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(54) **Title:** METHOD FOR ISOTHERMAL AMPLIFICATION OF NUCLEIC ACID TARGET SEQUENCES

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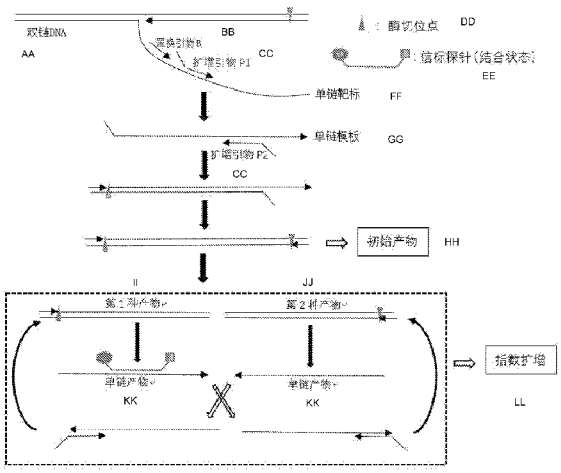

图 1

AA Double-strand DNA
BB Displacement primer
CC Amplification primer
DD Restriction enzyme cutting site
EE Beacon probe (binding state)
FF Single-strand target
GG Single-strand template
HH Initial product
II First product
JJ Second product
KK Single-strand product
LL Exponential amplification

(57) **Abstract:** The present invention provides a method for isothermal amplification of nucleic acid target sequences. The method is suitable for double-stranded DNA, single-stranded DNA, and single-stranded RNA, and comprises a combined reaction of nickase and strand displacement enzyme. In double-stranded DNA and single-stranded DNA detection, three primers and one probe are used, and in single-stranded RNA detection, three primers and one probe may be used, or two primers and one probe may be used. The probe is a molecular beacon, which does not degrade during an amplification process and is only used for specifically binding to a target fragment to provide a fluorescent signal to ensure the specificity of the reaction. In the present invention, the beacon probe that does not overlap with the primers in a binding region on a target sequence is used to determine the result in real time; the beacon probe has high specificity in binding to the target sequence; and it is unnecessary to open a tube after the reaction to further prevent generation of false positives. The reaction is carried out at a constant temperature and consumes short time, and the detection can be completed within 8 minutes, which is more in line with POCT detection requirements.

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— 包括国际检索报告(条约第21条(3))。

(57) 摘要：本发明提供一种等温扩增核酸靶序列的方法。该方法适用于双链DNA、单链DNA、单链RNA，包括切口酶和链置换酶的联合反应，在进行双链DNA和单链DNA检测时，采用3条引物和1条探针，而进行单链RNA检测时，可以为3条引物和1条探针，也可以是2条引物和1条探针。探针为分子信标，扩增过程中不发生降解，仅用于特异性结合目标片段，提供荧光信号，保证反应的特异性。本发明采用与引物之间在靶序列上的结合区域没有重叠的信标探针来实时判断结果，信标探针结合目标序列具有很强的特异性；同时反应后不开管，进一步避免假阳性的产生；反应在恒定温度下进行，耗时短，8min内即可完成检测，更符合POCT检测需求。

METHOD FOR ISOTHERMAL AMPLIFICATION OF NUCLEIC ACID TARGET SEQUENCES

TECHNICAL FIELD

The invention relates to the technical field of nucleic acid detection, and particularly, to a method for isothermally amplifying a target nucleic acid sequence.

BACKGROUND

Polymerase chain reaction (PCR) is the most commonly used nucleic acid amplification test (NAAT) technique. The classical reactions of the technology include three procedures: denaturation, renaturation and extension. PCR is a process requiring rapid temperature circulation, and requires a specific thermal cycler for high-precision temperature control, which consumes massive electric power. Meanwhile, PCR features a prolonged reaction time, which does not meet the requirement of point-of-care testing (POCT). Although products that can complete the reaction in 15-30 minutes have been known in recent years, such products are cost-inefficient due to complicated industrial designs.

In order to solve the numerous problems of PCR, a series of isothermal amplification techniques have been developed. Commonly used techniques are recombinase polymerase amplification (RPA), loop-mediated isothermal amplification (LAMP), strand displacement amplification (SDA), nicking enzyme amplification reaction (NEAR), transcription-mediated amplification (TMA), and the like.

RPA depends on three enzymes: a recombinase binding to a single-stranded nucleic acid, a single-stranded DNA binding protein, and a strand-displacing DNA polymerase. The recombinase recognizes a complementary sequence of the single-stranded nucleic acid and binds them, the single strand binding protein stabilizes the binding region, and the strand-displacing DNA polymerase extends the strand. The reaction is generally conducted at 37-42 °C within 15-30 min, and a special probe can be added to judge the result. RPA involves a large number of components and is thus cost-inefficient.

LAMP utilizes a strand-displacing enzyme to complete the reaction, and by designing 4 or 6 primers and the formation of stem-loop products, the reaction is continuously initiated at the stem-loop under the action of the strand-displacing enzyme. The process needs 30-45 min, and generally adopts dyes for judging the endpoint. LAMP reagents are cost-efficient, but the use of dyes may lead to false positive results and thus difficulties in designing the primer.

SDA uses specially modified nucleotides, endonucleases and strand-displacing DNA

polymerases, and requires 4 primers. The product with digestion sites at both ends is generated by reacting a melting primer and an amplification primer with the template. Since the end with the modified nucleotide cannot be cleaved by the endonuclease, nicks are generated in the product under the action of the endonuclease, and displacement and extension are conducted under the action of a strand-displacing DNA polymerase, thus achieving exponential amplification. The double-stranded DNA template requires melting and primer annealing before the enzyme is added for reaction. The reaction time is generally 30-60 min.

NEAR is similar to SDA, requiring nickases, strand-displacing enzymes, and only 2 primers. The distance between the 2 primers (3' ends) is 1-5 bases, and by the action of primer invasion, a product with nickase sites at both ends is formed. The product can be exponentially amplified under the action of the nickase and strand-displacing DNA polymerase. The product can be analyzed by using probes and dyes. Some products of this technique have been approved, but were reported with low sensitivity in SARS-CoV-2 nucleic acid detection. Because of the short distance between the primers, when the probes are used for real-time detection, false positive results may occur due to the homologous positions of the primers and the probes. The reaction time is about 12 min.

Transcription-mediated amplification (TMA) is conducted by reactions of reverse transcriptase and RNA polymerase, and the major product is RNA. The reaction time is 15-60 min.

CN104726549A discloses a novel detection method for isothermally amplifying double strands based on nickase, which adopts 3 primers. One of the amplification primers can be designed as a beacon probe, and the product is analyzed by staining, fluorescence, electrochemistry, colorimetry, and chemiluminescence. The detection time is 30-60 min. Methods other than fluorescence may easily lead to false positive results, and the labeled primers according to the patent may result in incorrect reactions. In addition, non-specific reactions in the labeled primers also bring false positive results. Due to factors of overlong reaction time, improper product analysis and the like, no products are on the market at present.

SUMMARY

The present invention is intended to solve the technical problems of prolonged detection time and poor specificity in the prior art.

In order to achieve the above intention, in a first aspect, the present invention provides a method for isothermally amplifying a target nucleic acid sequence, comprising:

I, preparing an initial product, comprising:

A1, for a single-stranded DNA target, complementing an amplification primer P1 and a displacement primer with the single-stranded target, and displacing an amplification product of the amplification primer P1 with the displacement primer while extending the amplification primer P1 along the single-stranded target under the action of a DNA polymerase, wherein the displaced product formed by extending the amplification primer P1 is used as a single-stranded template; or

A2, for a single-stranded RNA target, acquiring a single-stranded template through two ways of reaction:

(1) for a DNA polymerase having polymerase functionality, strand displacement functionality, and reverse transcription functionality, contacting an amplification primer P1, a displacement primer and the DNA polymerase with the single-stranded RNA, wherein the single-stranded RNA is reversely transcribed into a cDNA under the reverse transcription activity of the DNA polymerase, and the cDNA is displaced by the displacement primer to give a single-stranded template; or

(2) for a DNA polymerase with no reverse transcription functionality, adding a reverse transcriptase with RNase H activity, and contacting an amplification primer P1 and the reverse transcriptase with the single-stranded RNA, wherein the single-stranded RNA is reversely transcribed into a cDNA under the action of the reverse transcriptase to give a cDNA-RNA double-stranded complex product, and the RNA strand in the double-stranded complex product is hydrolyzed under the action of the RNase H activity of the reverse transcriptase to give a single-stranded template; and

B, complementing an amplification primer P2 with the single-stranded template formed in step A, extending the amplification primer P2 along the single-stranded template under the action of a DNA polymerase, digesting the extension product with a nickase, and extending and displacing at the nick to give a double-stranded initial product with 1 digestion site at each of the two ends; and

II, acquiring an exponential amplification signal, comprising:

C, contacting a nickase and a DNA polymerase with a double-stranded template, wherein a double-stranded nick is generated on the double-stranded template under the action of the nickase, and the DNA polymerase amplifies the strand from the nick and displaces the strand to give a single strand complementary to the amplification primer P1 or P2;

D, complementing the amplification primer P1 or P2 with the single strand formed in step C,

and extending the amplification primer under the action of a DNA polymerase to give two double-stranded products each having 1 digestion site;

E, contacting a nickase and a DNA polymerase with the two double-stranded products generated in step D, wherein nicks are generated on the double-stranded products under the action of the nickase, and the DNA polymerase amplifies the strands from the nicks and displaces the strands to give two single strand respectively complementary to the amplification primer P1 or P2; the single strands then contact with the amplification primer P1 or P2, and are extended under the action of the DNA polymerase to give double-stranded products; and

F, repeating step E to exponentially produce an amplification product;

wherein the above steps are conducted under an isothermal condition without denaturing the target sequence prior to the amplification;

steps C to F also comprise complementing the amplification system and a molecular beacon probe to give a fluorescent signal;

the amplification primers P1 and P2 sequentially comprise a stabilizing region, a nickase recognition site region and a base region complementary to the target sequence in the 5'-3' direction; wherein the stabilizing region has a length of 6-20 bp;

the displacement primer is completely complementary to the target sequence;

the molecular beacon probe is complementary to the target sequence or can be hybridized with the target sequence, and the molecular beacon probe does not overlap with the binding regions of the amplification primers P1 and P2 on the target sequence;

when for a single-stranded DNA target, the single-stranded target may be a single-stranded DNA and a single-stranded product obtained from a double-stranded DNA by contacting a nickase and a DNA polymerase with the double-stranded DNA, wherein a double-stranded nick is generated under the action of the nickase, and the DNA polymerase amplifies the strand from the nick and displaces the strand;

the DNA polymerase has strand displacement functionality;

the method is for non-diagnostic purposes.

Preferably, the base positions complementary to the target sequence on the amplification primers P1 and P2 are modified, and the mode of modification includes locked nucleic acid modification and methylation modification;

the distance between the 3' terminal bases of the amplification primers P1 and P2 on the target sequence is not less than 10 bp.

The molecular beacon may comprise conventional synthetic modifications similar to the primers described above.

Preferably, the molecular beacon has a length of 13-80 bp, and the binding position of the molecular beacon and the target sequence is a position close to the 5' end and not less than 12 bp to the 3' end.

Preferably, the amplification primer has a length of 17-40 bp; the displacement primer has a length of 10-30 bp and a GC% content of 20-80%; the probe has a length of 20-40 bp and a GC% content of 10-80%.

The method provided herein is a closed-tube real-time fluorescence detection. After the nucleic acid sample is loaded, the tube will not be open during the process, thus eliminating the possibility of product contamination due to sample exposure.

Preferably, the single-stranded target has a length of 30-100 bases;

the amplification is conducted at 37 °C to 70 °C;

the overall reaction time is 1-10 min; preferably, the reaction time of the method is not greater than 8 min. Positive and negative results are obtained within 8 min, and the positive result in a sample with a high concentration of the positive target sequence can be obtained within 1-2 min.

Preferably, the nickase is selected from at least one of Nt.AlwI, Nb.BbvCI, Nt.BbvCI, Nb.BsrDI, Nb.BsmI, Nt.BsmAI, Nt.BspQI, Nt.BstNBI, Nb.BtsI, and Nt.CviPII.

Preferably, the DNA polymerase is selected from one of Bst DNA polymerase, Bsu DNA polymerase, and phi29 DNA polymerase.

Preferably, the DNA polymerase is Bst 2.0 or Bst 3.0.

Preferably, one end of the molecular beacon probe is a fluorescent group, and the other end of the molecular beacon probe is a fluorescence-quenching group; the 5' end and the 3' end of the probe are partially complementary in sequence, and can form a stem-loop structure.

Preferably, the amplification reaction system comprises a Tris HCl buffer, BSA, NaCl, KCl, dNTP, Mg^{2+}, $(NH_4)_2SO_4$, and an additive.

Preferably, the additive comprises at least one of trehalose, betaine, dimethyl sulfoxide, gelatin,

Tween 20, Triton-x100, and NP-40.

In a second aspect, the present invention provides a kit for implementing the method, comprising at least the amplification primers P1 and P2, the displacement primer, the molecular beacon probe, and the amplification reaction system in the method.

Compared with the prior art, the method for isothermally amplifying a target nucleic acid sequence has the following beneficial effects:

1. The present invention provides a novel method for rapid isothermal amplification and detection of nucleic acid. The method is suitable for double-stranded DNA, single-stranded DNA, and single-stranded RNA, comprising a combined reaction of a nickase and a strand-displacing enzyme. The method adopts 3 primers and 1 probe for double-stranded DNA or single-stranded DNA assay, or 3 primers and 1 probe or 2 primers and 1 probe for single-stranded RNA assay. The probe is a molecular beacon, and will not be degraded in the amplification process. It is only used for specifically binding to a target fragment and provides a fluorescent signal, thereby ensuring the specificity of the reaction.

2. The whole reaction process is implemented under an isothermal condition, and does not require a denaturation of the target sequence before amplification, thus featuring simpler operation compared with the temperature-variable nucleic acid amplification and detection techniques.

3. According to the present invention, recognition site nucleic acid sequences for nickase digestion are introduced into both upstream and downstream amplification primers, such that both 5' and 3' ends of the generated double-stranded initial product are provided with one nickase digestion recognition site, which can effectively improve the reaction efficiency in the subsequent exponential amplification stage and allows a faster completion of the reaction. In addition, the present invention adopts locked nucleic acid-modified primers, such that the efficiency and stability of the binding of the primer and the template are better than those of conventional primers. Also, an additive for promoting the reaction efficiency and an upgraded DNA polymerase with strand displacement activity are added in the reaction system to further improve the reaction efficiency of the reaction system, such that the reaction time is shortened and the reaction can be completed within 8 min. Compared with conventional isothermal amplification reactions that require more than 30-60 min, the method provided herein is more compliant with the POCT detection requirement.

The present invention adopts a beacon probe that does not overlap with the binding region between primers in the target sequence and has strong specificity for binding the target sequence

to determine the result in real time, which avoids false positive results due to the use of staining or electrochemistry. The tube will not be opened after the reaction, thus further avoiding false positive results due to product contamination.

BRIEF DESCRIPTION OF DRAWINGS

FIG. 1 is a schematic of the detection of a double-stranded DNA according to a preferred embodiment of the present invention.

FIG. 2 is a schematic of the initial template formation in detecting a single-stranded DNA and single-stranded RNA according to a preferred embodiment of the present invention.

FIG. 3 is a schematic of the initial template formation in detecting a single-stranded RNA according to a preferred embodiment of the present invention.

FIG. 4 is a diagram illustrating the amplification effect in detecting a plasmid carrying human gene PSMB2 according to a preferred embodiment of the present invention.

FIG. 5 is a diagram illustrating the amplification effect in detecting a Mycoplasma pneumoniae sample according to a preferred embodiment of the present invention.

FIG. 6 is a diagram illustrating the amplification effect in detecting influenza B virus according to a preferred embodiment of the present invention.

FIG. 7 is a diagram illustrating the amplification effect in detecting canine parvovirus according to a preferred embodiment of the present invention.

FIG. 8 is a schematic of the self strand displacement amplification in a sample according to a preferred embodiment of the present invention.

FIG. 9 is a diagram illustrating the amplification effect of the self strand displacement amplification reaction in a sample according to a preferred embodiment of the present invention.

DETAILED DESCRIPTION

To help understand the present invention, a more detailed description is provided with reference to the accompanying drawings, which illustrate several examples of the present invention. However, the present invention can be implemented in various forms, and is not limited to the examples described herein. On the contrary, the examples are provided to completely illustrate the disclosure of the present invention.

The present invention provides a novel method for the rapid isothermal amplification and

detection of double-stranded DNA, single-stranded DNA, and single-stranded RNA. The method comprises:

an initial product generation stage (double-stranded initial product formation stage) and an exponential amplification signal acquisition stage.

1. In the initial product generation stage, there are slight differences depending on the template situation and the enzyme system used:

a) When the template is a double-stranded DNA, the nickase acts on the nickase digestion sites on the double-stranded DNA template to form nicks. The strand-displacing enzyme (DNA polymerase with strand displacement functionality) initiates the extension and strand displacement from the nicks to form single-stranded products. A primer with a single-stranded digestion site (F/R, or the amplification primer P1) and the displacement primer (B) bind to the single-stranded products to form single-stranded products with the digestion sites through extension and strand displacement. The other primer with a single-stranded digestion site (R/F, or the amplification primer P2) binds to the single-stranded products and is subjected to extension, digestion and strand displacement to form an initial product, i.e., a double-stranded initial product with 2 digestion sites at the two ends (FIG. 1).

b) When the template is a single-stranded DNA, the primer with a single-stranded digestion site (F/R) and the displacement primer (B) bind to the single-stranded product, and the initial product is formed by the same procedure as described above (FIG. 2).

c) When the template is a single-stranded RNA, there are 2 different ways to give the initial template. In the first method, when a reverse transcriptase with RNase H activity is used, a single strand with a digestion site is formed by reverse transcription and the action of RNase H without the use of displacement primer (B) as shown in FIG. 3, and the subsequent reaction is the same as that described above; in the second method, a reverse transcriptase (e.g., Bst 3.0) is used to generate a single strand with a digestion site by reverse transcription and the strand displacement functionality, and the process is similar to that of the single-stranded DNA template.

2. In the exponential amplification signal acquisition stage, the nickase cleaves the initial product to form two double-stranded DNAs with a digestion site on one side, as shown in the "exponential amplification" region of FIG. 4. The first product can generate a single-stranded product under the action of the nickase and the amplification enzyme, and the product further binds to the amplification primer and extends to form the second product; vice versa, the second product may also produce the first product. The two together lead to the exponential

amplification. The molecular beacon probe can bind to one of the single-stranded products, and the amplification signal can be acquired by a suitable fluorescence detection system.

When detecting a double-stranded DNA, a single-stranded DNA or single-stranded RNA, the method adopts 2 amplification primers, 1 displacement primer and 1 molecular beacon probe; when a single-stranded RNA is detected, the method may also adopt 2 amplification primers and 1 molecular beacon probe.

The molecular beacon has a length of 13-80 bp, and the binding position of the molecular beacon and the target sequence is a position close to the 5' end and not less than 12 bp to the 3' end.

The amplification enzyme used in the present invention has the functionality of synthesizing DNA using a DNA template and strand displacement functionality, and some amplification enzymes also have the functionality of reversely transcribing an RNA template into DNA.

The length of the specific region (excluding the sequences such as digestion sites introduced by primer amplification) in the initial product is 30-100 bp.

The molecular signaling probe binds to the single-stranded product without overlapping the binding region of the amplification primer on the single-stranded product. The distance between the 3' terminal bases of the amplification primers P1 and P2 on the target sequence is not less than 10 bp. In designing the primers and probes, sufficient specific sites should be ensured for binding of the probe to the target sequence without base overlap between the probe and the amplification primer on the target sequence.

The temperature is constant in the reaction process, and the reaction can be completed within 8 min.

The present invention adopts 3 primers and 1 beacon probe (2 primers and 1 probe can be used for detecting a single-stranded RNA), nickase, and strand-displacing DNA polymerase, and the nucleic acid amplification and the real-time fluorescence detection of the product can be completed within 8 min.

The method features isothermal amplification. The temperature is kept constant in the reaction at 37-70 °C.

The reaction time of the method is not greater than 8 min. Positive and negative results are obtained within 8 min, and the positive result in a sample with a high concentration of the positive target sequence can be obtained within 1-2 min. The method is a closed-tube real-time

fluorescence detection. After the nucleic acid sample is loaded, the tube will not be open during the process.

The primer is a single-stranded nucleotide polymer, and if necessary, the primer may comprise conventional synthetic modifications such as locked nucleic acid (LNA), methylation and the like. Of the 3 primers, 1 is a strand-displacing primer, and 2 are amplification primers. The strand-displacing primer is completely complementary to the template, and the amplification primer comprises 3 regions: a specific binding region, a digestion site region, and a stabilizing region.

The beacon probe is a single-stranded nucleotide polymer modified by a fluorescent group and a quenching group, and the 5' end and the 3' end of the artificial sequence are complementary to form a stem-loop structure. If necessary, the molecular beacon may comprise conventional synthetic modifications similar to the primers described above, and possibly, spacer modifications at the 5' and 3' ends to increase its length. The beacon probe and the primer have no overlaps on the target sequence, thus ensuring the specificity.

The nickase is a special enzyme for recognizing a specific sequence of a double-stranded DNA and forming a nick on the double-stranded DNA, such as Nt.AlwI, Nb.BbvCI, Nt.BbvCI, Nb.BsrDI, Nb.BsmI, Nt.BsmAI, Nt.BspQI, Nt.BstNBI, Nb.BtsI, Nt.CviPII, or other enzymes with the same functionality.

The strand-displacing DNA polymerase is a polymerase having nucleic acid 3' terminal polymerization activity and functionality of displacing nucleic acid in the polymerization direction. Such as Bst DNA polymerase (including Bst 2.0, Bst 3.0, and other upgraded products), large Bst DNA polymerase fragments, Bsu DNA polymerase, large Bsu DNA polymerase fragments, phi29 DNA polymerase, etc.

In addition to the above primers, probes and enzymes, the method also comprises various substances used in common nucleic acid amplification reactions, such as Tris HCl buffer, BSA, NaCl, KCl, dNTP, Mg^{2+}, $(NH_4)_2SO_4$ and other buffers and ionic components commonly used in reactions, and also comprises additives, such as trehalose, betaine, dimethyl sulfoxide, gelatin, Tween 20, Triton-x100, NP-40 and the like.

The following examples are intended to illustrate the present invention, rather than limiting the scope of the present invention. Unless otherwise indicated, the examples follow conventional experimental conditions, such as the Sambrook J & Russell DW, *Molecular Cloning: A Laboratory Manual*, 2001, or follow the conditions recommended by the manufacturer's instructions.

Example 1. Comparison of detections of plasmids carrying human gene PSMB2

The experimental group (the present invention) using upgraded DNA polymerases, primers introduced with locked nucleic acid labels and a reaction system added with a reaction enhancer, and the control groups using a low-grade version of DNA polymerase, primers without locked nucleic acid labels and a reaction system without a reaction enhancer were compared for the reaction time.

The amplification reaction system composition, additives and the modification of the primer used in the experimental group and the control groups are shown in Table 1:

Reaction system	The present invention	Control group 1	Control group 2	Control group 3	Control group 4
Amplification reaction system	Tris-HCl pH8.0, 50mM	Tris-HCl pH8.0, 50mM	Tris-HCl pH8.0, 50mM	Tris-HCl pH8.0, 50mM	Tris-HCl pH8.0, 50mM
	(NH4)2 SO4, 20mM	(NH4)2 SO4, 20mM	(NH4)2 SO4, 20mM	(NH4)2 SO4, 20mM	(NH4)2 SO4, 20mM
	MgCl2, 10mM	MgCl2, 10mM	MgCl2, 10mM	MgCl2, 10mM	MgCl2, 10mM
	NaCl, 30mM	NaCl, 30mM	NaCl, 30mM	NaCl, 30mM	NaCl, 30mM
	KCl, 10mM	KCl, 10mM	KCl, 10mM	KCl, 10mM	KCl, 10mM
	dNTP, 1mM	dNTP, 1mM	dNTP, 1mM	dNTP, 1mM	dNTP, 1mM
Additive	Betaine, 0.5 M	None	Betaine, 0.5 M	Betaine, 0.5 M	None
Primer and probe	PSMB2-B, 200mM	PSMB2-B, 200mM	PSMB2-B, 200mM	PSMB2-B, 200mM	PSMB2-B, 200mM
	PSMB2-F, 300mM	PSMB2-F, 300mM	PSMB2-F, 300mM	PSMB2-F, 300mM	PSMB2-F, 300mM
	PSMB2-R, 300mM	PSMB2-R, 300mM	PSMB2-R, 300mM	PSMB2-R, 300mM	PSMB2-R, 300mM
	PSMB2-P, 300mM	PSMB2-P, 300mM	PSMB2-P, 300mM	PSMB2-P, 300mM	PSMB2-P, 300mM
Nickase	Nt.BstNBI, 3U	Nt.BstNBI, 3U	Nt.BstNBI, 3U	Nt.BstNBI, 3U	Nt.BstNBI, 3U
DNA polymerase	Bst 2.0 warmstart, 4.8U	Bst 2.0 warmstart, 4.8U	Bst 1.0 warmstart, 4.8U	Bst 1.0 warmstart, 4.8U	Bst 1.0 warmstart, 4.8U
Whether the primer has a	Yes	No	Yes	No	No

modification ?					

Table 1

(1) Primer and probe sequences of the present invention:

The primer and probe sequences (5'-3') are as follows:

PSMB2-B (primer): CCCAGCACTTT

PSMB2-F (primer): TTCAGACTATTGAGTCTATTCTGACCAACAT

PSMB2-R (primer): GTCAGACTATTGAGTCTTCTCCCAGCTAAT

PSMB2-P (probe): ATGGTAGTAGAGACGGGGTTTTACCAT

Note: "A" is modified with LNA.

(2) Unmodified primers and probe are as follows:

The primer and probe sequences (5'-3') are as follows:

PSMB2-B (primer): CCCAGCACTTT

PSMB2-F (primer): TTCAGACTATTGAGTCTATTCTGACCAACAT

PSMB2-R (primer): GTCAGACTATTGAGTCTTCTCCCAGCTAAT

PSMB2-P (probe): ATGGTAGTAGAGACGGGGTTTTACCAT

The reactions in all groups were conducted at 55 °C and signals were acquired every 10 s with the instrument LightCycler 480II. Plasmid samples 1E5, 1E4, 1E3, 1E2, and 1E1 were tested. The results are shown in Table 1, and the test result profile of the present invention is shown in FIG. 4. Table 2. Comparison results of detections of plasmids carrying human gene PSMB2

Group	The present invention	Control group 1	Control group 2	Control group 3	Control group 4
Detection time	2.5-4min	5-9min	5.5-9min	7-10min	9-12min

Table 2

Therefore, the detection time of the present invention is significantly shorter than those of the control groups, suggesting that the present invention has a time advantage in the application of double-stranded DNA nucleic acid detection.

Example 2. Comparison of Mycoplasma pneumoniae detection in clinical samples

The experimental group (the present invention) using upgraded DNA polymerases, primers introduced with locked nucleic acid labels and a reaction system added with a reaction enhancer, and the control groups using a low-grade version of DNA polymerase, primers without locked nucleic acid labels and a reaction system without a reaction enhancer were compared for the reaction time.

The amplification reaction system composition, additives and the modification of the primer used in the experimental group and the control groups are shown in Table 3:

Reaction system	The present invention	Control group 1	Control group 2	Control group 3	Control group 4
Amplification reaction system	Tris-HCl pH8.0, 50mM	Tris-HCl pH8.0, 50mM	Tris-HCl pH8.0, 50mM	Tris-HCl pH8.0, 50mM	Tris-HCl pH8.0, 50mM
	(NH4)2 SO4, 20mM	(NH4)2 SO4, 20mM	(NH4)2 SO4, 20mM	(NH4)2 SO4, 20mM	(NH4)2 SO4, 20mM
	MgCl2, 8mM	MgCl2, 8mM	MgCl2, 8mM	MgCl2, 8mM	MgCl2, 8mM
	NaCl, 30mM	NaCl, 30mM	NaCl, 30mM	NaCl, 30mM	NaCl, 30mM
	KCl, 10mM	KCl, 10mM	KCl, 10mM	KCl, 10mM	KCl, 10mM
	dNTP, 1mM	dNTP, 1mM	dNTP, 1mM	dNTP, 1mM	dNTP, 1mM
Additive	Betaine, 0.5 M	None	Betaine, 0.5 M	Betaine, 0.5 M	None
Primer and probe	Mp-B, 200mM	Mp-B, 200mM	Mp-B, 200mM	Mp-B, 200mM	Mp-B, 200mM
	Mp-F, 400mM	Mp-F, 400mM	Mp-F, 400mM	Mp-F, 400mM	Mp-F, 400mM
	Mp-R, 400mM	Mp-R, 400mM	Mp-R, 400mM	Mp-R, 400mM	Mp-R, 400mM
	Mp-P, 300mM	Mp-P, 300mM	Mp-P, 300mM	Mp-P, 300mM	Mp-P, 300mM
Nickase	Nt.BstNBI, 3U	Nt.BstNBI, 3U	Nt.BstNBI, 3U	Nt.BstNBI, 3U	Nt.BstNBI, 3U
DNA polymerase	Bst 2.0 warmstart, 4.8U	Bst 2.0 warmstart, 4.8U	Bst 1.0 warmstart, 4.8U	Bst 1.0 warmstart, 4.8U	Bst 1.0 warmstart, 4.8U
Whether the primer has a modification?	Yes	No	Yes	No	No

Table 3

(1) Primer and probe sequences of the present invention:

The primer and probe sequences (5'-3') are as follows:

Mp-B (primer): CTCTCCACTAA

Mp-F (primer): CATAGACTTATGAGTCTTCTATTCGCTTC

Mp-R (primer): GTTAGACTTTTGAGTCTTCTTGCTCTGGT

Mp-P (probe): CGCAGCTGGTTACGGGAATACTGCG

Note: "A" is modified with LNA.

(2) Unlabeled primer and probe sequences are as follows:

The primer and probe sequences (5'-3') are as follows:

Mp-B (primer): CTCTCCACTAA

Mp-F (primer): CATAGACTTATGAGTCTTCTATTCGCTTC

Mp-R (primer): GTTAGACTTTTGAGTCTTCTTGCTCTGGT

Mp-P (probe): CGCAGCTGGTTACGGGAATACTGCG

The reactions in all groups were conducted at 55 °C and signals were acquired every 10 s with the instrument LightCycler 480II. 8 Mycoplasma pneumoniae samples were tested, as well as 8 other respiratory pathogens: influenza A virus, influenza B virus, Chlamydia pneumoniae, respiratory syncytial virus, human parvovirus B19, Staphylococcus aureus, human respiratory adenovirus, and rhinovirus. The results are shown in Table 4, and the test result profile of the present invention is shown in FIG. 5. The following table shows the comparison results of detection with Mycoplasma pneumoniae.

Group	The present invention	Control group 1	Control group 2	Control group 3	Control group 4
Detection time	3-7min	10-15min	14-19min	15-19min	18-22min
Non-specific results	0/8	2/8	0/8	1/8	2/8

Table 4

Therefore, the present invention has significant advantages in time and specificity for

double-stranded DNA nucleic acid detection.

Example 3. Comparison of influenza B virus (single-stranded RNA virus) detection in clinical samples

The experimental group (the present invention) using upgraded DNA polymerases, primers introduced with locked nucleic acid labels and a reaction system added with a reaction enhancer, and the control groups using a low-grade version of DNA polymerase, primers without locked nucleic acid labels and a reaction system without a reaction enhancer were compared for the reaction time.

The amplification reaction system composition, additives and the modification of the primer used in the experimental group and the control groups are shown in Table 5:

Reaction system	The present invention	Control group 1	Control group 2	Control group 3	Control group 4
Amplification reaction system	Tris-HCl pH8.0, 50mM	Tris-HCl pH8.0, 50mM	Tris-HCl pH8.0, 50mM	Tris-HCl pH8.0, 50mM	Tris-HCl pH8.0, 50mM
	(NH4)2 SO4, 20mM	(NH4)2 SO4, 20mM	(NH4)2 SO4, 20mM	(NH4)2 SO4, 20mM	(NH4)2 SO4, 20mM
	MgCl2, 8mM	MgCl2, 8mM	MgCl2, 8mM	MgCl2, 8mM	MgCl2, 8mM
	NaCl, 30mM	NaCl, 30mM	NaCl, 30mM	NaCl, 30mM	NaCl, 30mM
	KCl, 10mM	KCl, 10mM	KCl, 10mM	KCl, 10mM	KCl, 10mM
	dNTP, 1mM	dNTP, 1mM	dNTP, 1mM	dNTP, 1mM	dNTP, 1mM
Additive	Betaine, 0.5 M	None	Betaine, 0.5 M	Betaine, 0.5 M	None
Primer and probe	FluB-B, 200mM	FluB-B, 200mM	FluB-B, 200mM	FluB-B, 200mM	FluB-B, 200mM
	FluB -F, 400mM	FluB -F, 400mM	FluB -F, 400mM	FluB -F, 400mM	FluB -F, 400mM
	FluB -R, 400mM	FluB -R, 400mM	FluB -R, 400mM	FluB -R, 400mM	FluB -R, 400mM
	FluB -P, 300mM	FluB -P, 300mM	FluB -P, 300mM	FluB -P, 300mM	FluB -P, 300mM
Nickase	Nt.BstNBI, 3U	Nt.BstNBI, 3U	Nt.BstNBI, 3U	Nt.BstNBI, 3U	Nt.BstNBI, 3U
DNA polymerase	Bst 3.0, 6U	Bst 3.0, 6U	Bst 1.0, 6U	Bst 1.0, 6U	Bst 1.0, 6U

Whether the primer has a modification?	Yes	No	Yes	No	No

Table 5

(1) Primer and probe sequences of the present invention:

The primer and probe sequences (5'-3') are as follows:

FluB-B (primer): TGTTGCTAAACT

FluB-F (primer): CTACTGATGAGTCTTTTAGTGGAGGAT

FluB-R (primer): CCTTCATTGAGTCTTTTGAAGAGTGA

FluB-P (probe): ACGGCCATCGGATCCTCAAGCCGT

Note: "A" is modified with LNA.

(1) Unlabeled primer and probe sequences are as follows:

The primer and probe sequences (5'-3') are as follows:

FluB-B (primer): TGTTGCTAAACT

FluB-F (primer): CTACTGATGAGTCTTTTAGTGGAGGAT

FluB-R (primer): CCTTCATTGAGTCTTTTGAAGAGTGA

FluB-P (probe): ACGGCCATCGGATCCTCAAGCCGT

The reactions in all groups were conducted at 55 °C and signals were acquired every 10 s with the instrument LightCycler 480II. 8 influenza B virus samples were tested to verify the specificity of the reaction system, as well as 8 other respiratory pathogens: influenza A virus, Mycoplasma pneumoniae, Chlamydia pneumoniae, respiratory syncytial virus, human parvovirus B19, Staphylococcus aureus, human respiratory adenovirus, and rhinovirus. The results are shown in Table 6, and the test result profile of the present invention is shown in FIG. 6.

Therefore, the present invention has significant advantages in time and specificity for single-stranded RNA nucleic acid detection. Table 6 shows the comparison results of influenza B virus detection.

Group	The present	Control group 1	Control group 2	Control group 3	Control group 4

	invention				
Detection time	3-5min	7-10min	9-13min	11-15min	20-24min
Non-specific results	0/8	1/8	0/8	1/8	2/8

Table 6

Example 4. Comparison of canine parvovirus detection

The experimental group (the present invention) using upgraded DNA polymerases, primers introduced with locked nucleic acid labels and a reaction system added with a reaction enhancer, and the control groups using a low-grade version of DNA polymerase, primers without locked nucleic acid labels and a reaction system without a reaction enhancer were compared for the reaction time.

The amplification reaction system composition, additives and the modification of the primer used in the experimental group and the control groups are shown in Table 7:

Reaction system	The present invention	Control group 1	Control group 2	Control group 3	Control group 4
Amplification reaction system	Tris-HCl pH8.0, 50mM	Tris-HCl pH8.0, 50mM	Tris-HCl pH8.0, 50mM	Tris-HCl pH8.0, 50mM	Tris-HCl pH8.0, 50mM
	(NH4)2 SO4, 20mM	(NH4)2 SO4, 20mM	(NH4)2 SO4, 20mM	(NH4)2 SO4, 20mM	(NH4)2 SO4, 20mM
	MgCl2, 8mM	MgCl2, 8mM	MgCl2, 8mM	MgCl2, 8mM	MgCl2, 8mM
	NaCl, 30mM	NaCl, 30mM	NaCl, 30mM	NaCl, 30mM	NaCl, 30mM
	KCl, 10mM	KCl, 10mM	KCl, 10mM	KCl, 10mM	KCl, 10mM
	dNTP, 1mM	dNTP, 1mM	dNTP, 1mM	dNTP, 1mM	dNTP, 1mM
Additive	Betaine, 0.5 M	None	Betaine, 0.5 M	Betaine, 0.5 M	None
Primer and probe	CVP-B, 200mM	CVP-B, 200mM	CVP-B, 200mM	CVP-B, 200mM	CVP-B, 200mM
	CVP-F, 500mM	CVP-F, 500mM	CVP-F, 500mM	CVP-F, 500mM	CVP-F, 500mM
	CVP-R, 500mM	CVP-R, 500mM	CVP-R, 500mM	CVP-R, 500mM	CVP-R, 500mM
	CVP-P,	CVP-P,	CVP-P,	CVP-P,	CVP-P,

	300mM	300mM	300mM	300mM	300mM
Nickase	Nt.BstNBI, 3U	Nt.BstNBI, 3U	Nt.BstNBI, 3U	Nt.BstNBI, 3U	Nt.BstNBI, 3U
DNA polymerase	Bst 3.0, 6U	Bst 3.0, 6U	Bst 1.0, 6U	Bst 1.0, 6U	Bst 1.0, 6U
Whether the primer has a modification?	Yes	No	Yes	No	No

Table 7

(1) Primer and probe sequences of the present invention:

CVP-F (primer): GAACTTTTGAGTCTTTTACTATACACATC

CVP-R (primer): GAACTTTTGAGTCTTTTCCCAGTTTTCAT

CVP-B (primer): AGTCTTTGCAACCT

CVP-P (probe): CGCCAGGAAAAGTACCAGAATGGCG

Note: "A" is modified with LNA.

(2) Unlabeled primer and probe sequences are as follows:

CVP-F (primer): GAACTTTTGAGTCTTTTACTATACACATC

CVP-R (primer): GAACTTTTGAGTCTTTTCCCAGTTTTCAT

CVP-B (primer): AGTCTTTGCAACCT

CVP-P (probe): CGCCAGGAAAAGTACCAGAATGGCG

The reactions in all groups were conducted at 55 °C and signals were acquired every 10 s with the instrument LightCycler 480II. 5 canine parvovirus samples were tested, as well as 8 other respiratory pathogens: influenza A virus, Mycoplasma pneumoniae, Chlamydia pneumoniae, respiratory syncytial virus, human parvovirus B19, Staphylococcus aureus, human respiratory adenovirus, and rhinovirus. The results are shown in Table 4, and the test result profile of the present invention is shown in FIG. 7.

Therefore, the present invention has significant advantages in time and specificity for single-stranded DNA nucleic acid detection. The following Table 8 shows the comparison results of canine parvovirus detection.

Group	The present	Control	Control	Control	Control

	invention	group 1	group 2	group 3	group 4
Detection time	3.5-5min	8-12min	9-15min	13-17min	15-22min
Non-specific results	0/8	2/8	0/8	2/8	3/8

Table 8

Example 5. Self strand-displacing amplification of samples

When the sample is amplified using a strand-displacing enzyme and a nickase, the sample itself may undergo strand-displacing amplification due to the massive digestion sites present in the sample. The process is similar to the multiple displacement amplification, and the principle is shown in FIG. 8, with the exception that the use of primers and probes is not required. An exemplary reaction is as follows:

The following reaction system was prepared:

Tris-HCl pH 8.0, 50 mM

$(NH_4)_2SO_4$, 20 mM

MgCl2, 8 mM

NaCl, 30 mM

KCl, 10 mM

dNTP, 1 mM

Evagreen 1×

Nt.BstNBI, 3 U

Bst 3.0, 6 U

The reaction was conducted at 55 °C and signals were acquired every 1 min with the instrument LightCycler 480II in a total of 60 cycles. The samples were stock nucleic acid sample solutions extracted via throat swabs, and 10- and 100-fold diluted samples of the stock solutions in duplicate. The results are shown in FIG. 9. The amplification signals in the nucleic acid sample extracted via throat swabs were observed at about 12 min. When the strand-displacing enzyme and nickase are used in amplification, self amplification is inevitable. According to the staining method disclosed in CN104726549A, the false positive results cannot be avoided either after 30-60 min of reaction. The above examples are only specific and detailed descriptions of certain embodiments of the present invention, and should not be construed as limitations to the scope of

the present invention. It should be noted that various changes and modifications can be made by those skilled in the art without departing from the spirit of the present invention, and these changes and modifications are all within the protection scope of the present invention. Therefore, the protection scope of the present patent should be determined with reference to the appended claims.

What is claimed is:

1. A method for isothermally amplifying a target nucleic acid sequence, comprising:

I, preparing an initial product, comprising:

A1, for a single-stranded DNA target, complementing an amplification primer P1 and a displacement primer with the single-stranded target, and displacing an amplification product of the amplification primer P1 with the displacement primer while extending the amplification primer P1 along the single-stranded target under the action of a DNA polymerase, wherein the displaced product formed by extending the amplification primer P1 is used as a single-stranded template; or

A2, for a single-stranded RNA target, acquiring a single-stranded template through two ways of reaction:

(1) for a DNA polymerase having polymerase functionality, strand displacement functionality, and reverse transcription functionality, contacting an amplification primer P1, a displacement primer and the DNA polymerase with the single-stranded RNA, wherein the single-stranded RNA is reversely transcribed into a cDNA under the reverse transcription activity of the DNA polymerase, and the cDNA is displaced by the displacement primer to give a single-stranded template; or

(2) for a DNA polymerase with no reverse transcription functionality, adding a reverse transcriptase with RNase H activity, and contacting an amplification primer P1 and the reverse transcriptase with the single-stranded RNA, wherein the single-stranded RNA is reversely transcribed into a cDNA under the action of the reverse transcriptase to give a cDNA-RNA double-stranded complex product, and the RNA strand in the double-stranded complex product is hydrolyzed under the action of the RNase H activity of the reverse transcriptase to give a single-stranded template; and

B, complementing an amplification primer P2 with the single-stranded template formed in step A, extending the amplification primer P2 along the single-stranded template under the action of a DNA polymerase, digesting the extension product with a nickase, and extending and displacing at the nick to give a double-stranded initial product with 1 digestion site at each of the two ends; and

II, acquiring an exponential amplification signal, comprising:

C, contacting a nickase and a DNA polymerase with a double-stranded template, wherein a double-stranded nick is generated on the double-stranded template under the action of the nickase, and the DNA polymerase amplifies the strand from the nick and displaces the strand to give a single strand complementary to the amplification primer P1 or P2;

D, complementing the amplification primer P1 or P2 with the single strand formed in step C, and extending the amplification primer under the action of a DNA polymerase to give two double-stranded products each having 1 digestion site;

E, contacting a nickase and a DNA polymerase with the two double-stranded products generated in step D, wherein nicks are generated on the double-stranded products under the action of the nickase, and the DNA polymerase amplifies the strands from the nicks and displaces the strands to give two single strand respectively complementary to the amplification primer P1 or P2; the single strands then contact with the amplification primer P1 or P2, and are extended under the action of the DNA polymerase to give double-stranded products; and

F, repeating step E to exponentially produce an amplification product;

wherein the above steps are conducted under an isothermal condition without denaturing the target sequence prior to the amplification;

steps C to F also comprise complementing the amplification system and a molecular beacon probe to give a fluorescent signal;

the amplification primers P1 and P2 sequentially comprise a stabilizing region, a nickase recognition site region and a base region complementary to the target sequence in the 5'-3' direction; wherein the stabilizing region has a length of 6-20 bp;

the displacement primer is completely complementary to the target sequence;

the molecular beacon probe is complementary to the target sequence or can be hybridized with the target sequence, and the molecular beacon probe does not overlap with the binding regions of the amplification primers P1 and P2 on the target sequence;

when for a single-stranded DNA target, the single-stranded target may be a single-stranded DNA and a single-stranded product obtained from a double-stranded DNA by contacting a nickase and a DNA polymerase with the double-stranded DNA, wherein a double-stranded nick is generated under the action of the nickase, and the DNA polymerase amplifies the strand from the nick and displaces the strand;

the DNA polymerase has strand displacement functionality;

the method is for non-diagnostic purposes.

2. The method according to claim 1, wherein:

the base positions complementary to the target sequence on the amplification primers P1 and P2 are modified, and the mode of modification includes locked nucleic acid modification and methylation modification;

the distance between the 3' terminal bases of the amplification primers P1 and P2 on the target sequence is not less than 10 bp.

3. The method according to claim 1, wherein: the molecular beacon has a length of 13-80 bp, and the binding position of the molecular beacon and the target sequence is a position close to the 5' end and not less than 12 bp to the 3' end.

4. The method according to claim 1, wherein:

the single-stranded target has a length of 30-100 bases;

the amplification is conducted at 37 °C to 70 °C;

the overall reaction time is 1-10 min.

5. The method according to claim 1, wherein: the nickase is selected from at least one of Nt.AlwI, Nb.BbvCI, Nt.BbvCI, Nb.BsrDI, Nb.BsmI, Nt.BsmAI, Nt.BspQI, Nt.BstNBI, Nb.BtsI, and Nt.CviPII.

6. The method according to claim 1, wherein: the DNA polymerase is selected from one of Bst DNA polymerase, Bsu DNA polymerase, and phi29 DNA polymerase.

7. The method according to claim 1, wherein: the DNA polymerase is Bst 2.0 or Bst 3.0.

8. The method according to claim 1, wherein: one end of the molecular beacon probe is a fluorescent group, and the other end of the molecular beacon probe is a fluorescence-quenching group; the 5' end and the 3' end of the probe are partially complementary in sequence, and can form a stem-loop structure.

9. The method according to claim 1, wherein: the amplification reaction system comprises a Tris HCl buffer, BSA, NaCl, KCl, dNTP, Mg^{2+}, $(NH_4)_2SO_4$, and an additive.

10. The method according to claim 1, wherein: the additive comprises at least one of trehalose, betaine, dimethyl sulfoxide, gelatin, Tween 20, Triton-x100, and NP-40.

11. The method according to claim 1, wherein: the kit comprises the amplification primers P1 and P2, the displacement primer, the molecular beacon probe, and the amplification reaction system in the method according to any one of claims 1-10.

DRAWINGS

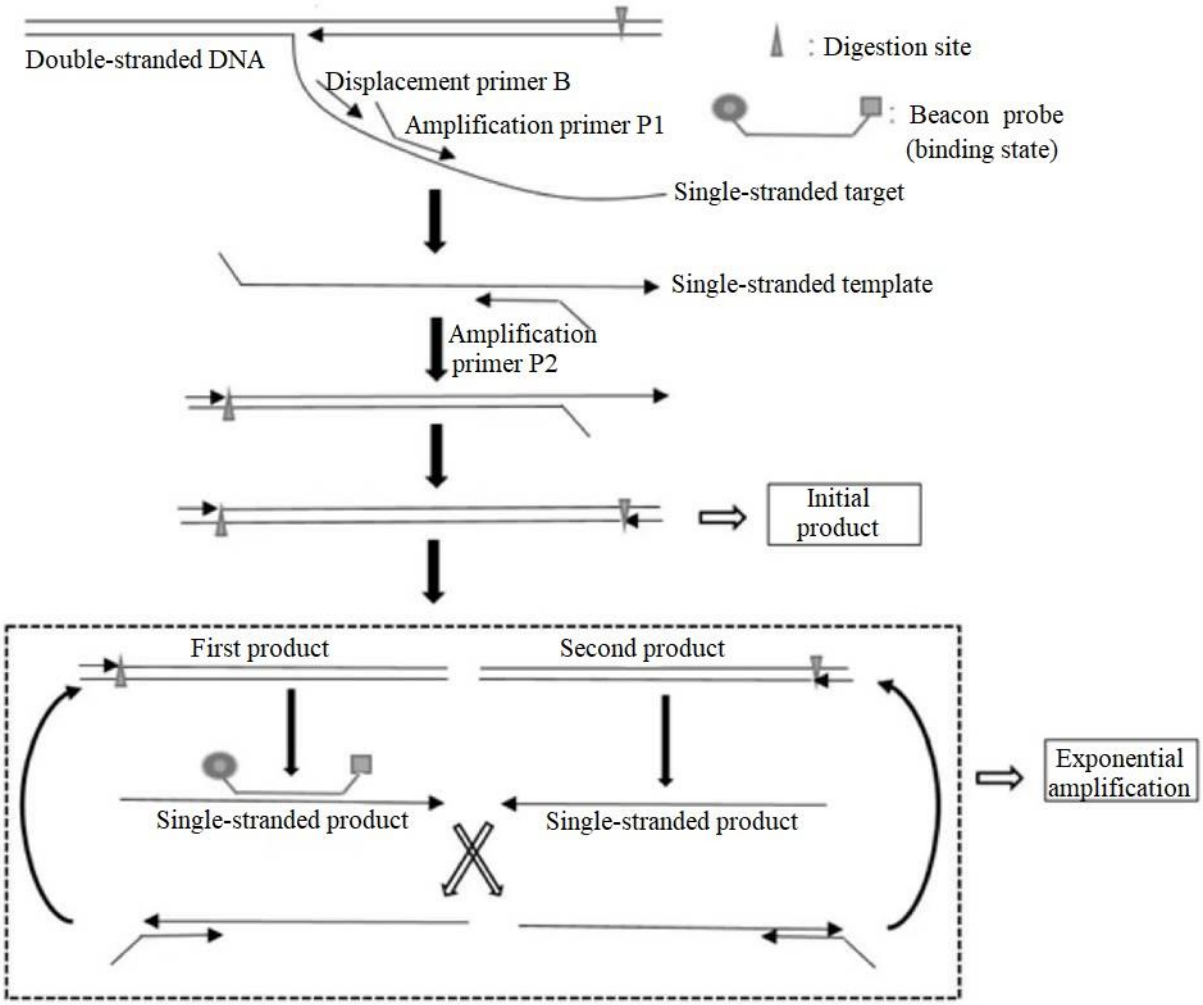

FIG. 1

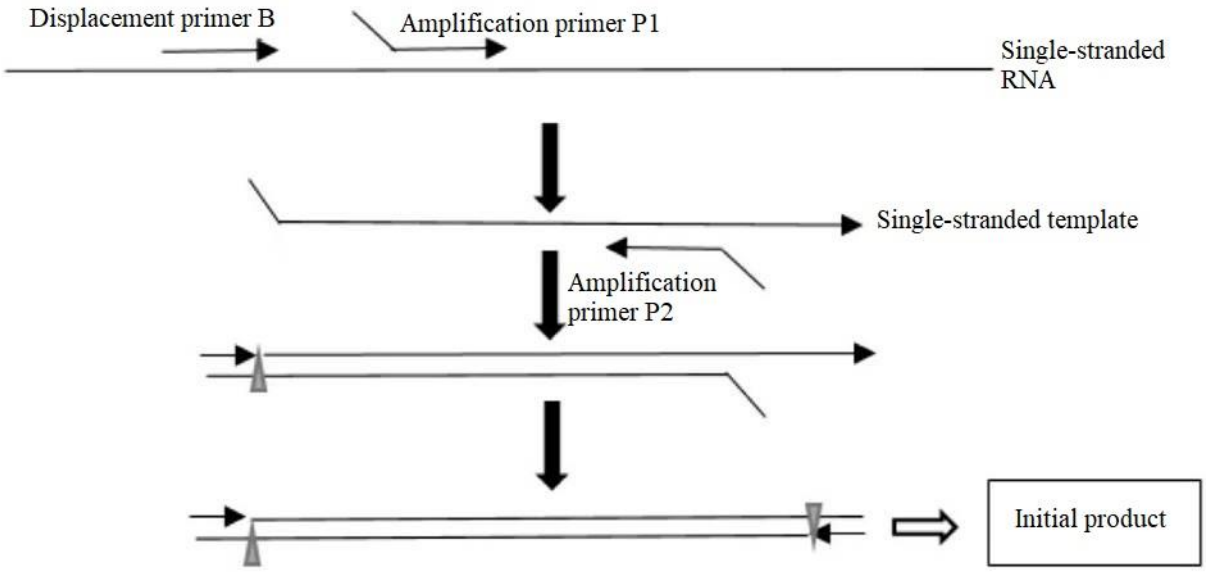

FIG. 2

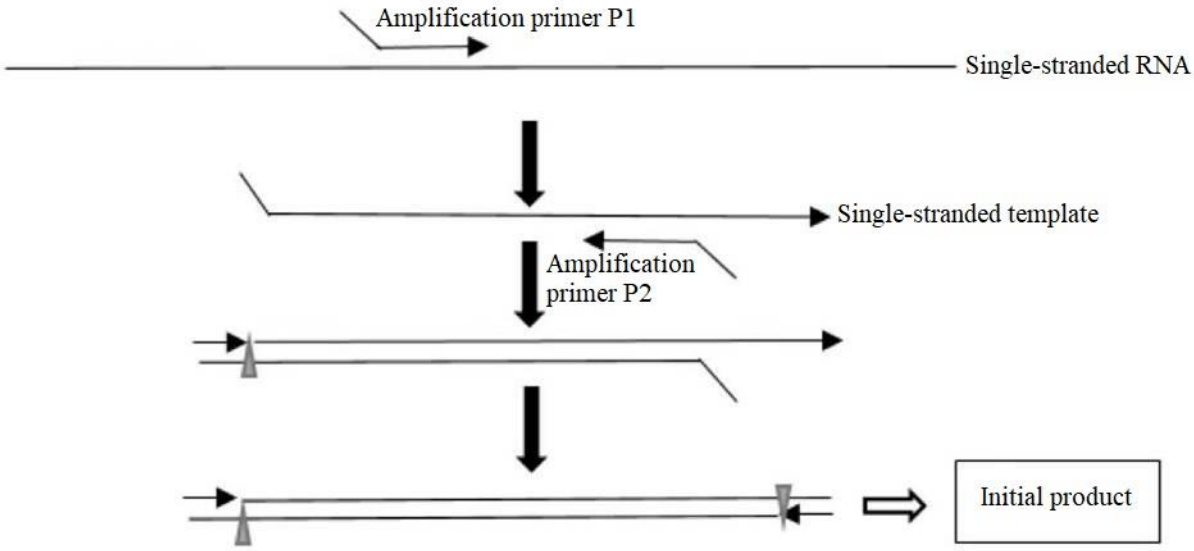
Amplification primer P1
Single-stranded RNA
Single-stranded template
Amplification
primer P2
Initial product

FIG. 3

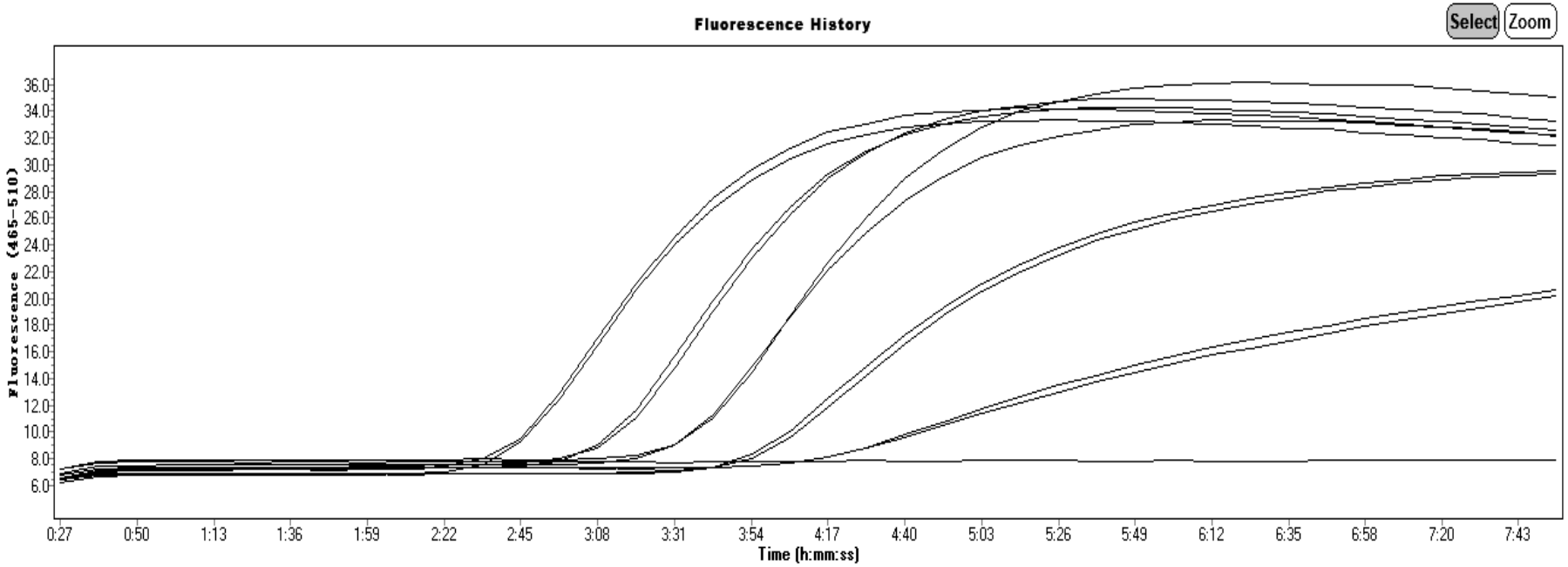
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FIG. 4

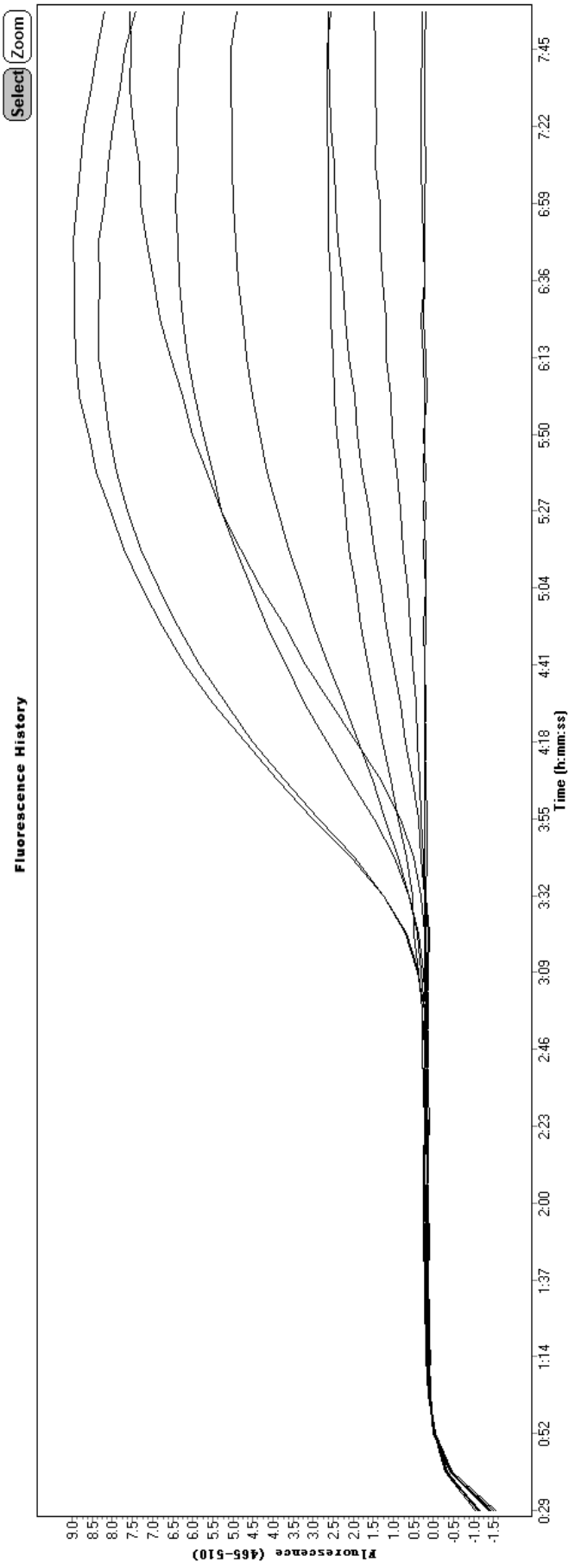
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FIG. 5

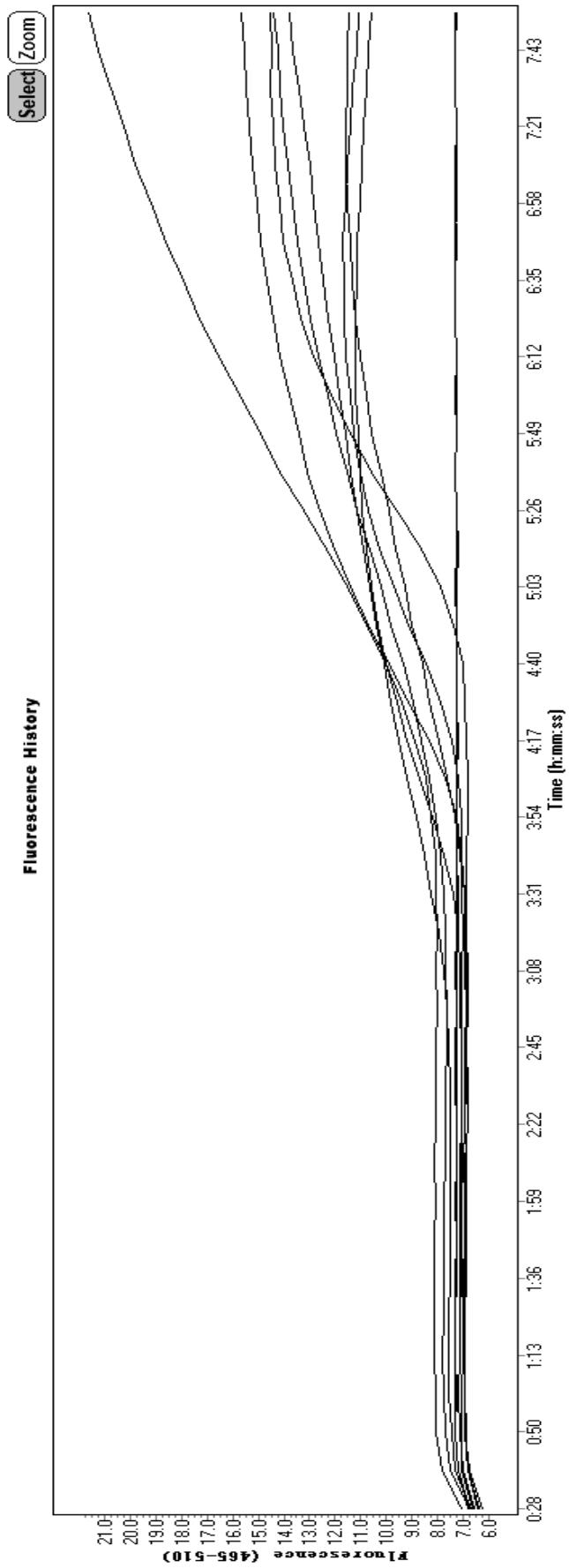
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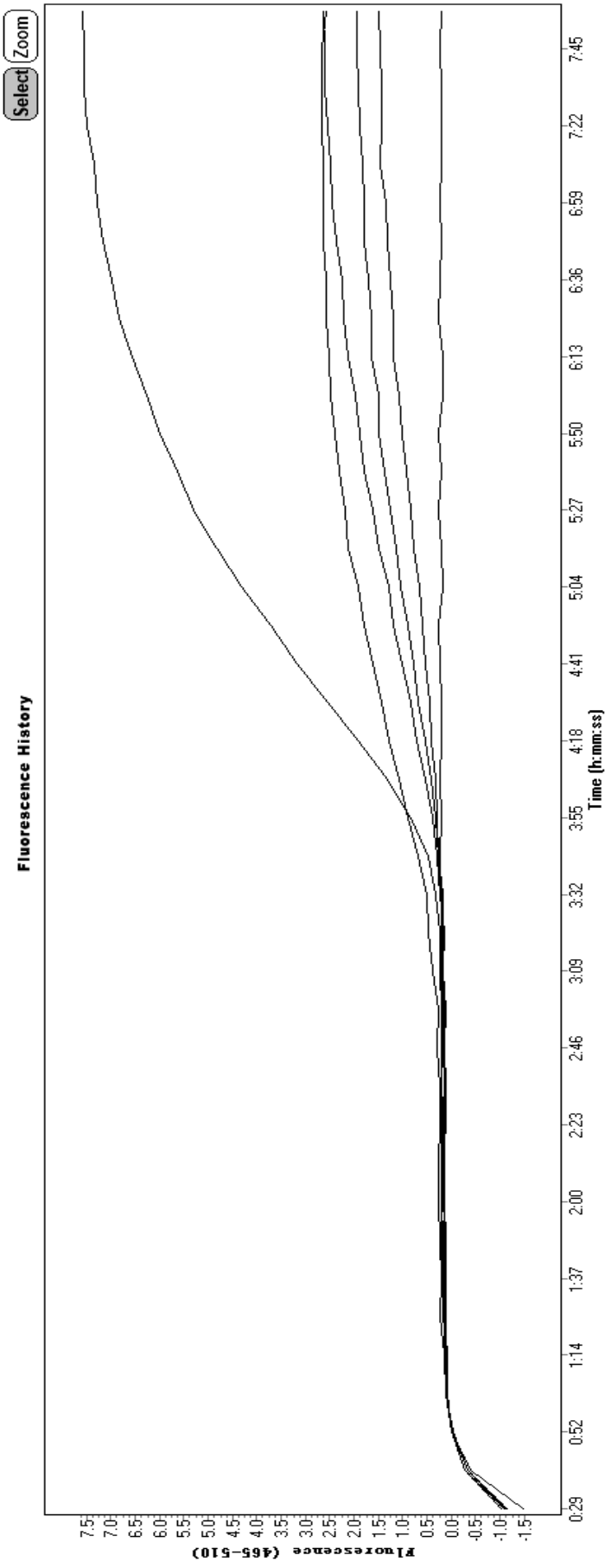
FIG. 6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Zoom
Fluorescence History
Fluorescence (465-510)
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Time (h:mm:ss)

FIG. 7

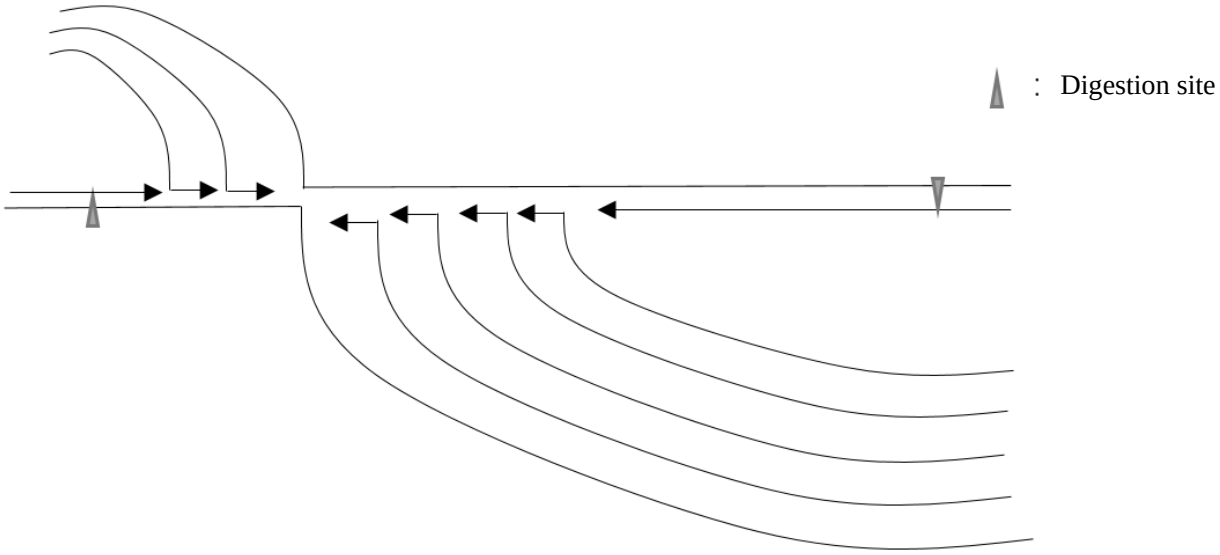

FIG. 8

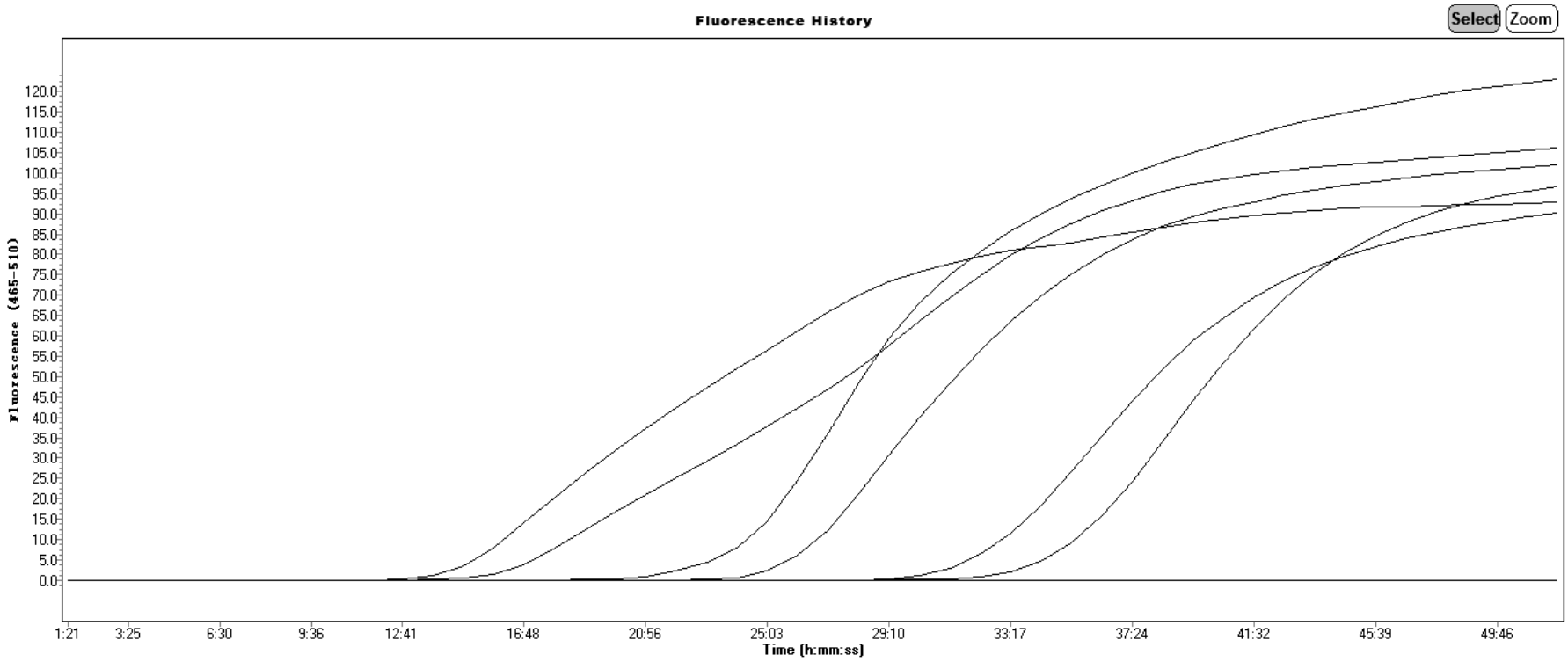
Fluorescence History
Select
Zoom
Fluorescence (465-510)
120.0
115.0
110.0
105.0
100.0
95.0
90.0
85.0
80.0
75.0
70.0
65.0
60.0
55.0
50.0
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
1:21
3:25
6:30
9:36
12:41
16:48
20:56
25:03
29:10
33:17
37:24
41:32
45:39
49:46
Time (h:mm:ss)

FIG. 9