Method and Kit for Nonspecific Replication of DNA Fragment

Field of invention

The invention belongs to the field of molecular biology, and in particular relates to a method and a kit for non-specific replication of DNA fragments.

Background of the invention

There are many methods for DNA amplification, but two particular approaches are commonly used. One is to use random primers to bind to any position of DNA and amplify, thereby obtaining a large number of DNA fragments of random length. The second is to connect the DNA fragments to the artificial header, and then use the DNA fragment with the header as a template for DNA amplification. The disadvantage of the former method is that the length of the amplified product DNA molecule is smaller than that of the original DNA molecule, the integrity of the DNA information is destroyed, the full length of the template molecule cannot be amplified, and the products of small fragments are excessive generated, which in turn lead to the insufficient amount of DNA template for conventional detection. For samples consisting of small fragments of DNA molecules, such as cell-free DNA samples, this method will make it difficult to obtain amplification products. Although the second method can obtain trace of the relatively complete amplified products of DNA molecules, a long artificial sequence is introduced at both ends of the product, which changes the sequence information of the original DNA molecules, and causes non-specific interference or loss of information for DNA sequence information dependent detections and research works, especially for NGS and other second-generation sequencing detection methods since the original DNA molecule itself with some exogenous DNA sequence information will cause great interference.

Summary of the invention

In view of the above problems in the prior art, the object of the present

invention is to provide a method and kit for non-specific replication of DNA fragments, and through the non-specific amplification of the DNA fragments that are expected to be replicated to obtain a large number of usable DNA fragments which are substantially identical to the initial DNA fragments that are expected to be replicated.

The present invention has been intensively studied to solve the above problems, and as a result, it was found that a large number of DNA fragments which are substantially identical to the initial DNA fragments that are expected to be replicated could be obtained by PCR amplification of DNA fragments with headers using PCR primers and the cleavage of the amplified products with enzymes cleaving reagents.

That is, the present invention includes:

1. A method of non-specific replication of DNA fragments, including:

STEP B: Adding headers to processed DNA fragments that are expected to be replicated to generate ligated DNA fragments with header sequences;

STEP C: Using primers that bind to the header sequences to amplify the ligated DNA fragments to obtain the PCR products.

STEP D: Breaking the PCR products to obtain the replicated DNA fragments which are substantially similar to the original DNA fragments that are expected to be replicated.

2. The method according to item 1, wherein the header contains breaking areas.

3. The method according to item 2, wherein the breaking areas are located near the connecting point between the header and the processed DNA fragments that are expected to be replicated.

4. The method according to item 3, wherein the breaking areas are cleavage sites.

5. The method according to item 4, wherein the cleavage sites contain at least 1bp nucleotide sequence.

6. The method according to item 4, wherein the cleavage sites contains at least one enzyme cutting site or nucleotide sequence with at least one Thymine nucleotide

(T base).

7. The method according to item 2, wherein the headers with breaking areas are annealing products of nucleotide sequences such as shown in SEQ ID NO: 1 and SEQ ID NO: 2.

SEQ ID NO: 1 sequence: 5'-$A_t(N)_a(N)_b$-3';

SEQ ID NO: 2 sequence: 5'-$(N)_c(N)'_a$T-3',

Wherein, N is one of the any four types of nucleotides, Adenine, Guanine, Cytosine and Thymine; wherein the nucleotide sequence of $(N)_a$ is the reverse-complement counterpart of $(N)'_a$; wherein t is 0 or 1; wherein a, b, and c are natural numbers over 5, respectively. Preferably, a is the number between 5-30, more preferably, a is the number between 10-20; preferably, b is the number between 5-20, more preferably, b is the number between 10-20; preferably c is the number between 10-20.

8. The method according to item 1, wherein the primers contain breaking areas,

9. The method according to item 8, wherein the breaking areas are located near the 3' terminal of the primers.

10. The method according to item 8, wherein the breaking areas are cleavage sites.

11. The method according to item 10, wherein the cleavage sites contain at least 1bp nucleotide sequence.

12. The method according to item 10, wherein the cleavage sites contains at least one enzyme cutting site or nucleotide sequence with at least one deoxyuridine nucleotide.

13. The method according to item 8, wherein the primers with breaking areas have a nucleotide sequence such as shown in SEQ ID NO: 3 and SEQ ID NO: 4.

SEQ ID NO: 3 sequence: 5'-$(N)_e(N)'_b(N)'_a$U (M) k -3';

SEQ ID NO: 4 sequence: 5'-$(N)_f(N)_c(N)'_a$U (M) g -3',

Wherein, N is one of the any four types of nucleotides, Adenine, Guanine,

Cytosine and Thymine; wherein M is a random nucleotide; wherein the nucleotide sequence of $(N)_a$ is the reverse-complement counterpart of $(N)'_a$; wherein the nucleotide sequence of $(N)_b$ is the reverse-complement counterpart of $(N)'_b$; wherein e, f, g and k are natural numbers; wherein a, b, and c are natural numbers over 5. Preferably, a is the number between 5-30, more preferably, a is the number between 10-20; preferably, b is the number between 5-20, more preferably, b is the number between 10-20; preferably c is the number between 10-20; preferably e is the number between 0-20, preferably f is the number between 0-20, more preferably, e is 0 and f is 0; preferably, g and k are the numbers between 0-5, more preferably, g and k are the numbers between 0-3.

14. The method according to item 1, wherein breakings of the PCR products are achieved through reagent cleavage process.

15. The method according to item 14, wherein the reagent cleavage is performed by cleavage reagents to break the PCR product at least once.

16. The method according to item 15, wherein the cleavage reagents contain at least an enzyme with cleavage function.

17. The method according to item 16, wherein the enzyme with cleavage function is selected from at least one of the groups consisting of restriction endonucleases, exonucleases, enzymes with deoxyuracil cleavage function and enzymes with single-strand specific cleavage function.

18. The method according to item 17, wherein the enzyme with deoxyuracil cleavage function is the Uracil-DNA Glycosylase (UDG enzyme) or the mixture of Endonuclease VIII and UDG; wherein the enzyme with single-strand specific cleavage function is the Mung Bean Nuclease and/or S1 Nuclease.

19. The method according to item 1, wherein the quantity of the DNA fragments expected to be replicated in step B is 0.1 to 100 ng, and

20. The method according to item 1, wherein the size of the DNA fragments desired to be replicated in step B is 80 to 1000 bp, preferably the size of the DNA

fragments expected to be replicated is 100~250bp.

21. The method according to item 1, wherein comprise the step A-2 performed before the step B: end-repairing the DNA fragments that expected to be replicated to obtain the processed DNA fragments that are expected to be replicated; or end-repairing the DNA fragments that are expected to be replicated and subsequently or simultaneously adding the Adenine nucleotide to the 3' end of the DNA fragments with blunt ends to obtain the processed DNA fragments that are expected to be replicated.

22. The method according to item 21, wherein comprise the step A-1 performed before the step A-2: the sample DNA is disrupted to obtain the DNA fragments that are expected to be replicated.

23. A usable DNA fragment obtained method, which through the method of non-specific replication of DNA fragment according to any one of item 1 to 22.

24. A method of DNA library construction, which is used to construct libraries using the usable DNA fragments according to item 23.

25. A method of DNA library construction according to item 24 also includes:

Step E: End-repairing the usable DNA fragments to receive DNA fragments with blunt ends;

STEP F: Adding the adenine nucleotide to the 3' end of the DNA fragments with blunt ends to obtain the DNA fragments with attached Adenine nucleotides at the 3' end.

STEP G: Adding headers to the DNA fragments with attached Adenine nucleotide at the 3' end to obtain a ligated DNA fragments with headers.

STEP H: Amplifying the DNA fragments with adapters by PCR amplification to obtain PCR amplified products to complete the library construction.

26. A kit for non-specific replication of DNA fragments, wherein the kit is used to implement any of the methods described in item 1-22, including: adding header reagents, wherein the adding header reagents include headers, PCR amplification

reagents, wherein the PCR amplification reagents include PCR primers, and/or cleavage reagents, wherein the cleavage reagents include enzymes with cleavage functions;

27. The kit according to item 25, wherein the headers are headers contain breaking areas; wherein the PCR primers are primers contain breaking areas; wherein the cleavage reagents are at least one enzyme with cleavage function.

28. The kit according to item 26, wherein in the headers is headers contain breaking areas, wherein the breaking areas on the header are located near the connecting point between the header and the processed DNA fragments that are expected to be replicated; wherein in the primers contain breaking areas, the breaking areas are located near the 3' terminal of the primers.

29. The kit according to item 26, wherein the breaking areas are cleavage sites. Preferably, wherein the cleavage sites contain at least 1bp nucleotide sequence; more preferably, the cleavage sites contains at least one enzyme cutting site or nucleotide sequence with at least one Thymine nucleotide.

30. The kit according to item 26, wherein the headers with breaking areas are annealing products of nucleotide sequences such as shown in SEQ ID NO: 1 and SEQ ID NO: 2.

31. The kit according to item 26, wherein the primers with breaking areas have nucleotide sequences such as shown in SEQ ID NO: 3 and SEQ ID NO: 4.

32. A preparation method of quality control product, standard product or calibration product. Wherein it uses non-specific DNA fragment replication method according to any one of the items between item 1-22.

33. A quality control product, standard product or calibration product obtained through the non-specific DNA fragment replication method according to any one of the items between item 1-22.

34. A diagnostic kit, wherein it consists of the quality control product, the standard product or the calibration product according to item 32.

Compared with the prior art, the present invention has the beneficial effects that the present invention efficiently replicates a DNA fragments that are desired to be replicated, and obtains a large number of usable DNA fragments which are substantially identical in size and sequence to the DNA fragments that are expected to be replicated.

Figure desciption

Figure 1 is a schematic diagram showing the analysis results of the Agilent 2100 on DNA sample No. 1 of the present invention;

Figure 2 is a schematic diagram showing the analysis results of the Agilent 2100 on the DNA amplification product of sample No. 1 of the present invention;

Figure 3 is a schematic diagram showing the analysis results of Agilent 2100 assay on enzyme digested DNA amplification product of sample No. 1 of the present invention;

Figure 4 is a schematic diagram showing the results of the duplication rate analysis of the sequencing data of sample No. 1-10 and the replication product No. 1-10;

Figure 5 is a schematic diagram showing the results of the mapping rate analysis of the sequencing data of sample No. 1-10 and the replication product No. 1-10;

Figure 6 is a schematic diagram showing the coverage fraction of the sequencing data of sample No. 1-10 and the replication product No. 1-10;

Figure 7 is a schematic diagram showing the results of signal distribution on all chromosomes of the original sample No. 5 and its enzyme digested sample;

Figure 8 is the calculated window-based depth value of the original sample No. 5 and the digested sample;

Figure 9 is a schematic illustration of the analysis results of the Agilent 2100 on DNA fragment sample a1 of the present invention;

Figure 10 is a schematic diagram showing the analysis results of the Agilent 2100 on the DNA amplification product of sample a1 of the present invention;

Figure 11 is a schematic diagram showing the analysis results of Agilent 2100 assay

on enzyme digested DNA amplification product of sample a1 of the present invention.

Detailed description of the invention

The present invention will be further described in detail below with figures and examples. It should be understood that the specific examples described herein are intended to explain the present invention, rather than to limit the present invention.

In one aspect, the present invention provides a method of non-specific replication of DNA fragments, (non-specific replication method of the present invention) including:

Step B: Adding headers to processed DNA fragments that are expected to be replicated to generate ligated DNA fragments with header sequences.

Step C: Using primers that bind to the header sequences to amplify the ligated DNA fragments to obtain the PCR products.

Step D: Breaking the PCR products to obtain usable DNA fragments which are substantially similar to the DNA fragments that are expected to be replicated.

'Non-specific replication' refers to amplification with little or no introduction of exogenous sequences and loss of template information, yielding a substantial number of products that are substantially identical to the original template sequences. Here, "substantially identical" means that as known to those skilled in the art, the replicated product has a very low ratio of foreign sequences introduced when compared with the original template nucleotide sequence and the size of the foreign sequences is as small as 0 bp or less than 5bp.

The source of the DNA fragments expected to be replicated in the step B is not particularly limited. It may be, for example, a large amount of samples of genomic DNA, or may be a trace amount of DNA sample (e.g., FFPE sample, cell free DNA in blood/body fluid, etc.). The size of the DNA fragments expected to be replicated is not particularly limited, and it may be any size known to those skilled in the art as an amplification template. Preferably, the size of the DNA fragments expected to be

replicated is 80 to 1000 bp, and more preferably, the size of the DNA fragments expected to be replicated is 100 to 250 bp.

Step A-2 may be performed before obtaining the DNA fragments expected to be replicated in said step B: end-repairing the DNA fragments expected to be replicated to obtain the processed DNA fragments expected to be replicated; or end-repairing the DNA fragments that are expected to be replicated and subsequently or simultaneously adding the Adenine nucleotide to the 3' end of the DNA fragments with blunt ends to obtain the processed DNA fragments that are expected to be replicated, DNA fragmentation may be performed before step A-2. Wherein the word "processed" in the said processed DNA fragments expected to be replicated may refers to, for example, a step of end repair, or steps of end repair and addition of Adenine base, etc., which are generally known methods to those skilled in the art for obtaining DNA fragments can be added headers.

According to the present invention, "breaking area" refers to an nucleotide sequence that can be recognized by any one of the cleaving means, such as a cleavage site or the like, which can be obtained by any cleaving method known to those skilled in the art. The said breaking areas on the header are located near the connecting point between the header and the processed DNA fragments that are expected to be replicated. The said "Cleavage site" refers to a nucleotide sequence that can be designed to allow for sequence-selective cleavage of both strands, such as a restriction enzyme cutting site and/or deoxyuracil nucleotide (also known as uracil deoxy nucleotide, deoxyuridine nucleotides, deoxy-Uracil or dU). Here, the said "Restriction Enzyme cutting site" refers to a specific nucleotide sequence on a DNA, and the restriction enzyme can recognize the sequence and cut the DNA molecule into two segments. Where appropriate, it can also be designed to allow selective cleavage of only one single strand. The said "nucleotide" refers to a deoxyribonucleotide, and in some special embodiments may also be a ribonucleotide. The length and exact sequence of the said cleavage site are not particularly limited and may be any desired

sequence, for example it may be designed to be cleaved by any selected enzyme.

The subsequent paragraphs describe various aspects of the invention in more detail. Each aspect of the invention may be combined with any other aspect or aspects of the invention, unless explicitly stated to the contrary. In particular, any feature that is referred to as particular, preferred or advantageous may also be combined with any other feature or features that are referred to as particular, preferred or advantageous.

In a particular example, the header described in step B is the product formed by annealing a nucleotide sequence such as SEQ ID NO: 1 and a nucleotide sequence such as SEQ ID NO: 2. Usually, the synthesized dry header primer powder is diluted to the concentration of 100 μM by using 1× annealing buffer, and then annealed in an equal volume, that is, 50 μL of each header primer is taken, and kept at 85℃ for 2 minutes after mixing, and the temperature is lowered by 1℃ per minute until 15℃ and incubate at 4℃ to obtain the header, or using methods and kits known in the art to prepare the header.

SEQ ID NO: 1 sequence: 5'- $A_t(N)_a(N)_b$-3';

SEQ ID NO: 2 sequence: 5'-$(N)_c(N)'_aT$-3',

The said primer with a cleavage site is the primer containing a deoxyuracil nucleotide, such as the nucleotide sequences shown in SEQ ID NO: 3 and SEQ ID NO: 4.

SEQ ID NO: 3 sequence: 5'-$(N)_e(N)'_{b(}(N)'_aU$ (M) k -3';

SEQ ID NO: 4 sequence: 5'-$(N)_f(N)_c(N)'_aU$ (M) g -3',

Wherein, N is one of the any four types of nucleotides, Adenine, Guanine, Cytosine and Thymine and M is a random nucleotide. Wherein $(N)_a$,$(N)_b$, $(N)_c$, $(N)_e$, $(N)_f$, $(N)'_a$, $(N)'_b$ are 7 independently selected segments from the sequence of A, T, C, G nucleotides and contain a, b, c, e, f, a, b Ns, repectively. The nucleotide sequence of $(N)_a$ is the reverse-complement counterpart of $(N)'_a$ and the nucleotide sequence of $(N)_b$ is the reverse-complement counterpart of $(N)'_b$. Wherein t is 0 or 1, when t equals to 0, there is 0 Adenine, and when t is 1, there is 1 Adenine. Wherein a, b, and c are

separate natural numbers with the value over 5. Preferably, a is the number between 5-30, more preferably, a is the number between 10-20; preferably, b is the number between 5-20, more preferably, b is the number between 10-20; preferably c is the number between 10-20; Wherein e and f are natural numbers, preferably e is the number between 0-20 and f is the number between 0-20, more preferably, e is 0 and f is 0; Wherein g and k are natural numbers, preferably, g and k are the numbers between 0-5, more preferably, g and k are the numbers between 0-3.

Preferably, step A-2 performed before the step of adding headers to processed DNA fragments desired to be replicated is included: The DNA fragments desired to be replicated is subjected to the end-repairing, and the header formed by annealing the primers containing nucleotide sequences such as SEQ ID NO: 2 and SEQ ID NO: 1 are used in the sequential step of adding the header to the DNA fragments. Or after end repairing the DNA fragments desired to be replicated and subsequently or simultaneously adding the Adenine nucleotide to the 3' end of the DNA fragments to obtain the processed DNA fragments, the header formed by annealing the primers containing nucleotide sequences such as SEQ ID NO: 2 and SEQ ID NO: 1 are used in the sequential step of adding the header to the DNA fragments.

Preferably, step B-1 is also included in said step B: pre-amplification of the DNA fragments with headers, for example, PCR amplification using reagents consisting PCR primers with nucleotide sequence shown in SEQ ID NO: 2 and SEQ ID NO: 5, KAPA HiFi HotStart, ReadyMix (KAPA), etc. The nucleotide sequence of SEQ ID NO: 5 is as follows:

SEQ ID NO: 5 sequence: 5'-$(N)_e(N)'_b(N)'_aT$-3'.

Wherein step C, reaction systems used for PCR amplification include Taq polymerase, Pfu polymerase, KAPA HiFi polymerase, KAPA HiFi Uracil+ system, KAPA 2G robust DNA polymerase system, and the like. The KAPA HiFi Uracil+ reaction system is preferred, and the KAPA 2G robust DNA polymerase system is more preferred.

Wherein step D, The cleavage reagent is an enzyme having a deoxyuracil cleavage function and an enzyme having a single-strand specific cleavage function. The enzyme with a deoxyuracil cleavage function includes a mixture of Endonuclease VIII and UDG. To make the enzymatic cleavage reaction more sufficient, the reaction conditions may be used as such, incubation at 30 to 50℃ for 5 to 60 minutes, preferably 20 to 40℃ for 20 to 40 minutes, more preferably 30 to 40℃ for 30 to 40 minutes.

And then use the enzyme, the enzyme having a single-strand specific cleavage function may be selected from a mung bean nuclease and/or an S1 nuclease, preferably an S1 nuclease. To more completely cleave headers from DNA amplified fragments, the reaction condition may be used as such, incubation at 20 to 50℃ for 0.1 to 2 hours, preferably 20 to 40℃ for 20 to 60 minutes, more preferably 25 to 35℃ for 25 to 35 minute.

Preferably, in the method of the present invention, a product purification step is further included between step B and step C and/or between step C and step D, and the purification step can be carried out by a conventional method in the art, for example, magnetic beads purification.

In an example of the invention, step B may comprise the use of headers with an enzyme cleavage site to ligate the treated DNA fragments that are desired to be replicated, wherein the method of adding the headers is known to those skilled in the art. In the method, the cleavage site is at the position where the headers is ligated to the processed DNA fragments desired to be replicated. The design of the headers is a method known to those skilled in the art, in which case the ligation reaction of the headers and DNA fragments can be carried out using conventional methods and kits known in the art.

Wherein step C, the fragment size of the nucleotide sequence of the primer is not longer than the size of the fragments of any single-stranded nucleotide sequence of the header and in the case known to those skilled in the art, the nucleotide sequence of

the primer can be bind to the DNA fragments with the headers and amplify the DNA fragments with headers to obtain the PCR product.

Wherein the step D, the breakings of the PCR product is achieved through reagent cleavage. In the present invention, the cleavage method may be, for example, biological, chemical or physical. In order to excise headers with cleavage sites in a PCR product to obtain DNA fragments which is substantially identical to the DNA fragments desired to be replicated, any suitable enzymatic, chemical or optical method can be used for the cleaving. Cleaving can result in partial or entire cutting on DNA chain. Any suitable enzyme includes, for example, restriction enzymes, exonucleases, enzymes with deoxyuracil cleavage function, and enzymes with single-strand specific cleavage function. Preferably, wherein the reagent cleavage refers as conducting the cleavage process at least once with the cleavage reagents.

Wherein the cleaving reagents comprise at least one enzyme with cleavage function; preferably, the enzyme with a cleavage function belongs to at least one of the groups below: restriction endonucleases, exonucleases, enzymes with deoxyuracil cleavage function, and enzymes with single-strand specific cleavage function; Wherein the cleavage reagent is the restriction enzyme, be the first to use the restriction enzyme for cleaving through the enzyme restriction site recognition, for example, MYLI enzyme is used to cleave the header as known to those skilled in the art.

Or the cleavage reagent said in step D is a restriction endonuclease and an enzyme with a single-strand specific cleavage function, such as a DpnII recognition site, in which case a corresponding enzyme to the recognition site (eg, HindIII enzyme) and an enzyme having a single-strand specific cleavage function are used. Wherein the enzyme with single-strand specific cleavage function is selected from mung bean nuclease and/or S1 nuclease, and the header can be excised to obtain usable DNA fragments.

In a particular example, the header used in step B is any header known to those

skilled in the art, the method of ligating the header is also any method that can be conceived by those skilled in the art, the primers used in step C can be bound to nucleotide sequences used in said step B, the principles of primer design are generally familiar to those skilled in the art, and PCR amplification can be carried out under the amplification conditions and amplification systems known in the art to obtain PCR products.

In this case, the digested reagent described in step D is an exonuclease, such as BAL31, and, where appropriate, the PCR product is digested to obtain DNA fragments that are substantially identical to the DNA fragments desired to be replicated. In the present invention, the usable DNA fragments can be obtained by selecting suitable reaction conditions depending on the type of different exonucleases.

The present invention further provides a type of available DNA fragments (usable DNA fragments of the present invention) which is obtained by a method of non-specific replication of DNA fragments. The size of the usable DNA fragments is 80 to 1000 bp, and preferably the size of the usable DNA fragments is 100 to 250 bp.

The present invention further provides a method for DNA library construction (method for DNA library construction of the present invention), using available DNA fragments are templates and also including:

STEP E: End-repairing the DNA fragments to received DNA fragments with blunt ends;

STEP F: Adding the adenine nucleotide to the 3' end of the DNA fragments with blunt ends to obtain DNA fragments with attached Adenine nucleotides at the 3' end.

STEP G: Adding headers to the DNA fragments with attached A nucleotides at the 3' end to obtain ligated DNA fragments with headers.

STEP H: Amplifying the DNA fragments with adapters by PCR amplification to obtain PCR amplified products to complete the library construction.

Preferably, wherein present invention, a product purification step is also included between step B and step C, between step C and step D, between step D and

step E, between step E and step F, between step F and step G, and/or between G and step H. Each step is carried out by conventional methods in the art.

The present invention also provides a kit for non-specific replication of DNA fragments (replication kit of the present invention), which can be used to practice the methods of the present invention. The replication kit includes:

Reagents for adding headers, including: headers;

Reagents for PCR amplification, including: primers;

Reagents for cleavage, including enzymes with the cleavage function.

In another aspect, the present invention also provides a kit for constructing a DNA library (the library construction kit of the present invention), which can be used to practice the methods of the invention.

The library construction kit of the present invention must contain the PCR amplification reagents for the replication of said ligated products after attaching the headers, including:

Reagents for ligating headers, including: headers.

Reagents for PCR amplification, the reagents comprising primers.

Reagents for cleaving, the reagents comprising enzymes with cleavage function.

Preferably, the library construction kit of the present invention may further comprise at least one or all selected items from below:

Reagents for end-repairing, such as T4 DNA polymerase, T4 PNK, Klenow fragment, and the corresponding ligation reaction buffer.

A reagent for adding Adenine to the 3' end, for example, Klenow fragment lacking exonuclease activity, a corresponding reaction buffer, and the like.

A reagent for ligating DNA fragments and headers, such as T4 DNA ligase and the corresponding ligation reaction buffer.

Reagent for purification, such as 1.8 x Ampure magnetic beads or the like.

In another aspect, the present invention also provides a method for preparing quality controls, standards or calibrators (method for preparing quality controls,

standards or calibrators of the invention), which are prepared by the method of non-specific replication of the present invention. The quality control products, standards or calibrators in the diagnostic reagents are the main tools for achieving accurate and consistent results of clinical testing and supervision, as well as the measurement physical standards for ensuring the effective transmission of quality values. Existing cell free DNA controls, standards, or calibrators require the collection of real plasma or tissue fluid, or mixing the real plasma sample with DNA from real diseased tissues etc. There are various drawbacks to the preparation methods. However, the method of present invention for preparing quality controls, standards or calibrators successfully solves these problems.

In another aspect, the invention also provides a type of quality control, standard or calibrator (the quality control, standard or calibrator of the present invention), which are obtained by the non-specific replication method of the present invention

In another aspect, the invention also provides a diagnostic reagent comprising the quality control, standard or calibrator of the present invention.

Example 1

1. Sample and primer preparation

1) The sequences of the primers A, B, and A' used are as follows, in which the primer A is phosphorylated at the 5' end and the primer sequences are as follows:

Primer ID	Primer name	Primer sequence (5'-3')
SEQ ID NO: 6	Primer A	GCTCGTTAGATCGTCGTGTAGGCTACCAGTGT
SEQ ID NO: 7	Primer B	CAGTGGACTTCAGACGTGTGATCTAACGAGCT
SEQ ID NO: 8	Primer A'	ACACTGGTAGCCTACACGACGATCTAACGAGCT

2) The primers used for amplification are primer a and primer b, wherein both primers a and b have a deoxyuracil at the 3' end, and the primer sequences are as follows:

Primer ID	Primer name	Primer sequence (5'-3')
SEQ ID NO: 9	Primer a	ACACTGGTAGCCTACACGACGATCTAACGAGCU

SEQ ID NO: 10	Primer b	CAGTGGACTTCAGACGTGTGATCTAACGAGCU

3) Header preparation (total volume 100μL)

The synthesized dry powder of primer A and primer B were diluted to a concentration of 100 μM using 1× annealing buffer, and then annealed after mixing together with an equal volume; 50 μL of primer A (100 μM) and 50 μL of primer B (100 μM) were added, and incubate at 85℃ for 2 minutes. The temperature was then lowered by 1℃ until 15℃, and hold at 4℃ to obtain the header.

2. DNA template pre-treatment

For 1 plasma sample, plasma cell free DNA was isolated as the original sample No. 1 (ie DNA desired to be replicated), and it was analyzed using Agilent 2100 Bioanalyzer and Agilent 2100 Highly Sensitive DNA Detection Chip (Agilent 2100). The size of the DNA fragments of sample No. 1 was mainly distributed at about 168 bp (Fig. 1), and 2 ng was taken for the following treatment:

1) DNA fragment End-repair

cell free DNA	2ng
10×PNK buffer	5μL
dNTP Solution Set（10mM）	1μL
T4 DNA polymerase	1μL
T4 polynucleotide kinase	1μL
Klenow fragment	1μL
Water	make up to 50μL
Total volume	50μL

Incubating at 20℃ for 30 minutes, followed by DNA purification using 1.8 × Ampure magnetic beads to recover purified DNA fragments, and dissolved in 32 μL of EB to obtain DNA fragments with blunt-end..

2) Adding the adenine nucleotide to the 3' end of the DNA fragments with blunt-end, the reaction system was as follows:

DNA fragments with blunt-end	32μL
10×blue buffer	5μL
dATP（1mM）	10μL

Klenow（3-5'exo-）	3μL
Total volume	50μL

Incubating at 37°C for 30 minutes, followed by DNA purification using 1.8 × Ampure magnetic beads to recover purified DNA fragments, and dissolved in 25 μL of EB to obtain DNA fragments with additional A at the 3' end.

3) Using the ligation reaction system to ligate headers with T nucleotide at the 3' end to the DNA fragments with A nucleotide at the 3' end. The ligation reaction mix was as follows:

DNA fragments with added A	25μL
2×Rapid ligation Buffer	28μL
Header	1μL
T4 DNA ligase	2μL
Total volume	56μL

Incubating at 20°C for 15 minutes, followed by DNA purification using 1.8 × Ampure magnetic beads to recover purified DNA fragments, and dissolved in 23 μL of EB to obtain DNA fragments ligated with headers.

4) PCR pre-amplification

Using the library PCR reaction system to amplify the DNA fragments with headers, the reaction system and condition were as follows:

Ligated DNA fragments with headers	23μL
Primer A'	1μL
Primer B	1μL
KAPA HiFi HotStart ReadyMix（KAPA Ltd.）	25μL
Total volume	50μL

PCR reaction condition:

Step	Reaction temperature	Reaction time
①	95°C	30s
②	95°C	10s

③	65℃	30s
④	72℃	30s
⑤	Go to ②	cycle: 15
⑥	72℃	7min
⑦	4℃	Hold

After the reaction, 1.8 × Ampure magnetic beads were used to purify and recover the purified PCR product, and dissolved in 50 μL of EB to obtain the primary amplification product.

3. PCR amplification

PCR amplification using the primary amplification product (or DNA fragments with headers) as the template and primer a and b. PCR reaction system and condition were as follows:

Primary amplification product	1ng
Primer a	1μL
Primer b	1μL
KAPA HiFi Uracil+	25μL
water	Make up to 50μL
Total volume	50μL

PCR reaction condition:

steps	Reaction temperature	Reaction time
①	95℃	30s
②	95℃	10s
③	65℃	30s
④	72℃	30s
⑤	Go to ②	cycle:15
⑥	72℃	7min
⑦	4℃	hold

After the reaction, 1.8 × Ampure magnetic beads were used to purify and recover the purified product, and dissolved in 50 μL of EB to obtain the PCR product containing deoxyuracil nucleotide. The Agilent 2100 was then used for fragment size

detection and concentration quantification. The results showed that the amplified fragment size is mainly distributed around 235 bp (Fig. 2).

4. Header cleavage and product recovery

1) Using the User enzyme to cleave the deoxyuracil in the PCR product containing the deoxyuracil nucleotide, the system is as follows:

PCR product	1μg
User enzyme	2μL
CutSmart Buffer	5μL
water	Make up to 50μL
Total volume	50μL

Incubating at 37°C for 30 minutes, followed by DNA purification using 1.8 × Ampure magnetic beads to recover purified DNA fragments, and dissolved in 30 μL of EB to obtain DNA fragments with nicks.

2) Digested DNA fragments with nicks using S1 nuclease, completely cleave the headers, the reaction system and the reaction conditions are as follows:

DNA fragments with nicks	30μL
S1 nuclease	1μL
S1 Buffer	5μL
water	Make up to 50μL
Total volume	50μL

Incubating at 30°C for 30 minutes, followed by DNA purification using 1.8 × Ampure magnetic beads to obtain replicated DNA (ie. a large number of available DNA fragments substantially identical to the sequence of cell free DNA fragments in the plasma of a pregnant woman).

5. DNA fragment size detection

The size of the replicated fragments was examined using Agilent 2100, and the results showed that the size was mainly distributed at about 166 bp (Fig. 3).

Figure 1 shows that the size of the DNA fragments of sample No. 1 is mainly distributed at about 168 bp, and this is also the size of the cell free DNA fragments extracted from the plasma of the pregnant woman. Figure 2 shows that the size of the

fragments after amplification is mainly distributed at about 253 bp. it is 66 bp longer than the length of sample No. 1 after ligating with the header and PCR amplification. The expected length of the amplified product is about 254 bp and the real size of the detected fragments was mainly distributed at about 253 bp, which is within the fluctuation range, indicating that the deoxyuracil-labeled primers were successfully introduced in the PCR amplification step, and the PCR product with deoxyuracil labeling was obtained. Figure 3 shows that the size of the replicated product is mainly distributed at 166 bp, and it differs from the original sample (sample No. 1) by a size of 2 bp, which is also within the fluctuation range, indicating that the replicated product is the PCR product of the headers was successfully cleaved.

6. DNA sequencing library construction

Take cell free DNA of the original sample No.1 as the control, and take 2 ng of the replicated product obtained in Step 4 to construct the DNA sequencing library according to the conventional method of library construction for second-generation sequencing recommended by Illumina, and perform sequencing. The sequencing type is 35 bp single-end and 3.5G data is obtained per sample. The sequencing results are as follows:

Table 1 High-throughput sequencing results analysis

	Original Sample (%)	Replicated product (%)
Dup rate	0.036	0.038
Align rate	0.783	0.790
Coverage	0.702	0.699

In which, the duplication rate (Dup rate): the ratio of the number of reads with identical sequences to reference genome.

maping rate: The ratio of the number of reads that can be completely aligned to the reference genome (hg19) to the total number of reads.

Coverage: The ratio of the target area covered by the sequencing reads to the whole genome area/the total target area after sequence alignment.

The table 2 shows: by comparing with the replicated product, the Dup rate of the

original sample No.1 and replicated product is 0.036% and 0.038% respectively, the difference is not significant, which indicating that the present invention does not increase the duplication rate due to PCR amplification and effectively replicates the original sample DNA; The original sample alignment ratio was 0.783%, and the replication product alignment ratio was 0.790%, indicating that the utilization level of sequencing data of the original sample and the replication product were basically the same, without significant difference; The coverage of the original sample was 0.702%, and the replication product coverage was 0.699%, that is, the ratio of the obtained data to the whole genome area was 0.702% and 0.699%, respectively, and the results were not significantly different.

Acording to the sequencing data of the original sample and the digested sample, the signal distribution on each chromosome is analyzed (Fig. 7). In Fig. 7, the x-axis is the chromosome number and the y-axis is the chromosome signal value. The so-called chromosome signal value is calculated as follows: after error-free alignment of sample reads with the human genome reference sequence, count the number of unique reads on each chromosome and obtain the ratio of the unique reads of each choromosome to the total number of unique reads, and this ratio is the signal value of the corrsponding chromosome. Group2_A is the original sample and Group2_B is the cut sample. Fig. 7 demonstrates that the signal values of the original sample and the digested sample are substantially identical on each chromosome, indicating that the number of Unique Reads positioned on each chromosome of the original sample and the digested sample is substantially the same. For further analysis, chromosome 3 of the original sample and the digested sample No. 5 were selected, the chromosome was divided into 4000 windows, each of which had a fragment size of 100 kb. As a result, as shown in Fig. 8, the depth of signal value distribution of the original sample and the digested sample are substantially the same throughout the window.

Example 2

On the basis of Example 1, 9 additional cell free DNA, numbered as 2-10, was

added as the original sample, the step 5-6 in Example 1 were carried out, and 2 ng of each of the 9 original samples was taken for the step 2-6 of Example 1. The use of the primers and various reaction conditions are as described in Example 1.

Figure 4-6 shows the results of the duplication rate, the alignment ratio, and the coverage of the original sample No. 1-10 after the treatment of step 5-6 (group 1) and step 2-6 (group 2): after the Welch's t-test analysis, t = 0.69842, df = 11.151, p-value = 0.4992, no significant difference was found between the two groups in the sequence duplication rate, indicating the original sample DNA was effectively replicated (Fig. 4); after the Welch's t-test analysis, t = 0.80577, df = 15.614, p-value = 0.4325, no significant difference was found between the two groups in the sequence alignment ratio, indicating the utilization level of the original sample group and the digested sample group was consistent (Fig. 5); after the Welch's t-test analysis, t = 0.54633, df = 16.409, p-value = 0.5922, no significant difference was found between the two groups in the sequence coverage (Fig. 6).

Example 3

1. Pre-treatment of the DNA template

1) Take 2ng of human genome and break the DNA into fragments of about 200bp via sonication (sample number a1). Agilent 2100 was used to analyze the sample No.a1 and the result showed that the DNA fragments of sample No. a1 is mainly distributed at 171bp (Fig. 7). End-repairing is performed, the reaction mixture and conditions are as follows:

DNAfragments	2ng
10×PNK buffer	5μL
dNTP Solution Set（10mM）	1μL
T4 DNA polymerase	1μL
T4 polynucleotide kinase	1μL
Klenow fragment	1μL
water	make up to 50μL
Total volume	50μL

Incubating at 20°C for 30 minutes, followed by DNA purification using 1.8 × Ampure magnetic beads to recover purified DNA fragments, and dissolved in 32 μL of EB to obtain blunt-end DNA fragments.

2) Adding the adenine nucleotide to the 3' end of the DNA fragments with blunt-end, the reaction system and conditions were as follows:

Blunt-end DNA fragments	32μL
10×blue buffer	5μL
dATP（1mM）	10μL
Klenow（3-5'exo-）	3μL
Total volume	50μL

Incubating at 37°C for 30 minutes, followed by DNA purification using 1.8 × Ampure magnetic beads to recover purified DNA fragments, and dissolved in 25 μL of EB to obtain DNA fragments with additional A at the 3' end.

3) Using the ligation reaction system to ligate headers with T nucleotide at the 3' end to the DNA fragments with A nucleotide at the 3' end. The ligation reaction mix was as follows:

DNA fragments with added A	25μL
2×Rapid ligation Buffer	28μL
Headers	1μL
T4 DNA ligase	2μL
Total volume	56μL

Incubating at 20°C for 15 minutes, followed by DNA purification using 1.8 × Ampure magnetic beads to recover purified DNA fragments, and dissolved in 23 μL of EB to obtain DNA fragments ligated with headers.

2. Amplification

Using the DNA fragments ligated with headers from the previous step as the template and obtain large quantity of amplified product with header sequences by PCR amplification with primer a and primer b

Using the Uracil-useable PCR system to add the uracil contained header into the

amplified DNA molecules. The reaction mix and conditions were as follows

DNA fragments with headers	23μL
Primer a	1μL
Primer b	1μL
KAPA HiFi Uracil+	25μL
Total volume	50μL

PCR reaction condition:

Step	Reaction temperature	Reaction time
①	95°C	30s
②	95°C	10s
③	65°C	30s
④	72°C	30s
⑤	Go to ②	cycle:17
⑥	72°C	7min
⑦	4°C	hold

After the reaction, 1.8 × Ampure magnetic beads were used to purify and recover the purified product, and dissolved in 50 μL of EB to obtain the PCR product containing deoxyuracil nucleotide. The Agilent 2100 was then used for fragment size detection and concentration quantification. The results showed that the of the PCR product containing deoxyuracil nucleotide is mainly distributed around 241 bp (Fig. 8).

3. Header cleavage and product recovery

1) Using the User enzyme to cleave the deoxyuracil in the PCR product containing the deoxyuracil nucleotide, the system is as follows:

PCR products	1ug
USER enzyme	2μL
CutSmart Buffer	5μL
water	make up to 50μL
Total volume	50μL

Incubating at 37°C for 30 minutes, followed by DNA purification using 1.8 ×

Ampure magnetic beads to recover purified DNA fragments, and dissolved in 30 μL of EB to obtain DNA fragments with nickes.

2) Digested DNA fragments with nickes using S1 nuclease, completely cleaved the headers, the reaction system and the reaction conditions were as follows:

DNA fragments with nickes	30μL
S1 nuclease	1μL
S1 Buffer	5μL
water	make up to 50μL
Total volume	50μL

Incubating at 30°C for 30 minutes, followed by DNA purification using 1.8 × Ampure magnetic beads to obtain the replicated product, i.e. the available DNA fragments.

4. DNA fragment size detection

The size of the replicated fragments and the original sample was examined using Agilent 2100, and the results showed that the size of the replicated fragments was mainly distributed at about 171 bp (Fig. 9).

Figure 9 shows that the DNA fragment size of sample a1 is mainly distributed at about 171 bp. Figure 10 shows that the fragment size distribution after amplification is about 241 bp. Figure 11 shows that the size of the replication product is mainly distributed at about 171 bp, which is basically the same as expected.

Figure 9 shows that the DNA fragment size of sample a1 is mainly distributed at about 171 bp, which is also the size of the extracted disrupted DNA fragments. Figure 10 shows that the size of the fragments after amplification is mainly distributed at about 241 bp. it is 66 bp longer than the length of sample No. 1 after ligating with the headers and PCR amplification. The expected length of the amplified product is about 237 bp and the real size of the detected fragments was mainly distributed at about 241 bp, which is within the fluctuation range, indicating that the deoxyuracil-labeled primers were successfully introduced during the PCR amplification step, and the PCR product with deoxyuracil labeling was obtained. Figure 11 shows that the size of the

replicated product is mainly distributed at 171 bp, and the fragment size of the replicated product is identical to that of the DNA fragments of the original sample (sample No. 1), indicating that the replicated fragments are the product of the PCR product after successful header cleavage.

The above description shows and describes preferred examples of the present invention. As mentioned above, it should be understood that the present invention is not limited to the forms disclosed herein, and should not be construed as an exclusion of other examples, but may be applied to various other combinations, modifications and environments, and may be altered within the scope of the inventive concepts described herein by the above teachings or techniques or knowledge in related fields. Alterations and variations made by the skilled person in the art without departing from the spirit and the scope of the present invention are intended to be included within the scope of the appended claims of the present invention.

Claims

1. A method of non-specific replication of DNA fragments, including:

Step B: Adding headers to processed DNA fragments that are expected to be replicated to generate ligated DNA fragments with headers;

Step C: Using primers that bind to the headers to amplify the ligated DNA fragments to obtain the PCR products;

Step D: Breaking the PCR products to obtain usable DNA fragments which are substantially similar to the DNA fragments that are expected to be replicated.

2. The method according to claim1, wherein the header contains breaking areas, wherein the breaking areas are located near the connecting point between the header and the processed DNA fragments that are expected to be replicated;

preferably, wherein the breaking areas are cleavage sites; preferably, wherein the cleavage sites contain at least 1bp nucleotide sequence; more preferably, wherein the cleavage sites contains at least one enzyme cutting site or nucleotide sequence with at least one Thymine nucleotide;

preferably, wherein the headers with breaking areas are annealing products of nucleotide sequences such as shown in SEQ ID NO: 1 and SEQ ID NO: 2.

3. The method according to claim 1, wherein the primers contain breaking areas, wherein the breaking areas are located near the 3' terminal of the primers;

preferably, wherein the breaking areas are cleavage sites;

preferably, wherein the cleaving sites contain at least 1bp nucleotide sequence; more preferably, the cleaving sites contains at least one enzyme cutting site or nucleotide sequence with at least one deoxyuridine nucleotide;

more preferably, wherein the primers with breaking areas have a nucleotide sequence such as shown in SEQ ID NO: 3 and SEQ ID NO: 4.

4. The method according claim 1, wherein in said step D the breakings of the PCR product are achieved through reagent cleavage; preferably, wherein the reagent cleavage is performed by cleavage reagents to break the PCR product at least once,

preferably, wherein the cleavage reagents contain at least an enzyme with

cleavage function,

preferably, wherein the enzyme with cleavage function is selected from one or more of the groups consisting of restriction endonucleases, exonucleases, enzymes with deoxyuracil cleavage function and enzymes with single-strand specific cleavage function,

preferably, wherein the enzyme with deoxyuracil cleavage function is the Uracil-DNA Glycosylase or a mixture of Endonuclease VIII and UDG; preferably, wherein the enzyme with single-strand specific cleavage function is the Mung Bean Nuclease and/or S1 Nuclease.

5. The method according to claim 1, wherein the quantity of the DNA fragments expected to be replicated in step B is 0.1 to 100 ng, and the size of the DNA fragments expected to be replicated is 80 to 1000 bp, preferably the size of the DNA fragments expected to be replicated is 100~250bp.

6. The method according to claim 1, comprise the step A-2 performed before the step B: end-repairing the DNA fragments expected to be replicated to obtain processed DNA fragments expected to be replicated; or end-repairing the DNA fragments expected to be replicated and subsequently or simultaneously adding the Adenine nucleotide to the 3' end of the DNA fragments to obtain the processed DNA fragments that are expected to be replicated,

preferably, wherein comprise a step A-1 performed before the step A-2: the sample DNA is disrupted to obtain the DNA fragments that are expected to be replicated.

7. A kind of usable DNA fragments obtained by the method of non-specific replication of the DNA fragments according to any one of claim 1 to 6.

8. A method of DNA library construction, which is used to construct libraries using the usable DNA fragments according to claim 7.

9. The method of DNA library construction according to claim 8, wherein the method includes the following steps performed after the step D:

Step E: End-repairing the usable DNA fragments to receive DNA fragments with blunt ends;

STEP F: Adding the adenine nucleotide to the 3' end of the DNA fragments with blunt ends to obtain the DNA fragments with attached Adenine nucleotides at the 3' end;

STEP G: Adding headers to the DNA fragments with attached Adenine nucleotides at the 3' end to obtain ligated DNA fragments with headers;

STEP H: Amplifying the DNA fragments with adapters by PCR amplification to obtain PCR amplified products to complete the library construction.

10. A kit for non-specific replication of DNA fragments, wherein the kit is used to implement any of the methods according to claims 1-6, including:

adding header reagents, wherein the adding header reagents include headers,

PCR amplification reagents, wherein the PCR amplification reagents include primers, and/or

cleavage reagents, wherein the cleavage reagents include enzymes with cleavage functions;

preferably, wherein the headers are headers contain breaking areas;

preferably, wherein the primers are primers contain breaking areas;

preferably, the cleavage reagents contain at least one enzyme with cleavage function.

11. The kit according to claim 10, wherein the headers is headers contain breaking areas, preferably the breaking areas located near the connecting point between the headers and the processed DNA fragments that are expected to be replicated. More preferably, wherein the headers contain breaking areas are annealing product of nucleotide sequences such as shown in SEQ ID NO: 1 and SEQ ID NO: 2;

wherein the primers contain breaking areas, preferably, wherein the breaking areas are located near the 3' terminal of the primers; more preferably, wherein the primers contain breaking areas have nucleotide sequences such as shown in SEQ ID NO: 3 and SEQ ID NO: 4;

wherein the cleavage reagents consist at least one enzyme with cleavage function.

12. The kit according to claim 11, wherein the breaking areas are cleavage sites;

preferably, wherein the cleavage sites contain at least 1bp nucleotide sequence;

more preferably, the cleavage sites contains at least one enzyme cutting site or nucleotide sequence with at least one Thymine nucleotide.