(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
11 April 2002 (11.04.2002)

PCT

(10) International Publication Number
WO 02/29081 A1

(51) International Patent Classification[7]: C12P 19/34, C12Q 1/68, C07H 21/02, 21/04, C12N 15/00

(21) International Application Number: PCT/US01/42409

(22) International Filing Date: 2 October 2001 (02.10.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/237,378 4 October 2000 (04.10.2000) US

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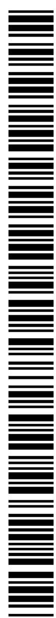

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**
— *with international search report*

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

WO 02/29081 A1

(54) Title: VECTOR FACILITATED SEQUENCE ADDITION

(57) Abstract: A technique is presented that utilizes linear plasmid DNA to add sequence to a previously amplified PCR product. This is accomplished by performing a secondary PCR with secondary primers wherein the secondary primers denote the endpoints of the sequence to be added. The method avoids the need for synthesizing long strands of nucleic with a DNA synthesizer and avoids the need for inefficient steps such as ligation and subcloning.

TITLE OF THE INVENTION

VECTOR FACILITATED SEQUENCE ADDITION

5 BACKGROUND OF THE INVENTION

[0001] The present invention is directed to a method for adding sequence to a polymerase chain reaction (PCR) product. More specifically, the present invention is directed to a technique that utilizes linear plasmid DNA to add sequence to a previously amplified PCR product. According to the present invention, this sequence addition is accomplished by performing a secondary PCR 10 with secondary primers wherein the secondary primers denote the endpoints of the sequence to be added. The method avoids the need for synthesizing long strands of nucleic with a DNA synthesizer and avoids the need for inefficient steps such as ligation and subcloning.

[0002] The publications and other materials used herein to illuminate the background of the invention or provide additional details respecting the practice, are incorporated by reference, and 15 for convenience are respectively grouped in the Bibliography.

[0003] A combination of many different discoveries has made manipulation of DNA routine in research laboratories. The discovery of restriction endonucleases which cleave DNA at specific base sequences, the discovery of plasmids and bacteriophage which can be used as cloning vectors, the development of automated DNA synthesizers and the development of amplification techniques 20 such as the polymerase chain reaction have made it very easy to prepare almost any sequence of DNA desired. Nonetheless, there is still a desire for improved methods. For example, although DNA synthesizers can be used to synthesize a DNA molecule of any desired base sequence, this can be quite expensive for very long molecules and errors in the base sequence are more likely to be present for very long molecules. Methods of using restriction endonucleases to cut out and subclone 25 desired fragments of DNA can overcome the problems associated with using DNA synthesizers, but such methods are limited by the necessity of having appropriate restriction sites at the desired locations.

[0004] Many books and articles have been published concerning techniques of nucleic acid manipulation. General texts include Ausubel et al. (1992), Maniatis et al. (1982), Sambrook et al. 30 (1989), and Glover (1985). One text on polymerase chain reaction (PCR) is by Innis et al. (1990). Several other amplification techniques have been developed since the original PCR method was developed, e.g., strand displacement amplification (Walker et al., 1992; Spargo et al., 1996) to name

just one. These methods are widely known by practitioners throughout the field of molecular biology and related fields.

[0005] It is an object of the present invention to provide an improved method for adding sequence to PCR product.

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SUMMARY OF THE INVENTION

[0006] The present invention discloses a method for adding vector sequence to a nucleic acid of interest. The method can be used to add large segments of nucleic acid from a vector onto a desired DNA fragment such as a fragment obtained by an amplification method. The method does 10 not require the synthesis of long pieces of DNA by an automated DNA synthesizer nor does it depend upon the use of appropriate restriction enzymes at desired locations, nor does it require the cloning of the nucleic acid into a vector or steps of ligation. Instead the method combines one step of adding short fragments of DNA to the nucleic acid of interest with an amplification technique and a second step to amplify the exact desired fragment of the combined vector plus nucleic acid of 15 interest. The result is that the nucleic acid of interest has portions of a vector added to both ends. This is accomplished without a need for cloning or subcloning into a host such as a bacterium.

[0007] This method is applicable to a variety of uses including, but not limited to, cloning a gene from a cDNA library into a vector, cloning a gene from an individual cDNA into a vector, constructing a cDNA library, and moving a library from one vector to a different vector.

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BRIEF DESCRIPTION OF THE FIGURES

[0008] Figures 1A-B illustrate one conceptual method of vector facilitated sequence addition which occurs via 1) filling-in a linearized plasmid, 2) denaturing the filled-in linearized plasmid and annealing with a primary PCR product, 3) extending the free 3' ends of the annealed product, and 25 4) performing PCR with specified primers. The numbers in parentheses above and below the sequences refer to SEQ ID NOs.

[0009] Figures 2A-B illustrate a second conceptual method of vector facilitated sequence addition which occurs via 1) annealing secondary primers with a linearized and denatured plasmid and extending the primers, 2) annealing the extended secondary primers with denatured primary 30 PCR product, 3) extending the ends of the annealed product, 4) denaturing and reannealing, 5) extending the 3' ends and 6) continuing with subsequent rounds of PCR. The numbers in parentheses above and below the sequences refer to SEQ ID NOs.

DETAILED DESCRIPTION OF THE INVENTION

[0010] "Vector Facilitated Sequence Addition" is a technique that utilizes linear DNA, preferably from a vector such as a plasmid, to add sequence (tails) to a previously amplified PCR product of interest. The method need not use plasmid DNA but can utilize any other linear or linearized DNAs (e.g., viral or phage DNA) with appropriate tails which can be made via restriction endonuclease digestion or which can be synthetically added to the DNA. Although one method described below begins with a circular plasmid which is linearized, it is also possible to begin with a linear nucleic acid. Furthermore, the method is applicable to using circular DNA directly without linearizing when said circular DNA encompasses a region which "poisons" amplification of the circular DNA, i.e., amplification of the circular DNA is inhibited because of the sequence of a portion of the DNA. Although not wishing to be bound by any theory, it is believed that often such "poisoned" circular nucleic acids linearly amplify to only a certain distance from each primer and these linearly amplified portions can then act as tails. The following discussion is based upon use with a circular plasmid.

[0011] The following description describes two methods which conceptually are occurring during the reaction process. In fact, a variety of reactions are occurring simultaneously during the reaction process, and the specific reactions described below are only two examples of what is occurring. The Applicants do not wish to be bound by the following description, rather the description is included only as an explanation to understanding some reactions which may be occurring when the physical reaction steps are performed. Whether the described reactions do or do not occur or whether additional, nondescribed reactions occur, is irrelevant to the fact that when the described method steps are followed the desired result is obtained.

[0012] The process is as follows. First a circular plasmid DNA is linearized with restriction endonucleases. Second, the template to be extended with plasmid specific sequence is prepared by PCR. The 5' end of each primer used to amplify the template contains additional sequence which is not homologous to the gene specific target sequence. These 5' ends of the primers are homologous to the linearized plasmid DNA and allow the primary PCR product to be extended in an extension step as part of a secondary polymerase chain reaction. It should be noted that it is irrelevant in which order the above two steps are performed, i.e., the template extension can be performed prior to plasmid linearization. Third, the secondary PCR utilizes the homology between the linearized plasmid DNA and the primary PCR product ends to add a specific plasmid sequence to the primary PCR product. Primers homologous to the plasmid sequence determine the number

of bases added. As PCR proceeds the specific plasmid sequence is added to the primary PCR product in two ways, either directly from the linear plasmid DNA or from the product produced from the linear plasmid during the secondary PCR. The end PCR product is the primary PCR product flanked 5' and 3' by sequence homologous to the plasmid and is suitable for use in homologous recombination cloning methods.

Step 1: Plasmid Linearization

[0013] The plasmid which is to be added in part to a nucleic acid of interest is linearized with restriction endonucleases. A wide variety of plasmids are known, many which include multicloning sites within them. Any desired plasmid can be used with this method, although those with multicloning sites may be simpler to work with because they have been engineered to contain a variety of single sites for a number of restriction endonucleases. In this example, the plasmid GBT.PN2 is digested with restriction enzymes *Eco*RI and *Pst*I. This causes the plasmid to be linearized with one end corresponding to an *Eco*RI recognition site and the other end corresponding to a *Pst*I recognition site. A short piece of DNA between these two restriction enzyme sites is cut out and, if desired, can be separated from the large linear remainder of the plasmid by any technique known to those of skill in the art, e.g., size separation through a molecular sieve or a gel, etc. The two ends of the large linear portion of the plasmid which is to be utilized is shown in part by the following sequences (note that each strand of double-stranded DNA is given its own SEQ ID NO (or SEQ ID NOs if in fragments) throughout this application):

*Eco*RI site

```
5'...CGCAGGAAACAGCTATGA...CCGCCACCATGG 3'      (SEQ ID NOs:1+2)
3'...GCGTCCTTTGTCGATACT...GGCGGTGGTACCTTAA 5'  (SEQ ID NOs:4+3)
```

*Pst*I site

```
    5' GTGAGAGCG...GTCGTGACTGGGAAAACA...3'     (SEQ ID NOs:5+6)
3' ACGTCACTCTCGC...CAGCACTGACCCTTTTGT...5'     (SEQ ID NOs:8+7)
```

The "..." represents additional bases in the sequence which are not shown.

[0014] The restriction endonuclease *Eco*RI generates a 5' overhang that can be filled in with a polymerase. The restriction endonuclease *Pst*I generates a 3' overhang that may not be filled in with a polymerase.

[0015] This linearized plasmid can be denatured to be used in a hybridization and extension reaction as part of a polymerase chain reaction with the DNA of interest as detailed in Step 3 below.

Step 2: Primary PCR Product Preparation

[0016] The nucleic acid of interest or target (e.g., a gene or gene fragment) is amplified using PCR techniques (see, e.g., U.S. Patent Nos. 4,683,195 and 4,683,202 and Innis et al., 1990). Modified specific primers are used to amplify the nucleic acid of interest or target. These modified primers possess additional sequence on their 5' ends homologous to the linear plasmid DNA of Step 1 above. These primer ends generate the homologous bases needed to add long tails to the primary PCR product from the linear plasmid in a secondary PCR.

[0017] The sequences of the primary PCR primers are:

FORWARD 5' CCACCATGGAATTA "TARGET" 3' (SEQ ID NO:9)

REVERSE 5' CGCTCTCACTGCA "TARGET" 3' (SEQ ID NO:8)

"TARGET" represents the target sequence of the nucleic acid to be amplified and cloned can be a gene, gene fragment, probe, primer or any desired nucleic acid sequence.

[0018] The forward and reverse primers are mixed with the nucleic acid of interest (TARGET) and a polymerase chain reaction or other amplification technique is performed by methods well known to those of skill in the art. The product which results is an amplified nucleic acid which consists of the original nucleic acid of interest with a tail at each end. The product is represented by:

`5' CCACCATGGAATTA--TARGET--TGCAGTGAGAGCG 3'` (SEQ ID NOs:9 + 10)

`3' GGTGGTACCTTAAT--TARGET'--ACGTCACTCTCGC 5'` (SEQ ID NOs:11 + 8)

where the term "TARGET" or "TARGET'"represents the nucleic acid of interest. This product is referred to as the primary PCR product.

[0019] The tail at the left end as represented is complementary to the filled-in *Eco*RI end of the linearized plasmid of Step 1 except that an extra "A:T" base pair is present abutting the TARGET. The reason for this additional base pair is that often upon filling in the overhang left by digestion with *Eco*RI an additional A can be added if a nonproofreading DNA polymerase is used.

By including the A:T basepair, the additional A, if added in the fill-in reaction, is automatically taken into account and all products are identical.

[0020] The tail at the right end as represented above is complementary to the *Pst*I end of the linearized plasmid of Step 1. The resulting amplified nucleic acid (the primary PCR product), upon denaturation, can renature with denatured linearized plasmid of Step 1. This is done in Step 3 described below.

Step 3: Addition of Vector Sequence to Primary PCR Product

[0021] The primary PCR product of Step 2 is denatured and mixed with linearized and denatured plasmid from Step 1 (the two DNAs may be mixed first and then denatured simultaneously). A secondary PCR will extend the 3' ends of the primary PCR product where they are homologous to the linear plasmid. Linear plasmid facilitates the extension of the primary PCR product in at least two ways. Two different methods are set forth below for mixing the products of Steps 1 and 2 to form a template for a final PCR to produce the desired nucleic acid of interest with a portion of the vector added to it.

A) Method One

[0022] In method one, the two ends of one strand of the linear plasmid are homologous to the two ends of one strand of the primary PCR product, the two ends of the second strand of the linear plasmid are homologous to the two ends of the second strand of the primary PCR product, and the desired sequence is added directly from the linear plasmid DNA. Long plasmid specific tails can be added to the primary PCR product through the homology found between the linear plasmid and the primary PCR products' ends. The amplified primary PCR product of Step 2 is denatured and mixed with denatured plasmid of Step 1 (or the two are mixed and simultaneously denatured). The ends of one strand of the primary PCR product of Step 2 can hybridize with the two free ends of one strand of the linearized plasmid of Step 1. At this step (following step B of Figure 1A) the plasmid is mostly single-stranded with double-stranded portions only occurring where the ends of the plasmid hybridize with the ends of the nucleic acid of interest, i.e., in the regions of the *Eco*RI and *Pst*I restriction enzyme sites. This partially single-stranded nucleic acid can be extended from both 3' ends to produce a double-stranded nucleic acid comprising the nucleic acid of interest (TARGET) between the *Eco*RI and *Pst*I sites.

[0023] The above process is illustrated in Figures 1A-B. In these Figures the linearized plasmid is shown at the top of the figure. The arrows at each end indicate that the strand continues and in fact, as shown, the 5' end of the top strand is shown on the right-hand side while the 3' end of the same strand is shown on the left-hand side. This is to represent the fact that a circular plasmid has been cut and is now linear. The "..." indicates additional DNA sequence but which sequence is not explicitly set out. Both strands of nucleic acid from the nucleic acid of interest will be used in this process with each one hybridizing to its complementary strand of the denatured plasmid.

[0024] All of the following steps as shown in Figures 1A-B can be performed as part of a single polymerase chain reaction and will all be occurring concurrently once the reaction has gone through the initial round. A polymerase chain reaction is performed as a series of cycles with each cycle including the steps of denaturation, reannealing and extension. However, for illustrative purposes, the various steps are shown as if they are separate steps being performed sequentially.

[0025] Step (i) of Figure 1A is a fill-in (or extension) reaction to fill in the 5' overhang of the *Eco*RI site in the linearized plasmid. If a nonproofreading DNA polymerase is used an additional A (shown in parentheses) may be added.

[0026] Step (ii) of Figure 1A includes the steps of denaturing the linearized and filled-in plasmid and denaturing the primary PCR product and reannealing the two. This step shows the "top" strand of the plasmid hybridizing with its complementary strand of the primary PCR product ("bottom" strand) and the "bottom" strand of the plasmid hybridizing with its complementary strand ("top" strand) of the primary PCR product. As shown, the single-stranded primary PCR product hybridizes to both the *Eco*RI end and the *Pst*I end of the single-stranded, linearized plasmid. Step (iii) is the extension of the free 3' ends thereby resulting in a nucleic acid which is blunt-ended at a definitive point on one side while continuing to an indefinite stop within the plasmid sequence (the stopping point of each individual molecule depending upon the fidelity of the enzymes involved) on the other end, with "TARGET" incorporated between the *Eco*RI and the *Pst*I sites.

[0027] Step (iv) is a standard PCR reaction utilizing the products of step (iii) and the two primers shown. Complementary strands of each of the two products of step (iii), specifically the strands labeled as "Plasmid top strand" and "Plasmid bottom strand" at the top of Figure 1B, hybridize with each other to form a product which acts as a template for the two primers (SEQ ID NOs:1 and 7) to allow exponential amplification of this product. Other strands may also hybridize but will not lead to exponential amplification and these are not illustrated. The primers are complementary to a portion of the linearized plasmid. By "portion" is meant a continuous stretch

of bases of length equal to or approximately equal to the length of the primer which is complementary to said portion, e.g., if a 15 base primer is used then "portion" can refer to 15 bases of the plasmid which are complementary to the primer or to any part of the plasmid to which the primer will specifically hybridize under the PCR annealing conditions. The resulting product (the secondary PCR product) is the amplified PCR product shown at the bottom of Figure 1B. Further rounds of PCR continue to amplify this final product. The primers used in step (iv) can be selected to give the exact ends desired from the plasmid. The final product is a linear nucleic acid comprising the nucleic acid of interest (TARGET) flanked on each side by a segment of plasmid.

B) Method Two

[0028] The second method of producing the final product differs conceptually from the first method described above. Again a primary PCR product is prepared as in method one and again the secondary plasmid specific primers are:

FORWARD 5' CGCAGGAAACAGCTATGA 3' (SEQ ID NO:1) and

REVERSE 5' TGTTTTCCCAGTCACGAC 3' (SEQ ID NO:7)

These primers are homologous to the plasmid in the regions indicated by lowercase letters:

*Eco*RI site

```
5'...cgcaggaaacagctatga...CCGCCACCATGG 3'        (SEQ ID NOs:1+2)
3'...gcgtcctttgtcgatact...GGCGGTGGTACCTTAA 5'    (SEQ ID NOs:4+3)
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*Pst*I site

```
   5' GTGAGAGCG...gtcgtgactgggaaaaca...3'        (SEQ ID NOs:5+6)
3' ACGTCACTCTCGC...cagcactgacccttttgt...5'       (SEQ ID NOs:8+7)
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[0029] In this second method, the plasmid specific primers generate a plasmid specific product from the linear plasmid DNA. The 3' ends of the plasmid specific PCR products are homologous to the 3' extendable ends of the primary PCR product. Therefore, the plasmid specific product serves as a template for the addition of a long plasmid specific tail to the primary PCR product. The plasmid itself or an elongated primer resulting from annealing and extension of the secondary primers during the PCR can hybridize with the denatured primary PCR product. Figures 2A-B illustrate the case using elongated secondary PCR primers. As with Figures 1A-B, all

reactions shown in Figures 2A-B can be occurring concurrently within a single PCR but for explanatory purposes are shown as individual sequential steps.

[0030] The top of Figure 2A again shows the linearized plasmid. Upon denaturation of the plasmid and annealing with secondary PCR primers (step (i)), the primers anneal to the plasmid and are extended to the ends of the *Eco*RI site and the *Pst*I site by DNA polymerase which is present in the PCR reaction. Step (i) can be considered to be a linear amplification step. In step (ii), upon denaturation and reannealing, the elongated secondary PCR primers can anneal with the denatured primary PCR product to form the two products shown. The 3' ends are extended in step (iii) to yield the two distinct products shown. Each of these effectively is the primary PCR product with one of the two elongated secondary primers added in its double-stranded form. In step (iv), another round of denaturation and annealing occurs. Several products are possible. The two products shown resulting from step (iii) can be reformed (these are not shown following step (iv)) but these could not be exponentially amplified by the primers which are present if they were in isolation from each other. Two other possible products in this round are those shown after step (iv). The upper product cannot be elongated but the lower product can be elongated as shown by step (v). The resulting product is the primary PCR product with both elongated secondary PCR primers added, one at each end. Successive rounds of PCR (step (vi)) with this product and the secondary PCR primers result in exponential amplification of the product shown resulting from step (v). This is the secondary PCR product and it is identical to the product obtained by method 1 as shown in Figure 1B.

[0031] As noted above, other reactions are simultaneously occurring with those shown in Figures 2A-B. Rather than annealing with elongated secondary PCR primer, the primary PCR product can anneal with one end of the linearized plasmid. The end result from PCR will be identical as that shown in Figures 2A-B. Those of skill in the art can easily work through the steps to prove this point. It is also possible for a linearized plasmid to anneal its *Eco*RI tail to one primary PCR product and its *Pst*I tail to a second primary PCR product or to even form concatemers. Once again, the final exponentially amplified product of PCR will be identical to that shown in Figures 2A-B. All such reactions are in fact occurring simultaneously in a single tube. Furthermore, although the two "methods" described above are described as though they are separate methods, in fact they are occurring simultaneously within a single test tube.

[0032] The Figures illustrate that regardless of the exact steps which occur, a single desired exponentially amplified product is obtained. As those of skill in the art recognize, other products will also be formed during the PCR, but the majority product formed is the desired product as

outlined above and this can be purified from other products which may form by gel electrophoresis or other desired means. If desired, more than one nucleic acid of interest and/or more than one plasmid could be used simultaneously to yield a mixture of products, for example the method can be used to create cDNA libraries or to transfer libraries from one vector to another vector.

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EXAMPLE

[0033] The present invention is further detailed in the following Example, which is offered by way of illustration and is not intended to limit the invention in any manner. Standard techniques well known in the art or the techniques specifically described below are utilized.

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EXAMPLE 1

<u>Vector Facilitated Sequence Addition</u>

[0034] Step 1: Circular plasmid GBT.PN2 is linearized with *Eco*RI and *Pst*I according to manufacturers' instructions.

15 [0035] Step 2: In Step 2, tails homologous to the *Eco*RI and *Pst*I tails of the linearized plasmid are added to the gene of interest or target by a primary PCR reaction. For a nucleic acid of interest of sequence:

5' ACGATAGAGACTTTAGAAGCCCGGTTGGACCGCGTAGACGAGTC 3' (SEQ ID NO:12)

3' TGCTATCTCTGAAATCTTCGGGCCAACCTGGCGCATCTGCTCAG 5' (SEQ ID NO:13)

20 the primers for the primary PCR are:

5' ccaccatggaattaACGATAGAGACTT 3' (SEQ ID NO:14) and

5' cgctctcactgcaGACTCGTCTACGC 3' (SEQ ID NO:15),

where the lower case letters indicate added tails and the upper case letters indicate the nucleic acid of interest or "TARGET". Performing PCR using any set of conditions well known to those of skill

25 in the art (see, e.g., U.S. Patent Nos. 4,683,195 and 4,683,202 and Innis et al., 1990) yields the primary PCR product of SEQ ID NOs:16 and 17:

5' ccaccatggaattaACGATAGAGACTTTAGAAGCCCGGTTGGACCGCG (cont'd)

3' ggtggtaccttaatTGCTATCTCTGAAATCTTCGGGCCAACCTGGCGC (cont'd)

TAGACGAGTCtgcagtgagagcg 3' (SEQ ID NO:16)

30 ATCTGCTCAGacgtcactctcgc 5' (SEQ ID NO:17)

[0036] Step 3: In this step a secondary PCR is performed. The secondary primers are used at 0.4 µM, and the dNTP mix is 0.2 mM of each dNTP. Twelve nanograms of GBT.PN2 is used per 12 µL reaction. The primary PCR product is diluted 20 fold in the final secondary PCR reaction (i.e., if a 20 µL secondary PCR were being performed then it would contain 1 µL of the primary
5 PCR product of step 2). The buffer consists of 10% (w:v) sucrose, 0.5 mM tartrazine, 10 mM Tris·HCl, pH 8.3, 50 mM KCl, 5 mM $MgCl_2$, and 0.01% (v:v) Tween 20 plus the primers, dNTPs and plasmid as noted above. Also, DNA polymerase is present as a mixture of two polymerases, these being 0.38 units of Amplitaq Gold polymerase (Perkin-Elmer) plus 0.008 units of cloned PFU polymerase (Stratagene). The PCR conditions are an initial denaturation at 95°C for 10 minutes
10 followed by 35 cycles of 95°C for 20 seconds, 54°C for 30 seconds and 72°C for 4 minutes. The final cycle is followed by holding at 72°C for an additional 5 minutes and then the samples are placed at 10°C. During this secondary PCR all of the steps shown by Figures 1A-B and 2A-B occur and produce the desired product. Using secondary primers of:

5' CGCAGGAAACAGCTATGA 3' SEQ ID NO:1

15 5' TGTTTTCCCAGTCACGAC 3' SEQ I D NO:7

will yield the final desired amplified molecule:

```
         (1)                                (16)
5' cgcaggaaacagctatga...ccaccatggaattaACGATAGAGACTTTAGAAGCCC
3' gcgtcctttgtcgatact...ggtggtaccttaatTGCTATCTCTGAAATCTTCGGG
         (4)                                (17)

         (16 cont'd)                        (6)
GGTTGGACCGCGTAGACGAGTCtgcagtgagagcg...gtcgtgactgggaaaaca 3'
CCAACCTGGCGCATCTGCTCAGacgtcactctcgc...cagcactgacccttttgt 5'
         (17 cont'd)                        (7)
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[0037] While the invention has been disclosed in this patent application by reference to the details of preferred embodiments of the invention, it is to be understood that the disclosure is intended in an illustrative rather than in a limiting sense, as it is contemplated that modifications will
30 readily occur to those skilled in the art, within the spirit of the invention and the scope of the appended claims.

BIBLIOGRAPHY

Ausubel FM, et al. (1992). Current Protocols in Molecular Biology, (John Wiley and Sons, New York, New York).

Glover, D (1985). DNA Cloning, **I** and **II** (Oxford Press).

5 Innis MA, et al. (1990). PCR Protocols: A Guide to Methods and Applications (Academic Press, San Diego).

Maniatis T, et al. (1982). Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.).

Sambrook J, et al. (1989). Molecular Cloning: A Laboratory Manual, 2nd Ed. (Cold Spring
10 Harbor Laboratory, Cold Spring Harbor, N.Y.).

Spargo CA, et al. (1996). "Detection of M. tuberculosis DNA using thermophilic strand displacement amplification." *Mol. Cell. Probes* **10**:247-256.

Walker GT, et al. (1992). "Strand displacement amplification - an isothermal, in vitro DNA amplification technique." *Nucl. Acids Res.* **20**:1691-1696.

15 U.S. Patent No. 4,683,195

U.S. Patent No. 4,683,202

WHAT IS CLAIMED IS:

1. A method for adding portions of a circular nucleic acid to ends of a nucleic acid of interest comprising the steps of:
(a) linearizing said circular nucleic acid to form a linear nucleic acid;
5 (b) adding tails to said nucleic acid of interest to form a tailed nucleic acid of interest, wherein said tails are complementary to said circular nucleic acid; and
(c) performing an amplification reaction comprising combining said linear nucleic acid, said tailed nucleic acid of interest and a pair of primers wherein said primers are complementary to a portion of said circular nucleic acid.

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2. The method of claim 1 wherein step (c) is a polymerase chain reaction.

3. The method of claim 1 wherein said linearizing is performed with two restriction endonucleases.

15

4. The method of claim 1 wherein said tails are complementary to ends of said linear nucleic acid.

5. The method of claim 2 wherein two DNA polymerases are present.

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6. The method of claim 1 wherein said nucleic acid of interest comprises more than one sequence of nucleic acid.

7. The method of claim 1 wherein said method is used to create a DNA library.

25

8. A method for adding portions of a first nucleic acid, wherein said first nucleic acid is linear, to ends of a second nucleic acid wherein said second nucleic acid is a nucleic acid of interest comprising the steps of:
(a) digesting said first nucleic acid of interest with one or more restriction endonucleases to
30 produce a digested nucleic acid;
(b) adding tails to said second nucleic acid to form a tailed second nucleic acid, wherein said tails are complementary to said plasmid; and

(c) performing an amplification reaction comprising combining said digested nucleic acid, said tailed second nucleic acid and a pair of primers wherein said primers are complementary to a portion of said first nucleic acid.

9. The method of claim 8 wherein step (c) is a polymerase chain reaction.

10. The method of claim 8 wherein said linearizing is performed with two restriction endonucleases.

11. The method of claim 8 wherein said tails are complementary to ends of said digested nucleic acid.

12. The method of claim 9 wherein two DNA polymerases are present.

13. The method of claim 8 wherein said nucleic acid of interest comprises more than one sequence of nucleic acid.

14. The method of claim 8 wherein said method is used to create a DNA library.

15. A method for adding portions of a circular nucleic acid to ends of a nucleic acid of interest comprising the steps of:

(a) adding tails to said nucleic acid of interest to form a tailed nucleic acid of interest, wherein said tails are complementary to said circular nucleic acid;

(b) performing an amplification reaction comprising combining said circular nucleic acid, said tailed nucleic acid of interest and a pair of primers wherein said primers are complementary to a portion of said circular nucleic acid.

16. The method of claim 15 wherein two DNA polymerases are present.

17. The method of claim 15 wherein said nucleic acid of interest comprises more than one sequence of nucleic acid.

18. The method of claim 15 wherein said method is used to create a DNA library.

19. The method of claim 15 wherein said circular nucleic acid is recent to exponential amplification by polymerase chain reaction.

```
            (1)                 (2)                          (5)          (6)
← CGCAGGAAACAGCTATGA...CCGCCACCATGG 3'            5' GTGAGAGCG...GTCGTGACTGGGAAAACA →
← GCGTCCTTTGTCGATACT...GGCGGTGGTACCTTAA 5'    3' ACGTCACTCTCGC...CAGCACTGACCCTTTTGT →
            (4)                 (3)                          (8)          (7)

                          (i)  ↓  Fill-in

            (1)                 (18)                         (5)          (6)
← CGCAGGAAACAGCTATGA...CCGCCACCATGGAATT(A) 3'     5' GTGAGAGCG...GTCGTGACTGGGAAAACA →
← GCGTCCTTTGTCGATACT...GGCGGTGGTACCTTAA 5'    3' ACGTCACTCTCGC...CAGCACTGACCCTTTTGT →
            (4)                 (3)                          (8)          (7)

                          (ii)  ↓  Denature and anneal with Primary PCR Product

            (1)                 (18)                         (5)          (6)
←CGCAGGAAACAGCTATGA...CCGCCACCATGGAATT(A) 3'    5' GTGAGAGCG...GTCGTGACTGGGAAAACA → (Plasmid top)
                     3' GGTGGTACCTTAAT-TARGET-ACGTCACTCTCGC 5' (Primary transcript bottom strand)
                                (11)                     (8)

                       (9)                          (10)
                      5' CCACCATGGAATTA-TARGET-TGCAGTGAGAGCG 3' (Primary transcript top strand)
← GCGTCCTTTGTCGATACT...GGCGGTGGTACCTTAA 5'   3' ACGTCACTCTCGC...CAGCACTGACCCTTTTGT →(Plasmid bottom)
        (4)                (3)                          (8)          (7)

                     (iii)  ↓  Extend free 3' ends
```

FIGURE 1A

```
       (1)                  (18)                     (10)
← CGCAGGAAACAGCTATGA...CCGCCACCATGGAATTA-TARGET-TGCAGTGAGAGCG 3'  (Plasmid top strand)
← GCGTCCTTTGTCGATACT...GGCGGTGGTACCTTAAT-TARGET-ACGTCACTCTCGC 5'  (Primary transcript bottom strand)
       (4)                  (19)                     (8)

             (9)                 (10)           (6)
     CCACCATGGAATTA-TARGET-TGCAGTGAGAGCG...GTCGTGACTGGGAAAACA →  (Primary transcript top strand)
     GGTGGTACCTTAAT-TARGET-ACGTCACTCTCGC...CAGCACTGACCCTTTTGT →  (Plasmid bottom strand)
            (11)                 (8)            (7)

                        (iv) ↓  Perform PCR with 5'CGCAGGAAACAGCTATGA3' (SEQ ID
                                NO:1) and 5' TGTTTTCCCAGTCACGAC 3' (SEQ ID NO:7)

       (1)                  (18)                     (10)          (6)
5' CGCAGGAAACAGCTATGA...CCGCCACCATGGAATTA-TARGET-TGCAGTGAGAGCG...GTCGTGACTGGGAAAACA 3'
3' GCGTCCTTTGTCGATACT...GGCGGTGGTACCTTAAT-TARGET-ACGTCACTCTCGC...CAGCACTGACCCTTTTGT 5'
       (4)                  (19)                     (8)           (7)
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FIGURE 1B

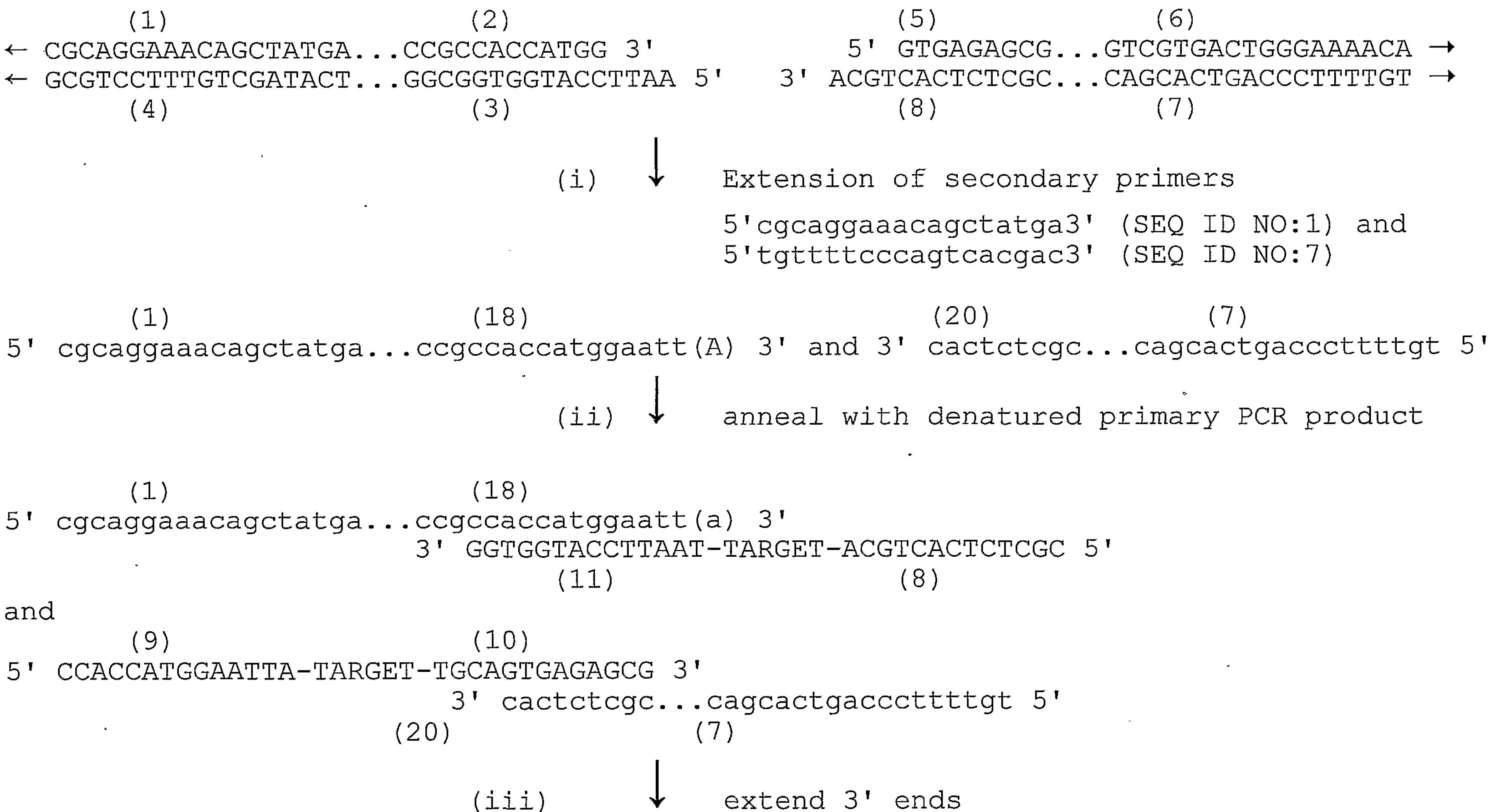


Figure 2A

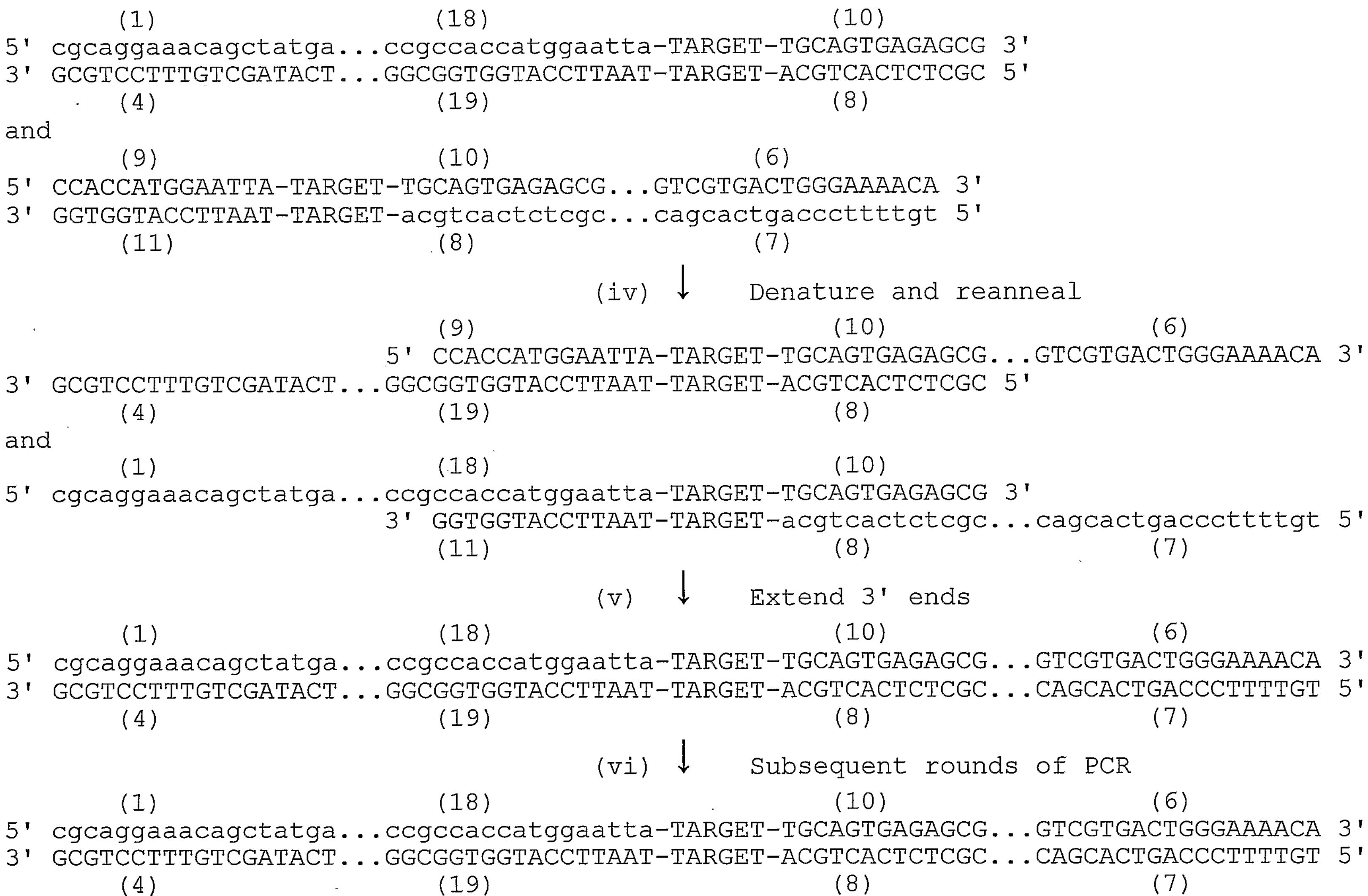


FIGURE 2B

SEQUENCE LISTING

<110> Myriad Genetics, Inc.
McBride, Celeste
Oliphant, Arnold R

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# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/42409

**A. CLASSIFICATION OF SUBJECT MATTER**
IPC(7) : C12P 19/34; C12Q 1/68; C07H, 21/02, 21/04; C12N, 15/00
US CL : 435/6, 91.2
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/6, 91.2, 194, 536/23.1, 23.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | WALKER et al., Strand displacement amplification-an isothermal, in vitro DNA amplification technique, Nucleic Acids Research, April 1992, Vol 20, No. 7, pages 1691-1696, see entire document. | 1-19 |
| Y | NOTOMI et al., Loop-mediated isothermal amplification of DNA, Nucleic Acids Research, June 2000, Vol 28, No. 12, pages e63, see entire document. | 1-19 |
| Y | US 5,856,144 A (MEIRENDORF et al.) 5 January 1999, (05.01.1999), see entire document. | 1-19 |
| Y | US 5,580,759 A (YANG et al.) 3 December 1996 (03.12.1996), see entire document. | 1-19 |
| A | US 5,989,872 A (LUO et al.) 23 November 1999 (23.11.1999), see entire document. | 1-19 |

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 11 January 2002 (11.01.2002) | 18 JAN 2002 |

| Name and mailing address of the ISA/US | Authorized officer |
|---|---|
| Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231 | Ponnathapy Achutamurthy |
| Facsimile No. (703)305-3230 | Telephone No. (703) 308-0196 |

**Continuation of B. FIELDS SEARCHED Item 3:**
EAST, MEDLINE, CAPLUS, BIOSIS, EMBASE, JAPIO, PATOSWO, PATOSEP
search terms: nucleic acid, modification, vector facilitated, sequence addition, polymerase chain reaction, amplification, sequence extension, addition of sequence