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Cheeseman

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[54] **METHOD FOR SEQUENCING POLYNUCLEOTIDES**

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Related U.S. Application Data

[63] Continuation of Ser. No. 393,586, Aug. 14, 1989, abandoned.

[51] **Int. Cl.**[5] **C12Q 1/68**; C12P 19/34; C07H 21/04

[52] **U.S. Cl.** **435/6**; 536/24.33; 536/25.3; 935/77; 935/78

[58] **Field of Search** 435/6, 91; 536/27, 25.3, 536/24.33; 935/77, 78

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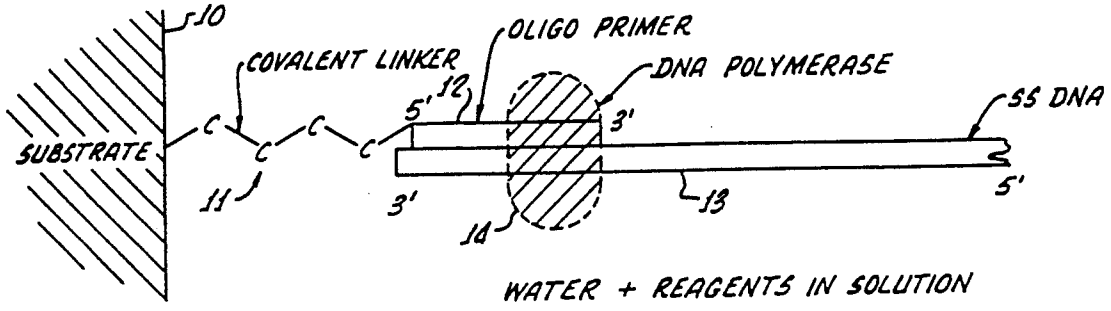
[57] **ABSTRACT**

A method is provided for determining the sequence of nucleotides on a single strand DNA molecule. The single strand DNA molecule is attached to a leader oligonucleotide and its complementary strand to a solid state support. Fluorescently-labeled 3'-blocked nucleotide triphosphates, with each of the bases A, G, C, T having a different fluorescent label, are mixed with the bound DNA molecule in the presence of DNA polymerase. The DNA polymerase causes selective addition of only the complementary labeled NTP, thus identifying the next unpaired base in the unknown DNA strand. The 3'-blocking group is then removed, setting the system up for the next NTP addition and so on. The sequence is repeated until no more fluorescently-labeled NTPs can be detected as being added by the polymerase.

10 Claims, 2 Drawing Sheets



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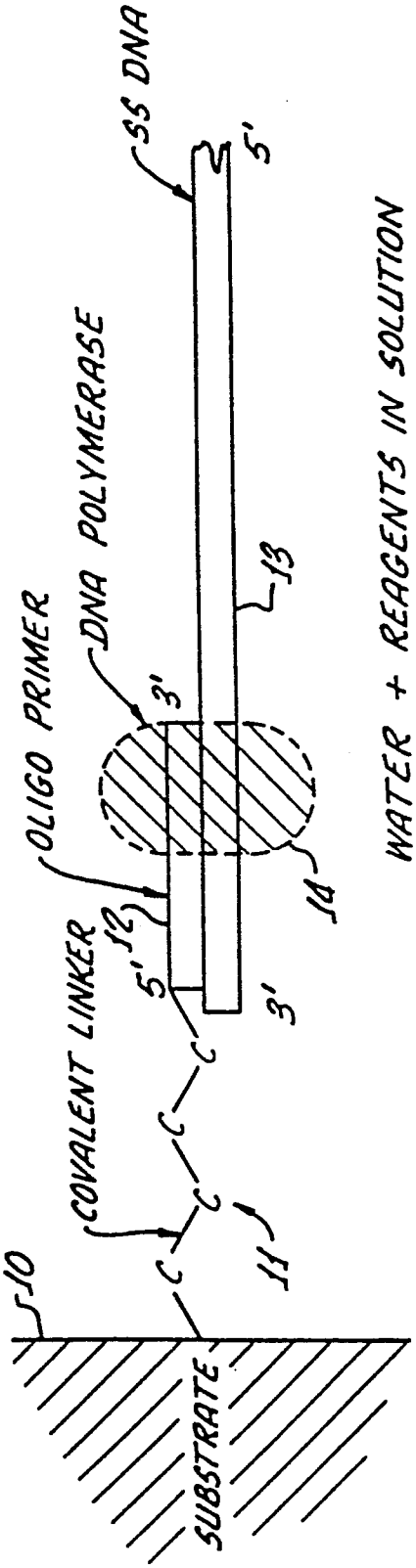

FIG. 1.

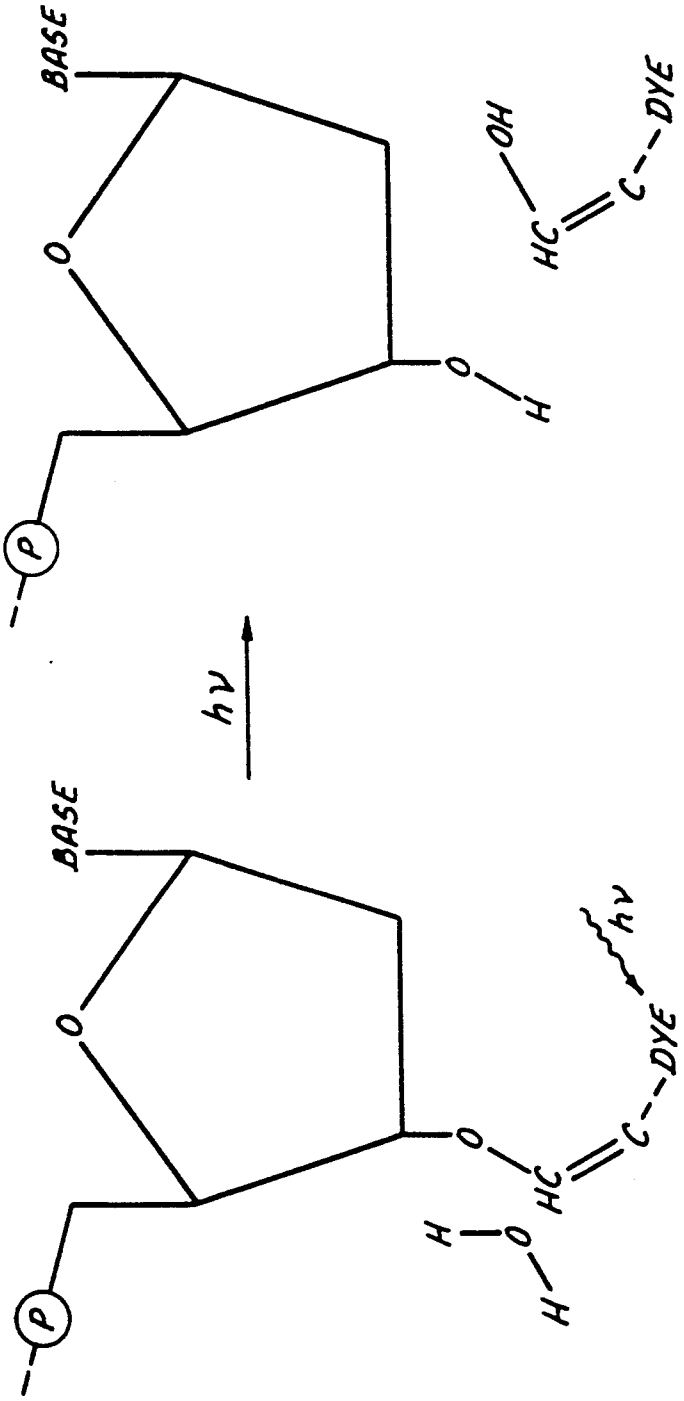

FIG. 2.

METHOD FOR SEQUENCING POLYNUCLEOTIDES

This application is a continuation of U.S. application Ser. No. 07/393,586 filed Aug. 14, 1989 now abandoned.

The present invention is directed to a method for sequencing DNA molecules.

BACKGROUND OF THE INVENTION

The present invention provides a method for determining the nucleotide sequence of DNA molecules (referred to herein as the nucleotide base sequence or simply the base sequence). Several methods are known for sequencing DNA molecules such as methods of F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.*, 74, 5463 (1977), and A. M. Maxam and W. Gilbert, *Proc. Natl. Acad. Sci. U.S.A.*, 74, 560 (1977). These known methods use various means for producing labeled fragments of DNA, each of which terminates with a known base (A, G, C or T). These fragments are then separated by length, typically by an electrophoretic gel, utilizing a different gel strip for each type of terminal base. The DNA sequence is then read from the gel strips. As a variation, instead of using the same label for each fragment (such as a fluorescent dye or radioactive label) J. M. Prober, et al., Science, 238, 336–341, Oct. 1987, and C. Connell et al., BioTechniques, Vol. 5, No. 4, 342–348 (1987), use a different dye to label each of the different base termination fragments so there is a different dye associated with A, G, C and T termination. This modification allows a single gel to be used, however, it also introduces new problems due to the effect of the different dyes on fragment mobility.

A limitation of the prior methods is that they are apparently limited by the rate at which the fragments may be separated and are also limited by the number of bases that can be sequenced in a given run by the resolution obtainable on the gel. The separation rate is inherently limited, for example, by thermal distortion of the gel caused by electrical heating, and thus the identification can only be obtained as often on average as about a few bases per minute. Also the resolution on the gel is a maximum of about 1,000 bases, with improvement in this resolution not being likely because of band compression effects, and because there are interactions between the DNA strands which dominate over the length effect of very long strands, thus confusing the signal for long fragments.

The present invention provides an improvement over these prior art methods.

It is thus an object of the present invention to provide a method of DNA identification in which the rate limiting step is essentially the rate of a polymerase reaction, which is usually on the order of at least 60 bases per second, or limited by the rate in which the reagents can be delivered to the reaction site, whichever is slower.

It is another object of the present invention to provide a method of DNA sequencing in which the accuracy does not depend upon the length of the DNA molecule to be sequenced but, rather on the signal-to-noise ratio of the detection means, which is very low using optical detection methods. Such high sensitivity detection means provide the advantage that only very small quantities of DNA are necessary, typically, less than a million molecules.

It is yet another object of the present invention to provide a DNA sequencing which is unambiguous even in short sequences of identical bases, which are difficult to distinguish by prior art methods.

Another object of the present invention is to provide a novel method for DNA sequencing in which the reagents for detection comprises a single mixture of bases, and does not require four separate preparations (one for each base) as required by methods of the prior art.

These and other objects of the present invention will be apparent from the following description, the appended claims and from practice of the invention.

SUMMARY OF THE INVENTION

The present invention provides a method for determining the nucleotide sequence of a single strand DNA molecule comprising the steps of:

(a) providing a set of identical single strand DNA molecules (ssDNA) comprising at the 3′ end a leader sequence, the leader sequence comprising a region recognizable by a DNA polymerase for initiation of replication;

(b) providing an oligonucleotide complementary to at least a portion of the leader sequence, and capable of forming a stable double stranded DNA hybrid therewith;

(c) covalently attaching the 3′ end of the leader sequence, the 5′ end of the ssDNA or an end of the oligonucleotide to a solid support;

(d) forming a stable double strand DNA hybrid bound to the solid support, the hybrid comprising the oligonucleotide and the single stranded DNA molecule with the leader sequence and the bound hybrid acting as a primer for DNA polymerase replication;

(e) exposing the hybrid bound to the solid support to a DNA polymerase in the presence of fluorescently-labeled 3′-blocked derivatives of the four nucleotide 5′-triphosphates of 2′-deoxyadenosine, 2′-deoxyguanosine, 2′-deoxycytidine and 2′-deoxythymidine, where each of the four nucleotide 5′-triphosphate (NTPs) derivatives is labeled with a fluorescent label distinguishable by fluorescent detection means from the other three labels on the other three nucleotide 5′-triphosphate derivatives, under conditions whereby the polymerase will add the appropriate complementary nucleotide 5′-triphosphate derivative to the oligonucleotide;

(f) separating any unused NTP derivatives from the solid supported DNA hybrid and the support;

(g) identifying the labeled NTP derivative added to the double stranded DNA by optical detection means; thereby identifying its complementary deoxynucleotide present in the single stranded DNA molecule;

(h) removing the fluorescent label and 3′ blocking group from the labeled NTP derivative of step (g) to expose the normal OH group in the 3′-position;

(i) separating the freed blocking group and label (which may be associated with the blocking group) from the solid supported double stranded DNA hybrid;

(j) repeating steps (e) through (i) through a plurality of cycles until labeled NTPs can no longer be added to the oligonucleotide; whereby the result of each cycle identifies the next deoxynucleotide in sequence in the single stranded DNA molecule.

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