as or partially the same as the first universal sequence or the second universal sequence of the capture oligonucleotide.

- 10 In the present application, the amplification reaction based on the universal primer employs a target molecule having defined sequence information at the 5'-end as a template. The target molecule can be a target molecule that inherently has defined sequence information at the 5'-end, or a target molecule having defined sequence information at the 5'-end that is generated by treatment of a chemical factor or a biological method.

- 15 In the present application, the type of the nucleic acid sequence having defined sequence information at the 5'-end includes a normal nucleic acid sequence, a modified nucleic acid sequence, a single nucleotide mutation, sequence transposition, sequence deletion, sequence recombination and other different types.

Preferably, the target molecule that inherently has defined sequence information at the 5'-end includes, such as mature microRNA, precursor microRNA, cfDNA (cell free DNA), etc.

- 20 Preferably, the target molecule having defined sequence information at the 5'-end can be obtained through cleavage or blocking with a chemical factor, including any one or a combination of at least two of a short oligonucleotide chain complementary to the target sequence and modified with a polypeptide nucleic acid(s) (PNA), a short oligonucleotide chain complementary to the target sequence and modified with a locked nucleic acid(s) (LNA), a short oligonucleotide chain complementary to the target sequence and modified with methoxyl, a short
25 oligonucleotide chain complementary to the target sequence and modified with RNA, a short oligonucleotide chain complementary to the target sequence and modified with 2-fluoro, a short oligonucleotide chain complementary to the target sequence and modified with a reverse base, a short oligonucleotide complementary to the target sequence and modified with a phosphate group, or a short oligonucleotide complementary to the target sequence and modified with a thio

group, etc.

Preferably, the target molecular intermediate having defined sequence information at the 5'-end can be obtained through cleavage or blocking with a biological method, including any one or a combination of at least two of an AP nucleic acid exonuclease, an AP lyase, a uracil-DNA glycosylase (UDG), a nucleic acid restriction endonuclease, a methylation-dependent nucleic acid restriction endonuclease, a methylation-sensitive nucleic acid restriction endonuclease, a nicking enzyme, a giant nuclease, a zinc finger nuclease (ZFN), a transcription activator-like effector nuclease (TALEN), or CRISPR-Cas, etc.

In the present application, the amplification reaction can only occur with the joint participation of the capture oligonucleotide, the universal primer and/or the specific primer, which is briefly described as follows. In an amplification system involving dual universal primers, the universal primer used has a nucleic acid sequence that is the same as or partially the same as the first universal sequence or the second universal sequence of the capture oligonucleotide. When there is no target molecule having the defined 5'-end sequence, PCR amplification reaction cannot be started. When the target molecule having the defined 5'-end sequence exists in the system, an extension reaction will be initiated by the capture oligonucleotide to form an intermediate product having a hairpin structure, which will act as a template for an amplification reaction of the universal primer. At the same time, the extended capture oligonucleotide can self-fold and form a hairpin structure only when the extended sequence generated by the extension reaction is complementary pair with the folding sequence, thus effectively ensuring specificity of the reaction. The extended sequence and the folding sequence can be completely complementary or partially complementary.

Preferably, the kit further includes a specific primer.

In the present application, when the capture oligonucleotide only includes the first universal sequence, the folding sequence and the binding capture sequence, but does not include a second universal sequence, that is, in a single universal primer amplification system, an universal primer and a target molecule-specific primer are used as an upstream primer and a downstream primer of PCR respectively, which further improves specificity and sensitivity of the reaction. In the method of the present application, the Ct values obtained by detecting target molecules with the same concentration but different sequences are basically the same, thus proving that the method can ensure the equivalent amplification performance for different target molecules.

Preferably, the kit further includes a detection probe.

Preferably, the detection probe is labeled with a fluorescent group and/or a quenching group.

5 In the present application, the detection probe labeled with a fluorescent group and/or a quenching group is used to hybridize and complement an amplification product, which is beneficial to realize real-time quantitative detection of the amplification product.

Preferably, the fluorescent group is labeled at the 5'-end of the detection probe.

Preferably, the quenching group is labeled at the 3'-end of the detection probe.

10 Preferably, the fluorescent group includes any one of FAMTM (carboxyfluorescein), VICTM (green fluorescent protein), JOETM (2,7-dimethyl-4,5-dichloro-6-carboxyfluorescein), TETTM (tetrachloro-6-carboxyfluorescein), CYTM3 (maleimide 3), CYTM5 (maleimide 5), ROXTM (X-rhodamine maleimide), Texas RedTM (sulforhodamine 101 acid chloride) or LC RED460TM.

Preferably, the quenching group includes any one of BHQ1TM (black hole quencher 1), BHQ2TM, BHQ3TM, Dabcyl or Tamra (carboxyl tetramethylrhodamine).

15 In a third aspect, the present application provides a method for nucleic acid detection, which includes binding a target molecule with a capture oligonucleotide and starting specific linear amplification, and performing nucleic acid amplification detection based on a universal primer.

Preferably, steps of binding a target molecule with a capture oligonucleotide and starting specific linear amplification are as follows:

20 (1) allowing the binding of a target molecule having a defined 5'-end sequence to the binding capture sequence of a capture oligonucleotide;

(2) subjecting the capture oligonucleotide to an extension reaction with the target molecule as a template, and adding nucleotides complementary to the target molecule at the 3'-end of the capture oligonucleotide, to form an extended capture oligonucleotide;

25 (3) allowing the extended sequence of the extended capture oligonucleotide to bind to the intramolecular folding sequence, to form a product having a semi-hairpin structure; and

(4) subjecting the product having a semi-hairpin structure to an extension reaction, and adding nucleotides complementary to the intramolecular first universal sequence at the 3'-end, to form a product having a complete hairpin structure.

5 Preferably, steps of performing nucleic acid amplification detection based on a universal primer are as follows:

performing exponential amplification using the product having a complete hairpin structure as a template and using a universal primer and/or a specific primer, to obtain an amplification product.

10 Preferably, the universal primer has a nucleic acid sequence that is the same as or partially the same as the first universal sequence or the second universal sequence of the capture oligonucleotide.

Preferably, the exponential amplification includes a polymerase chain reaction, which may be, for example, any one of ordinary PCR, qPCR or digital PCR.

15 Preferably, the method further includes a step of binding the amplification product to the detection probe and detecting.

The present application further provides a method for nucleic acid amplification, which includes the following steps:

20 (1) contacting a target molecule having a defined 5'-end sequence with the capture oligonucleotide provided in the application, thereby the binding capture sequence of the capture oligonucleotide binds to the target molecule;

(2) subjecting the capture oligonucleotide to an extension reaction with the target molecule as a template, thereby nucleotides complementary to the target molecule are added at the 3'-end of the capture oligonucleotide to form an extended capture oligonucleotide;

25 (3) incubating the extended capture oligonucleotide under a condition allowing intramolecular self-folding, thereby a product having a semi-hairpin structure is formed via complementary base pairing between the extended sequence and the folding sequence of the extended capture oligonucleotide; and

(4) subjecting the product having a semi-hairpin structure to an extension reaction, thereby nucleotides complementary to the first universal sequence are added at the 3'-end to form a product having a complete hairpin structure.

In some specific embodiments, the method further includes: performing exponential
5 amplification using the product having a complete hairpin structure as a template and using a universal primer(s) and/or a specific primer(s), to obtain an amplification product. In some more specific embodiments, the universal primers has the nucleic acid sequence that is the same as or partially the same as the first universal sequence or the second universal sequence of the capture oligonucleotide.

10 Compared with the prior art, the present application has the following beneficial effects.

(1) The kit for nucleic acid detection of the present application does not need additional steps such as ligation reaction and chemical treatment of amplification products, and can realize high specificity and high sensitivity multiplex detection of nucleic acids as long as the target molecule has defined sequence information at the 5'-end.

15 (2) In the kit for nucleic acid detection of the present application, the capture oligonucleotide and the universal primer are specially designed and cooperated with each other, and in the presence of the target molecule, the target molecule initiates an extension reaction initiated by the capture oligonucleotide to form a product having a hairpin structure, which is used as a template for the amplification reaction of the universal primer and/or the specific
20 primer, so that the false positive problem is effectively avoided.

(3) In the present application, only when the extended 3'-end sequence of the capture oligonucleotide is complementary with the folding sequence, the capture oligonucleotide can be initiated to self-fold and form a hairpin structure, thus effectively ensuring specificity of the reaction.

25 (4) When detecting different target molecules, the kit for nucleic acid detection of the present application only needs to design the folding sequence and the binding capture sequence of the capture oligonucleotide according to the target molecule, while keeping the first universal sequence unchanged, thus greatly reducing the interference among multiplex primers for multiple target amplification and improving the amplification sensitivity.

(5) The kit for nucleic acid detection of the present application achieves the purpose of signal multiplication through the exponential amplification process. It can well meet the requirement of sensitivity in DNA/RNA detection. In addition, the exponential amplification is carried out only using the extended capture oligonucleotide and universal primers. It achieves the purpose of equivalent amplification of multiplex target molecules on the premise of keeping the number and concentration of universal primers unchanged. Thus, the deviation of amplification efficiency caused by sequence differences is avoided.

(6) The kit for nucleic acid detection of the present application is easy to operate.

BRIEF DESCRIPTION OF DRAWINGS

FIG. 1 (A) shows a schematic diagram of amplification with dual universal primers, and FIG. 1(B) shows a schematic diagram of amplification with a single universal primer;

FIG. 2 shows a schematic diagram of constructing a target molecule having a defined 5'-end sequence;

FIG. 3 shows results of qPCR detection of miRNA27a and let-7a;

FIG. 4 shows results of agarose gel detection of multiplex human genes;

FIG. 5 shows results of detection of rifampicin 526 resistance mutations in *Mycobacterium tuberculosis*;

FIG. 6 shows results of qPCR detection of Septin 9 gene;

FIG. 7 shows results of ddPCR detection of Septin 9 gene;

FIG. 8(A) shows results of sensitivity detection of Septin 9 gene in methylated samples at different concentrations, and FIG. 8(B) shows results of sensitivity detection of RASS-F1 gene in methylated samples at different concentrations;

FIG. 9(A) shows results of detection specificity of a common capture oligonucleotide, and FIG. 9(B) shows results of detection specificity of an LNA-modified capture oligonucleotide; and

FIG. 10 shows results of qPCR detection of RASS-F1 gene.

DETAILED DESCRIPTION

In order to further illustrate the technical methods adopted in the present application and technical effects, the present application will be further explained with embodiments and drawings. It can be understood that the detailed description herein are only used to explain the present application, but not to limit the present application.

Embodiments that do not indicate specific techniques or conditions are performed with reference to the techniques or conditions described in consensus literature in the art, or the product specification. The reagents or instruments used without indicating the manufacturer are all conventional products that can be purchased through regular channels.

10 **Example 1 Basic principle of amplification in the present application**

The present application includes two modes: a mode of dual universal primers and a mode of single universal primer. Mechanisms are shown in FIG. 1(A) and FIG. 1(B).

In the amplification involving dual universal primers, the capture oligonucleotide includes four regions: a first universal sequence U1s, a folding sequence T1s, a second universal sequence U2s and a binding capture sequence T2a. The capture oligonucleotide firstly captures a target molecule that has a defined 5'-end sequence via the binding capture sequence T2a. Then, the capture oligonucleotide is subjected to an extension reaction using the target molecule as a template, and a strand T1a complementary to the folding sequence T1s is added at the 3'-end to obtain an extended capture oligonucleotide. T1a and T1s are complementary to each other either completely or partially, as long as T1a and T1s can initiate intramolecular self-folding and form a product having a semi-hairpin structure. The product having a semi-hairpin structure is further extended under the polymerases, and a strand U1a complementary to the first universal sequence U1s is added at the 3'-end to obtain a product having a hairpin structure. The product is detected via PCR amplification by using universal primers which are the same as or partially the same as the first universal sequence U1s and the second universal sequence U2s, respectively. Specifically, a universal primer U1s (herein, the U1s is used for convenience, the actual sequence may not be exactly the same as U1s) binds to the U1a region of the product having a hairpin structure in the annealing stage then extends. When there is a nucleic acid extension blocking site between the second universal sequence and the folding sequence of the product having a hairpin structure, the extension stops at the nucleic acid extension blocking site to form a partial double-stranded structure. The product formed by the extension of the universal primer U1s is

exactly the PCR template for the universal primers U1s and U2s, and can be used for subsequent PCR amplification detection. When there is no nucleic acid extension blocking site between the second universal sequence and the folding sequence of the product having a hairpin structure, the universal primer U1s extends to form a complete double-stranded structure, and a partial region of the newly generated double-stranded structure is the PCR template for the universal primers U1s and U2s, and can be detected via subsequent PCR amplification.

In the amplification involving a single universal primer, the capture oligonucleotide mainly includes three regions: a first universal sequence U1s, a folding sequence T1s and a binding capture sequence T3a. The capture oligonucleotide firstly captures a target molecule having a defined 5'-end sequence via the binding capture sequence T3a. Then, the capture oligonucleotide is subjected to an extension reaction using the target molecule as a template, and a strand T1a complementary to the folding sequence T1s is added at the 3'-end to obtain an extended capture oligonucleotide. T1a and T1s are complementary to each other either completely or partially, as long as T1a and T1s can initiate intramolecular self-folding and form a product having a semi-hairpin structure. The product having a semi-hairpin structure is further extended under the polymerases, and a strand U1a complementary to the first universal sequence U1s is added at the 3'-end to obtain an intermediate product having a hairpin structure. The intermediate product is detected via PCR amplification by using a universal primer which is the same as or partially the same as the first universal sequence U1s and a primer specific for the target molecule. Specifically, a universal primer U1s (herein, the U1s is used for convenience, but the actual sequence may not be exactly the same as U1s) binds to the U1a region of the product having a hairpin structure in the annealing stage then extends. When there is a nucleic acid extension blocking site between the binding capture sequence and the folding sequence of the product having a hairpin structure, the extension stops at the nucleic acid extension blocking site to form a partial double-stranded structure. The product formed by the extension of the universal primer U1s is exactly the PCR template for the universal primer U1s and the specific primer T2a, and can be detected via subsequent PCR amplification. When there is no nucleic acid extension blocking site between the second universal sequence and the folding sequence of the product having a hairpin structure, the universal primer U1s extends to form a complete double-stranded structure, and a partial region of the newly generated double-stranded structure is the PCR template for the universal primer U1s and the specific primer T2a, and can be detected via subsequent PCR amplification.

Example 2 Construction of a product having a defined 5'-end sequence

In the method of the present application, a sample with a defined 5'-end sequence is required to initiate the extension reaction mediated by the capture oligonucleotide. A strategy for constructing a product with a defined 5'-end sequence is shown in FIG. 2: (1) for example, the extension of DNA polymerase is blocked with a nucleic acid extension blocker designed for the target molecule, so as to obtain a product having a defined 5'-end sequence; (2) specific enzyme cutting sites in the target molecule are digested with different cutting enzymes, so as to obtain a product having a defined 5'-end sequence; (3) or, specific amplification is performed using a low concentration of a specific primer designed for the target molecule, so as to obtain a product having a defined 5'-end sequence.

10 **Example 3 qPCR detection of miRNA 27a and let-7a**

MicroRNA (miRNA) is a kind of endogenous noncoding single-stranded small molecular RNA with a length of 19-25 nucleotides, which is ubiquitous in eukaryotic cells and controls the activity of more than 50% coding genes.

In this example, miRNA27a and let-7a were detected, and steps are as follows.

15 (1) MiRNA27a and let-7a sequences were synthesized artificially.

(2) Capture oligonucleotides, universal primers and detection probes of miRNA27a gene and let-7a gene were simultaneously added into a reaction system containing the miRNA27a sequence and the let-7a sequence. miRNA27a and let-7a were detected by PCR. The PCR amplification system was: template RNA, 5 nM of capture oligonucleotides, 150 nM of a first universal primer, 150 nM of a second universal primer, 150 nM of detection probes, 1 U of a reverse transcriptase, 1U of Taq polymerase, 200 μ M of dNTP, 4.5 mM of MgCl₂ and 2 $\times$ PCR buffer, with a final volume of 20 μ L. The PCR reaction procedure was 42°C for 1h, inactivation and pre-denaturation at 94°C for 15min; 10 cycles of 94°C for 10 s and 66°C for 90 s; and, 40 cycles of 94°C for 10 s and 65°C for 20 s. Real-time PCR was performed on ROCHE instrument (480), and the corresponding fluorescence values were collected.

The combination of the universal primer, the capture oligonucleotide and the detection probe included:

first universal primer (SEQ ID NO: 1)

5-GCGATCCGCACCGTCAATTCTG;

second universal primer (SEQ ID NO: 2)

5-GAGGTCCGATCCATCCAGACC;

capture oligonucleotide used for let-7a (SEQ ID NO: 3)

5-CCGCACCGTCAATTCGTGAGGTAGTAGG-spacer-CCGATCCATCCAGACCTTTAA
5 CTATACAAC;

let-7a detection probe (SEQ ID NO: 4)

5-FAM-TGAGGTAGTAGGT-MGB;

capture oligonucleotide used for miRNA27a (SEQ ID NO: 5)

5-CCGCACCGTCAATTCGTTCACAGTGGC-spacer-CCGATCCATCCAGACCTTTGCG
10 GAACTTAG;

miRNA27a detection probe (SEQ ID NO: 6)

5-ROX-GTTCACAGTGGC-MGB.

The detection results are shown in FIG. 3. The miRNA27a curve is the amplification curve of miRNA27a, and the let 7a curve is the amplification curve of let 7a. It follows that according to the present application, miRNA 27a and let-7a can be simultaneously detected using universal primers, and Ct values are the same, that is, the equivalent amplification of the two miRNAs is realized.

Example 4 Multiplex Detection of Human Gene

In this example, human gene DNA was detected, and steps are as follows.

20 (1) Genomic DNA of Hela cell line was extracted, diluted by gradient and used as a template.

(2) The genome was subjected to an enzyme digestion reaction with a restriction endonuclease AluI. The reaction system was 2 μ L of 10 $\times$ enzyme digestion buffer and different concentrations of genomic DNA, with a total of 20 μ L. The reaction was incubated at 37°C for 1

hour. After the enzyme digestion reaction, the system was heated to 85°C and incubated for 10 minutes to thermally inactivate AluI.

(3) Capture oligonucleotide for each gene and universal primers were added into the enzyme digestion system, respectively. PCR amplification was performed. The PCR amplification system included: an enzyme digested DNA template, 5 nM of capture oligonucleotides, 150 nM of a first universal primer, 150 nM of a second universal primer, 1 U of Taq polymerase, 200 µM of dNTP, 4.5 mM of MgCl₂ and 2×PCR buffer, with a final volume of 20 µL. The PCR reaction procedure was: pre-denaturation at 94°C for 5 min; 10 cycles of 94°C for 10 s and 66°C for 90 s; and, 40 cycles of 94°C for 10 s and 65°C for 20 s. Real-time PCR was performed on ROCHE instrument (480), and products of PCR were subjected to agarose gel electrophoresis.

The combination of the capture oligonucleotide and the universal primer includes:

first universal primer (SEQ ID NO: 7)

5-ACTGGACGAGCTGATTTACG;

second universal primer (SEQ ID NO: 8)

5-CGCAATCAGGCAATACCATAGC;

capture oligonucleotide 1 (SEQ ID NO: 9) used for IL4 (template length: 86 bp)

5-ACTGGACGAGCTGATTTACGCTGCTAGAGAAGTTGA-spacer-CGCAATCAGGCAATACCATAGCGGCTGCCACTGACCACCA;

capture oligonucleotide 2 (SEQ ID NO: 10) used for GAPDH (template length: 104 bp)

5-ACTGGACGAGCTGATTTACGCTGCTTAGACGCTGGA-spacer-CGCAATCAGGCAATACCATAGCGTCGCGGGAGGCTGCT;

capture oligonucleotide 3 (SEQ ID NO: 11) used for Homo sapiens actin alpha 1 (template length: 137 bp)

5-ACTGGACGAGCTGATTTACGCTTCCCGTTCTCAGCCTTGACGCAATCAGGCAATACCATAGCCATTGACCTCAACTACATGG;

capture oligonucleotide 4 (SEQ ID NO: 12) used for 18S ribosomal 1 (template length: 200 bp)

5-ACTGGACGAGCTGATTTACGCTCTTTCTCGATTCCGTGGG-spacer-ATCAGGCAA
TACCATAGCGTCCCTCTAAGAAGTTGG;

5 capture oligonucleotide 5 (SEQ ID NO: 13) used for 18S ribosomal 2 (template length: 300 bp)

5-ACTGGACGAGCTGATTTACGCTACCCGCCCAGAACTAGA-spacer-CGCAATCA
GGCAATACCATAGCCACCAGGCCGGAGCCATTG;

10 The results are shown in FIG. 4. Lane 1 was 20 bp ladder, Lane 2 was an amplification product of *Saimiri Sciurius* Interleukin 4 (IL4) gene, Lane 3 was an amplification product of *Homo sapiens* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, Lane 4 was an amplification product of *Homo sapiens* actin alpha 1 gene, Lane 5 was an amplification product of 18S ribosomal 1, Lane 6 was an amplification product of 18S ribosomal 2, and Lane 7 was a result of multiplex amplification, wherein all results were positive.

15 This indicates that the present application provides a sensitive and specific multiplex detection of different human-derived genes with universal primers.

Example 5 Detection of rifampicin resistance mutations in *Mycobacterium tuberculosis*

20 Multiplex detection of multi-position resistance mutations was performed on rifampicin resistance mutations in *Mycobacterium tuberculosis* (mutation at position 526 of the rpoB gene) in combination with a mismatch repair enzyme and universal primers.

Steps are as follows.

25 (1) 100 nM artificial sequence completely complementary to a wild genome was added into a synthesized mutant plasmid of *Mycobacterium tuberculosis*. The system was heated to 95°C and incubated for 10 minutes. The system was cooled to 60°C and hybridization incubation was performed for 30 minutes. The artificial sequence was hybridized with the mutant plasmid to produce a mismatch. The system was slowly cooled to room temperature to obtain a hybridized product of the artificial sequence and the genome.

(2) The hybridized product obtained in step (1) was subjected to an enzyme digestion reaction with a mismatch enzyme T7E1. The reaction system was 2 μ L of 10 $\times$ enzyme digestion buffer and different concentrations of the hybridized product, with a total of 20 μ L. The reaction was incubated at 37°C for 1 hour. After the enzyme digestion reaction, the system was heated to 85°C and incubated for 10 minutes to thermally inactivate the mismatch enzyme.

(3) A corresponding capture oligonucleotide, universal primers and a detection probe were added into the system of the enzyme digestion reaction and a system of wild plasmid without enzyme digestion treatment, respectively. Mutation state of position 526 was detected by PCR. The PCR amplification system included: an enzyme digestion DNA template, 5 nM of a capture oligonucleotide, 150 nM of a first universal primer, 150 nM of a second universal primer, 150 nM of a detection probe, 1 U of Taq polymerase, 200 μ M of dNTP, 4.5 mM of MgCl₂ and 2 $\times$ PCR buffer, with a final volume of 20 μ L. The PCR reaction procedure was pre-denaturation at 94°C for 5 min; 10 cycles of 94°C for 10 s and 66°C for 90 s; and 40 cycles of 94°C for 10 s and 65°C for 20 s. Real-time PCR was performed on ROCHE instrument (480), and the corresponding fluorescence values were collected.

First universal primer (SEQ ID NO: 8)

5-CGCAATCAGGCAATACCATAGC.

Second universal primer (SEQ ID NO: 14)

5-GAGCGCAGGTCGTACTCG.

Capture oligonucleotide for 526 mutation (SEQ ID NO: 15)

5-CAGGCAATACCATAGCTTGTGGGTCAACCCCGACAGAGCGCAGGTCGTACTCG
TGCACCAGCCAGCTGAG.

Detection probe for 526 mutation (SEQ ID NO: 16)

5-FAM-CCAATTCATGGACCAGAACAAC-MGB.

Artificial sequence (SEQ ID NO: 17)

5-GGGGTTGACCCACAAGCGCCGACTGTCG.

The results are shown in FIG. 5. Curve A is an amplification curve of the mutant plasmid and curve a is an amplification curve of the wild plasmid.

This indicates that the present application can provide a sensitive and specific detection for rifampicin resistance mutations in *Mycobacterium tuberculosis* by using universal primers with the help of suitable artificial sequences.

Example 6 qPCR detection of human Septin 9 gene methylation

Septin 9 gene, located on chromosome 17q25.3, is a member of Septin gene family, which is involved in regulating cell biological processes such as cytokinesis and cell cycle.

Because of the diversity of cells, DNA of biological samples obtained by the existing technology is often a mixture of un-methylated DNA and methylated DNA. For example, the sources of DNA in cancer tissues include cancer cells with DNA methylation and normal cells without DNA methylation. The main source of circulating cell-free DNA (cfDNA) in peripheral blood is normal white blood cells without DNA methylation. However, with the apoptosis and decay of cancer cells, DNA content from the cancer cell entering peripheral blood is generally less than 0.1%. Therefore, a method for detecting methylated DNA of Septin 9 gene need to be able to effectively distinguish methylated DNA from unmethylated DNA and specifically detect methylated DNA in samples.

In this example, the methylated DNA of human Septin 9 gene was detected, and steps are as follows.

(1) Genomic DNA of Jurkat cell line and Hela cell line were extracted respectively, and methylation status of Septin 9 gene was identified by sequencing;

(2) The genomic DNA of Jurkat cell line, Hela cell line and negative control (NC) were subjected to an enzyme digestion reaction with a methylation-dependent restriction endonuclease *GlaI*. The reaction system was 2 μ L of 10x enzyme digestion buffer and different concentrations of genomic DNA, with a total of 20 μ L. The reaction was incubated at 37°C for 1 hour. After the enzyme digestion reaction, the system was heated to 85°C and incubated for 10 minutes to thermally inactivate *GlaI*.

(3) A capture oligonucleotide, a universal primer and a detection probe of Septin 9 gene were added into the enzyme digestion reaction systems respectively. Methylation status of Septin

9 gene was detected by PCR. The PCR amplification system included an enzyme digestion DNA template, 5 nM of the capture oligonucleotide, 150 nM of the universal primer, 150 nM of a specific primer, 150 nM of the detection probe, 1 U of Taq polymerase, 200 μM of dNTP, 4.5 mM of MgCl₂ and 2×PCR buffer. The PCR reaction procedure was pre-denaturation at 94°C for 5 min; 10 cycles of 94°C for 10 s and 66°C for 90 s; and 40 cycles of 94°C for 10 s and 65°C for 20 s. Real-time PCR was performed on ROCHE instrument (480), and the corresponding fluorescence values were collected.

The combination of the capture oligonucleotide, the universal primer and the detection probe includes:

capture oligonucleotide (SEQ ID NO: 18)

5-TGTCAGCCAACGGTATTCGTTGACCGCGGGCTCGCCGCTGCCCTCCGC;

universal primer (SEQ ID NO: 19)

5-GCCTGTCAGCCAACGGTATTC;

specific primer (SEQ ID NO: 20)

5-CGACCCGCTGCCCACCAG;

detection probe (SEQ ID NO: 21)

5-FAM-CCATCATGTCGGACCC-MGB;

Human Septin 9 gene is shown in SEQ ID NO: 22:

5-GCGCCG//GTTGACCGCGGGGTCCGACATGATGGCTGGTGGGCAGCGGGTCGCGC
GGAGGGCAGCGGCGAGGAA;

wherein, the underline is the methylation position, and // is the cleaving site.

It was found by sequencing that the Septin 9 gene in the Jurkat cell line genome was unmethylated, while the Septin 9 gene in the Hela cell line genome was methylated. The methylation status of Septin 9 gene in different concentrations of methylated samples and un-methylated samples was detected by qPCR. As shown in FIG. 6, 140 copies and 28 copies are

amplification curves of Septin 9 methylated samples, and are 140 copies/reaction and 28 copies/reaction of Septin 9 methylated gene samples respectively, 16000 copies is an amplification curve of the un-methylated sample And NC is an amplification curve of double distilled water (dd H₂O)

- 5 This indicates that the present application can provide a sensitive and specific detection for methylated DNA in SEPTIN 9 gene.

Example 7 ddPCR detection of Septin 9 gene methylation

- 10 Compared with Example 6, the Septin 9 gene methylation detection was performed on a NaicaTM crystal digital PCR instrument from STILLA Company with a methylation-dependent restriction endonuclease *GlaI*, and other conditions were the same as Example 6.

The results are shown in FIG. 7, which shows the copy number of the Septin 9 gene in different concentrations of methylated samples. Left graph shows a 100 copies/reaction scatter plot, and right graph shows a 10 copies/reaction scatter plot. In the FIG. 7, scatter plots above the dotted line are positive droplets, and scatter plots below the dotted line are negative droplets.

- 15 This indicates that the present application can realize the detection of methylated DNA in Septin 9 gene with high sensitivity on a digital PCR instrument.

Example 8 Dual detection of human Septin 9 gene methylation and RASS-F1 gene methylation

- 20 In this example, Jurkat DNA was treated with a methyltransferase and was used as a methylation positive standard, in which CG site was 5mCG. A dual detection of human Septin 9 gene methylation and RASS-F1 gene methylation was performed. *GlaI* was used as a methylation-dependent restriction endonuclease. Nuclease-free water was used as a negative control. For the Septin 9 gene, the capture oligonucleotide was SEQ ID NO: 18, the universal primer was SEQ ID NO: 19, the specific primer was SEQ ID NO: 20 and the detection probe was SEQ ID NO: 21. For the RASS-F1 gene, the capture oligonucleotide was SEQ ID NO: 23, the universal primer was SEQ ID NO: 19, the specific primer was SEQ ID NO: 24 and the detection probe was SEQ ID NO: 25.

Capture oligonucleotide (SEQ ID NO: 23)

5-TGTCAGCCAACGGTATTCGCCCAGCGGGTGCGAAGCACGGGCCCAAC.

Specific primer (SEQ ID NO: 24)

5-CCATGTCGGGGGAGCCTG.

Detection probe (SEQ ID NO: 21)

5 5-VIC-CCATCATGTCGGACCC-MGB.

Detection probe (SEQ ID NO: 25)

5-FAM-CTCCCGCAGCTCAATG-MGB.

10 FIG. 8(A) shows results of sensitivity detection of the Septin 9 gene in different concentrations of methylated samples. 140 copies/reaction and 28 copies/reaction are shown from left to right, respectively. FIG. 8(B) shows results of sensitivity detection of RASS-F1 gene in different concentrations of methylated samples. 140 copies/reaction and 28 copies/reaction are shown from left to right, respectively.

This indicates that the present application can provide a sensitive and specific detection for methylated DNA in the Septin 9 gene and the RASS-F1 gene simultaneously.

15 **Example 9 Optimization of methylation site detection specificity of human Septin 9 gene**

20 In this example, Jurkat DNA was treated with a methyltransferase and was used as a methylation positive standard of human Septin 9 gene. Jurkat DNA was used as a methylation negative standard of human Septin 9 gene. Nuclease-free water was used as a negative control nc. The sample was digested with a methylation-dependent restriction endonuclease *GlaI*. A common capture oligonucleotide or an LNA-modified capture oligonucleotide, respectively, and a universal primer (SEQ ID NO: 19), a specific primer (SEQ ID NO: 20) and a detection probe (SEQ ID NO: 21) were used for amplification detection (FAM channel was used for the common capture oligonucleotide; VIC channel was used for the LNA-modified capture oligonucleotide).

The capture oligonucleotide includes:

25 common capture oligonucleotide (SEQ ID NO: 26)

5-GCCTGTCAGCCAACGGTATTCGTTGACCGCGGGCTCGCCGCTGCCCTCCGC;

LNA Modified Capture Oligonucleotide (SEQ ID NO: 27)

5-GCCTGTCAGCCAACGGTATTCGTTGACC+G+CGCTCGCCGCTGCCCTCCGC (the base after + represents that the base was modified with a locked nucleic acid);

5 and human Septin 9 gene is shown in SEQ ID NO: 22.

The results are shown in FIG. 9(A) and FIG. 9(B). Both capture oligonucleotides showed positive results in 120 copies and 12 copies of positive templates. However, in the system of the common capture oligonucleotide, when the un-methylated genome (jurkat DNA) was added to 100 ng, non-specific amplification was caused as a result of the binding and extension of the synthetic by-products of the capture oligonucleotide to the target molecule. After LNA modification was introduced into the folding sequence of the capture oligonucleotide, the amplification efficiency was ensured (120 copies and 12 copies was able to be detected), and non-specific amplification was avoided in a high concentration (100 ng) of the un-methylated genome. The mechanism of inhibiting non-specific amplification is that when the folding sequence is modified with a nucleic acid analogue, the extension of the synthetic by-product of the primer can be inhibited while ensuring the complementary base pairing in the folding region. Therefore, it can be seen from the results that the modification to the folding sequence with a nucleic acid analogue can improve the specificity of the detection system.

Example 10 Amplification results for folding regions with not exactly the same sequences

20 In this example, methylated DNA of human RASS-F1 gene was detected, and steps are as follows.

(1) Jurkat DNA was treated with a methyltransferase to construct a completely methylated positive genome, and the methylation status of RASS-F1 gene was identified by sequencing.

25 (2) The methylation-positive Jurkat genomic DNA and a negative control were subjected to an enzyme digestion reaction with a methylation-dependent restriction endonuclease *GlaI*, respectively. The reaction system was 2 μ L of 10 $\times$ enzyme digestion buffer and different concentrations of genomic DNA, with a total of 20 μ L. The reaction was incubated at 37°C for 1 hour. After the enzyme digestion reaction, the system was heated to 85°C and incubated for 10 minutes to thermally inactivate *GlaI*.

(3) A capture oligonucleotide, a universal primer, a specific reverse primer and a detection probe of RASS-F1 gene were added into the system of the enzyme digestion reaction respectively. Methylation status of RASS-F1 gene was detected by PCR. The PCR amplification system included an enzyme digestion DNA template, 5 nM of the capture oligonucleotide, 150 nM of the universal primer, 150 nM of the specific primer, 150 nM of the detection probe, 1 U of Taq polymerase, 200 μ M of dNTP, 4.5 mM of $MgCl_2$ and 2 $\times$ PCR buffer, with a total volume of 20 μ L. The PCR reaction procedure was pre-denaturation at 94°C for 5 min; 10 cycles of 94°C for 10 s and 66°C for 90 s; and 40 cycles of 94°C for 10 s and 65°C for 20 s. Real-time PCR was performed on ROCHE instrument (480), and the corresponding fluorescence values were collected.

The combination of the capture oligonucleotide, the universal primer and the detection probe included:

capture oligonucleotide (SEQ ID NO: 28)

5-GCCTGTCAGCCAACGGTATTCGCCCAG+C+GGTTTTTGCCAG/spacer

18/GCGAAGCACGGGCCCAAC (the base after + represents that the base is modified with a locked nucleic acid);

universal primer (SEQ ID NO: 19)

5-GCCTGTCAGCCAACGGTATTC;

specific primer (SEQ ID NO: 29)

5-CCATGTCGGGGGAGCCTGAG;

detection probe (SEQ ID NO: 30)

5-FAM-CTCCCGCAGCTCAATG-MGB;

human RASS-F1 gene is shown in SEQ ID NO: 31:

5-GCGC//GCCAGCGGGTGCCAGCTCCCGCAGCTCAATGAGCTCAGGCTCCCCCG
ACATGGCCCCGGTTGGGCCCGTGCTTCGCTGG;

wherein, the underline was the methylation position, and // was the cleaving site.

In this example, the folding region of the capture oligonucleotide is not completely the same as the 5'-end sequence of the target molecule. qPCR was performed to detect the methylation status of RASS-F1 gene in different concentrations of methylated positive samples. As shown in FIG. 10, curves 1, 2 and 3 are amplification curves of RASS-F1 methylated samples, which are 120 copies/reaction, 40 copies/reaction and 28 copies/reaction respectively, and the negative control is an amplification curve with ddH₂O.

This indicates that the folding region of the capture oligonucleotide in the present application only needs to be partially the same as the 5'-end sequence of the target molecule to enable the amplification.

To sum up, in the present application, a sample having a defined 5'-end sequence is amplified by a specially designed capture oligonucleotide to form a product having a hairpin structure, which is used as a template for an amplification reaction of a universal primer, thus achieving the detecting of different target molecules by multiplex PCR amplification with universal primers, avoiding the deviation of amplification efficiency caused by sequence diverse, having good specificity and being suitable for popularization and application.

The applicant declares that although the embodiments are used in the present application to illustrate the detailed methods of the present application, the present application is not limited to the detailed methods, which means that the present application does not necessarily rely on the detailed methods to be implemented. It should be clear to those skilled in the art that any improvement to the present application, equivalent substitution of each raw material and addition of auxiliary components for the product of the present application, or selection of specific methods, etc., all fall within the protection scope and disclosure scope of the present application.

SEQUENCE LISTING

<110> SHANGHAI HEALZONE BIOTECHNOLOGY CO., LTD

<120> MULTI-AMPLIFICATION METHOD

<130> BY21DX1657FPPC-CN

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What is claimed is:

1. A capture oligonucleotide for nucleic acid amplification, comprising a first universal sequence, a folding sequence and a binding capture sequence in sequence from 5'-end to 3'-end;

wherein,

5 the folding sequence is at least partially the same as a 5'-end sequence of a target molecule;
and

the binding capture sequence is complementary to a non-5'-end sequence of the target molecule.

10 2. The capture oligonucleotide according to claim 1, wherein the capture oligonucleotide further comprises a second universal sequence;

preferably, the second universal sequence is located at the 5'-end of the binding capture sequence.

3. The capture oligonucleotide according to claim 1 or 2, wherein, the capture oligonucleotide further comprises a nucleic acid extension blocking site;

15 preferably, the nucleic acid extension blocking site is located at the 3'-end of the folding sequence;

preferably, the nucleic acid extension blocking site is located at the 5'-end of the second universal sequence;

20 preferably, the nucleic acid extension blocking site is modified with any one or a combination of at least two of Spacer, a thio group or a uracil base.

4. The capture oligonucleotide according to any one of claims 1 to 3, wherein the folding sequence is modified with a nucleic acid analogue;

25 preferably, the nucleic acid analogue comprises any one or a combination of at least two of a peptide nucleic acid(s), a locked nucleic acid(s), a transposed base(s), 2'-O,4'-C-methylene bridged RNA, 2'-O-methyl RNA or 2'-fluoro RNA.

5. A kit for nucleic acid amplification, comprising the capture oligonucleotide according to any one of claims 1 to 4.

6. The kit according to claim 5, wherein the kit further comprises a pretreatment reagent of the target molecule;

5 preferably, the pretreatment reagent of the target molecule comprises any one or a combination of at least two of a site-specific cleaving nuclease, a nucleic acid extension blocker or a specific primer;

preferably, the site-specific cleaving nuclease comprises a exonuclease and/or a endonuclease;

10 preferably, the endonuclease comprises any one or a combination of at least two of a methylation-dependent restriction endonuclease, a methylation-sensitive restriction endonuclease or a mismatch repair enzyme;

preferably, the nucleic acid extension blocker comprises a peptide nucleic acid(s) and/or a locked nucleic acid(s);

15 preferably, the specific primer has a modified group at the 5'-end;

preferably, the modified group comprises a phosphate group and/or a thio group.

7. The kit according to claim 5 or 6, wherein the kit further comprises a universal primer;

20 preferably, the universal primer has a nucleic acid sequence that is the same or partially the same as the first universal sequence or the second universal sequence of the capture oligonucleotide;

preferably, the kit further comprises a specific primer.

8. The kit according to any one of claims 5 to 7, wherein the kit further comprises a detection probe;

25 preferably, the detection probe is labeled with a fluorescent group and/or a quenching group;

preferably, the fluorescent group is labeled at the 5'-end of the detection probe;

preferably, the quenching group is labeled at the 3'-end of the detection probe;

preferably, the fluorescent group comprises any one of FAM, VIC, JOE, TET, CY3, CY5, ROX, Texas Red or LC RED460;

5 preferably, the quenching group comprises any one of BHQ1, BHQ2, BHQ3, Dabcyl or Tamra.

9. A method for nucleic acid amplification, comprising:

(1) contacting a target molecule having a defined 5'-end sequence with the capture oligonucleotide according to any one of claims 1 to 4, thereby the binding capture sequence of
10 the capture oligonucleotide binds to the target molecule;

(2) subjecting the capture oligonucleotide to an extension reaction with the target molecule as a template, thereby nucleotides complementary to the target molecule are added at the 3'-end of the capture oligonucleotide to form an extended capture oligonucleotide;

(3) incubating the extended capture oligonucleotide under a condition allowing
15 intramolecular self-folding, thereby a product having a semi-hairpin structure is formed via complementary base pairing between the extended sequence and the folding sequence of the extended capture oligonucleotide; and

(4) subjecting the product having a semi-hairpin structure to an extension reaction, thereby nucleotides complementary to the first universal sequence are added at the 3'-end to form a
20 product having a complete hairpin structure.

10. The method according to claim 9, wherein the method further comprises: performing an exponential amplification using the product having a complete hairpin structure as a template and using a universal primer and/or a specific primer, to obtain an amplification product;

preferably, the universal primer has a nucleic acid sequence that is the same as or partially
25 the same as the first universal sequence or the second universal sequence of the capture oligonucleotide;

preferably, the exponential amplification comprises a polymerase chain reaction;

preferably, the method further comprises steps of allowing the amplification product to bind to a detection probe and detecting.