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Gupte et al.

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[45] **Date of Patent: Jul. 11, 2000**

[54] **METHOD FOR SEQUENCING BOTH STRANDS OF A DOUBLE STRANDED DNA IN A SINGLE SEQUENCING REACTION**

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[21] Appl. No.: **08/925,277**

[22] Filed: **Sep. 8, 1997**

[51] **Int. Cl.**[7] **C12P 19/34**; C07H 21/04

[52] **U.S. Cl.** **435/6**; 435/91.2; 536/24.3; 536/24.33; 536/26.6

[58] **Field of Search** 536/24.33, 24.3, 536/26.6; 435/91.2, 6

[56] **References Cited**

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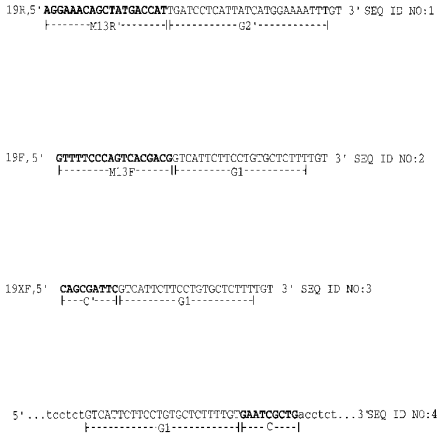
Attorney, Agent, or Firm—Rothwell, Figg, Ernst & Kurz PC

[57] **ABSTRACT**

A method is presented which uses a unique opposite strand joining strategy during PCR of an original DNA to generate a product which, when sequenced with a single sequencing primer yields the sequence of both strands of the original DNA. The PCR primers include 1) a modified oligomer corresponding to the 5' end of a first strand of the DNA to be amplified wherein said modified oligomer includes the reverse complementary sequence to a sequence within said first strand of DNA and a specific PCR priming sequence which will specifically hybridize to a portion of the DNA to be amplified and 2) a second oligomer corresponding to the 5' end of the second strand of the DNA to be amplified and which contains the priming sequence for the second strand of the DNA and will specifically hybridize to a portion of the DNA to be amplified. During PCR an intermediate product is formed where one end of one strand loops around to hybridize to its complement on the same strand. This results in a hairpin structure which elongates using its own strand as a template to form a double sized product that contains the sequence of both original strands. Upon denaturation this yields single strands with the single strands having the sequence of both of the original strands included in tandem. Sequencing these single strands using a single primer, e.g., a primer complementary to the second oligomer, yields the sequences of both strands of the DNA of interest.

6 Claims, 3 Drawing Sheets



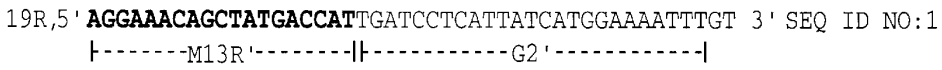


FIG. 1A

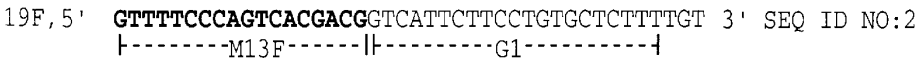


FIG. 1B

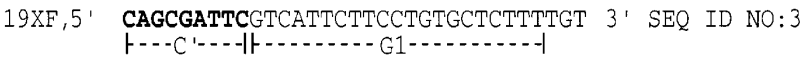


FIG. 1C

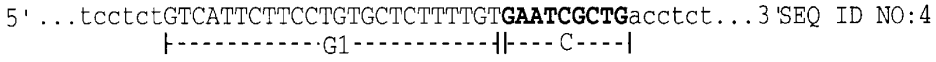


FIG. 1D

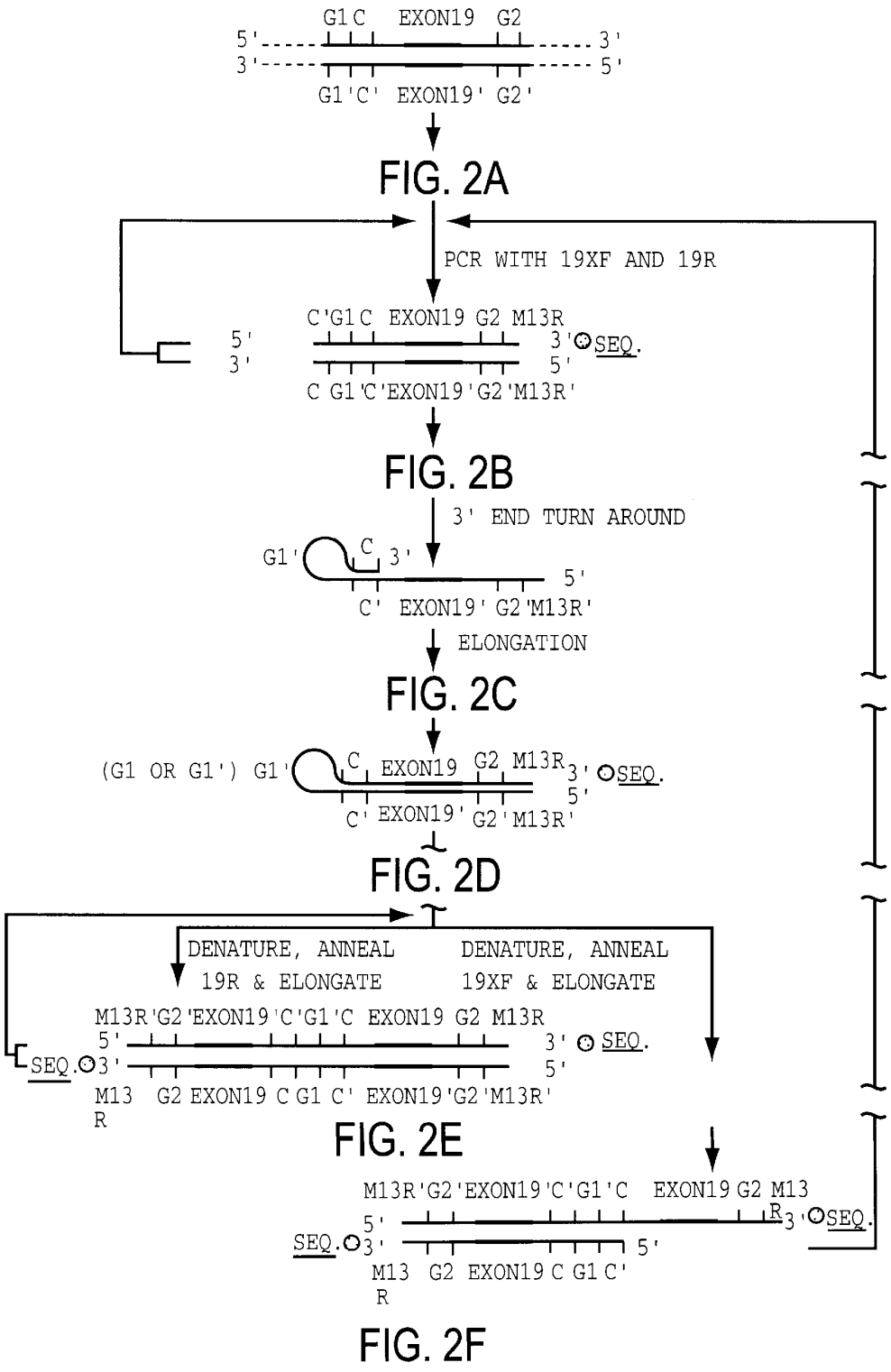


FIG. 2A

FIG. 2B

FIG. 2C

FIG. 2D

FIG. 2E

FIG. 2F

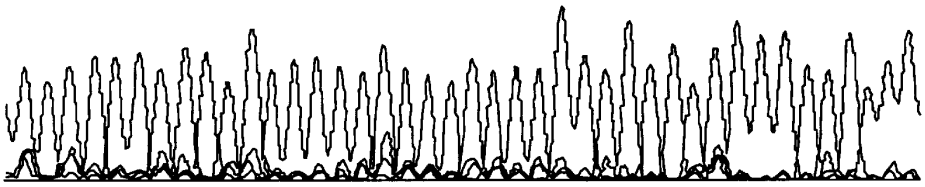


FIG. 3A

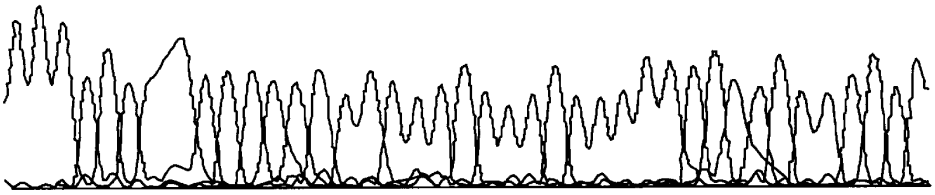


FIG. 3B

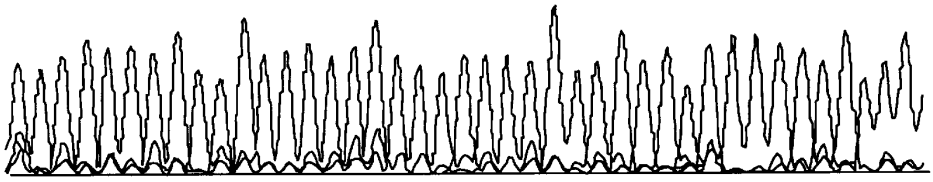


FIG. 3C

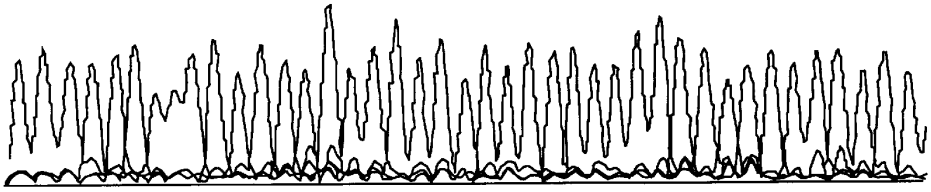


FIG. 3D

METHOD FOR SEQUENCING BOTH STRANDS OF A DOUBLE STRANDED DNA IN A SINGLE SEQUENCING REACTION

BACKGROUND OF THE INVENTION

Sequencing of nucleic acids is an extremely important and widely used technique. It is used for a variety of purposes. One such purpose is to identify whether mutations within genes of known sequence are present in a sample of DNA taken from a person. This is especially important in diagnosing whether the person may have a disease which is known to be associated with specific mutations in the gene being analyzed. When this type of testing is performed, it is common to sequence both strands of DNA to minimize any errors which may occur in the sequencing. To date, when sequencing both strands by the Sanger dideoxy method there has been a requirement to use one primer to sequence the sense strand and a second primer to sequence the antisense strand of the double-stranded DNA. The two strands have been sequenced in separate sets of reactions. The present invention is a technique by which both strands of DNA are sequenced in a single set of reactions using only a single primer. This method allows one to use fewer reactions for obtaining the data. This is especially important for laboratories which will be processing many samples. The use of fewer reactions will decrease the cost of analysis.

DNA sequencing methods were developed during the 1970s by Maxam and Gilbert (1977) and by Sanger (1977). The Sanger method which uses dideoxy nucleotides to terminate newly synthesized DNA strands is most commonly used and has been adapted such that it can be used with fluorescent markers rather than radioactivity. One variation is a technique called cycle sequencing in which DNA sequencing is combined with polymerase chain reaction (PCR). Chadwick et al. (1996) teach a variation of cycle sequencing in which a mutant Taq DNA polymerase is utilized.

The polymerase chain reaction itself is only one of a number of different methods now available for amplifying nucleic acids. Some of the other methods include ligase chain reaction (Wu and Wallace, 1989), Strand Displacement Amplification (SDA) (Walker, U.S. Pat. No. 5,455,166 (1995); Walker et al., 1992), thermophilic SDA (Spargo et al., 1996), and 3SR or NASBA (Compton, 1991; Fahy et al., 1991).

The instant invention is a method of using a specially designed oligomer which contains a reverse complement sequence along with a standard primer during PCR. This generates a double stranded DNA product such that when it is denatured one end of the resulting single stranded DNA loops around to form an intrastrand stem-loop structure. This structure is then elongated thereby producing a double-stranded DNA but wherein the two strands are joined by a loop. This method is referred to as opposite strand joining PCR. When denatured this product forms a single-stranded DNA which contains both strands of the original DNA. When this resulting single-stranded DNA is sequenced it yields the sequence of both strands of the original double-stranded DNA.

A similar stem-loop DNA structure was used as a template for PCR amplification by Jones et al. (1992). The Jones et al. reference describes a "panhandle PCR" method. This technique introduced a self-complementary portion into the target DNA strand by ligation. The goal of panhandle PCR is to amplify unknown sequence by generating a stem loop template structure for PCR whereas one of the goals of opposite strand joining PCR is to amplify known sequence by generating a stem-loop structure during PCR and then sequencing both strands of the longer product in one sequencing reaction. Another use for opposite strand joining PCR is in denaturing gradient gel electrophoresis techniques wherein the use of this technique can form a covalently bonded hairpin loop which can replace the use of a GC clamp. Yet another use for opposite strand joining PCR is simply the use of the method effectively to join together the two strands of any double stranded DNA into a single strand of DNA for any desired purpose.

SUMMARY OF THE INVENTION

A method is presented which uses a unique opposite strand joining strategy during PCR of an original DNA to generate a product which, when sequenced with a single sequencing primer yields the sequence of both strands of the original DNA. The PCR primers include 1) a modified oligomer corresponding to the 5' end of a first strand of the DNA to be amplified wherein said modified oligomer includes the reverse complementary sequence to a sequence within said first strand of DNA and a specific PCR priming sequence which will specifically hybridize to a portion of the DNA to be amplified and 2) a second oligomer corresponding to the 5' end of the second strand of the DNA to be amplified and which contains the priming sequence for the second strand of the DNA and will specifically hybridize to a portion of the DNA to be amplified. During PCR an intermediate product is formed where one end of one strand loops around to hybridize to its complement on the same strand. This results in a hairpin structure which elongates using its own strand as a template to form a double sized product that contains the sequence of both original strands. Upon denaturation this yields a single strand having the sequence of both of the original strands included in tandem. Sequencing these single strands using a single primer, e.g., a primer complementary to the second oligomer, yields the sequences of both strands of the DNA of interest.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. **1**A–**1**D show the primer design used in the Example. FIG. **1**A shows the sequence of primer **19**R which consists of the −28M13 reverse DET primer sequence (shown in bold) which is 5' to the gene specific sequence G**2**'. FIG. **1**B shows the sequence of primer **19**F which consists of the −40M13 forward DET sequence (shown in bold) which is 5' to the gene specific sequence G**1**. FIG. **1**C shows the sequence of the opposite strand joining primer **19**XF which consists of a short reverse complemented genomic sequence C' (shown in bold) which is 5' to the gene specific sequence G**1** used in primer **19**F. FIG. **1**D shows the genomic sequence in the region of the opposite strand joining primer. The gene specific sequence G**1** (shown in nonbolded upper case letters) used in both the **19**F and **19**XF primers is 5' of sequence C (shown in bold upper case letters). It is this genomic region C which is reverse complemented (and therefore called C') and placed 5' to the gene specific sequence G**1** in the opposite strand joining primer **19**XF.

FIGS. **2**A–**2**F illustrate the opposite strand joining strategy. Throughout these figures, all the strands labeled •SEQ are substrates for dye primer sequencing.

FIG. **2**A shows genomic DNA in the region of exon **19**. This is shown as four sections on each strand with one strand having G**1**, C, the exon **19** containing region, and G**2** and the opposite strand being designated with primes, e.g., G**1**', C',

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