World Intellectual Property Organization
International Office

PCT International Application Laid Open [WIPO seal]
According to Patent Cooperation Treaty

(51) Int'l Patent Classification 6
C12Q 1/68, C12M 1/00, G01N 33/50, C12N 15/10 **A1**

(11) Int'l KOKAI No. **WO98/33939**

(43) Int'l KOKAI Date August 6,1998

(21) Int'l Application No. PCT/JP97/00239

(22) Int'l Application Date January 31, 1997

(71) Applicant (all countries except the United States)
Hitachi, Ltd. [JP/JP] 4-6 Kanda Surugadai, Chiyoda-ku, Tokyo 101 (JP)
(72) Inventor; and
(75) Inventor/Applicant (the United States only)
ANAZAWA, Takeshi [JP/JP]
UEMATSU, Chihiro [JP/JP]
3-8-1 Koigakubo, Kokubunji-shi, Tokyo 185 (JP)
OKANO, Kazunori [JP/JP]
5-17-2-402 Honcho, Shiki-shi, Saitama-ken 353 (JP)
KAMBARA, Hideki [JP/JP]
1-4-3 Kitanodai, Hachioji-shi, Tokyo 192 (JP)
(74) Agent
OGAWA, Katsuo, patent attorney
c/o Hitachi, Ltd., Tokyo (JP)
1-5-1 Marunouchi, Chiyoda-ku, Tokyo 100

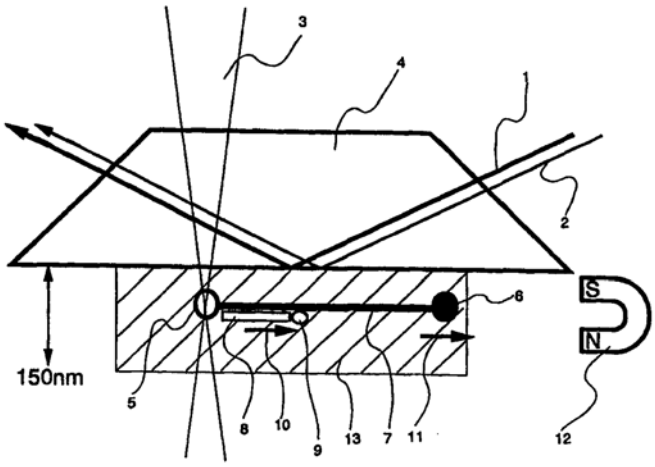
(81) Designated countries: CN, JP, KR, US, European Patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Attached KOKAI documents
International Search Report

(54) Title of Invention: Method for Determining Nucleic Acid Base Sequences and Apparatus for Determining Nucleic Acid Base Sequences

(57) Abstract

A method for determining a DNA base sequence which comprises fixing in an extended state a molecule of a single-stranded sample DNA (7) carrying a bead (5) at one end thereof and a magnetic bead (6) at the other end within the visual field of a fluorescence microscope by means of a magnetic force (11) and a laser trap (3); binding a primer (8) thereto; effecting an extension reaction (10) with a polymerase to thereby cause the incorporation of a single chemically modified nucleotide (9) alone labeled with a fluorescent substance differing from base species to base species; measuring exclusively the fluorescent substance thus incorporated as a fluorescent microscopic image by the evanescent irradiation (13) with an excitation laser (1); determining the base species from the fluorescent substance; liberating the fluorescent substance with which the incorporated nucleotide has been labeled by the evanescent irradiation (13) with an ultraviolet laser (2); and then effecting the step of the incorporation of the next nucleotide followed by repeating these steps. Thus, the base sequence can be determined by using a single DNA molecule, and a DNA consisting of several hundred bases or more can be efficiently sequenced.



Illumina Ex. 1073
IPR Petition - USP 10,435,742

DOCKET ALARM
Find authenticated court documents without watermarks at docketalarm.com.

(57) Abstract

A single molecule of single-stranded sample DNA (7) having a bead (5) at one end thereof and a magnetic bead (6) at the other end thereof is elongated and fixed in the field of view of a fluorescence microscope by a magnetic force (11) and a laser trap (3), a primer (8) is bound thereto, and an elongation reaction (10) is conducted with polymerase. Only a single chemically modified nucleotide (9) that is labeled with a fluorophore that is different for each base type is incorporated. Only the single fluorophore incorporated is measured, by evanescent irradiation from an ultraviolet laser (2), as a fluorescence microscopic image, and the base type is determined from the fluorophore type. By the evanescent irradiation (13) from the excitation laser (1), the fluorophore that labels the incorporated nucleotide is released, and the next nucleotide is incorporated. DNA base sequences are determined by repeating this process. Base sequence determination can be performed using a single DNA molecule, and base sequences can be efficiently determined for DNA having several hundreds of thousands of bases or more.

Codes used for identifying PCT member nations listed on page 1 of the pamphlet for the international application laid open on the basis of the PCT (reference information)

AL Albania	DK Denmark	KZ Kazakhstan	PT Portugal
AM Armenia	EE Estonia	LC Saint Lucia	RO Romania
AT Austria	ES Spain	LI Liechtenstein	RU Russian Federation
AU Australia	FI Finland	LK Sri Lanka	SD Sudan
AZ Azerbaijan	FR France	LR Liberia	SE Sweden
BA Bosnia and Herzegovina	GA Gabon	LS Lesotho	SG Singapore
	GB United Kingdom	LT Lithuania	SI Slovenia
BB Barbados	GE Georgia	LU Luxembourg	SK Slovakia
BE Belgium	GH Ghana	LV Latvia	SL Sierra Leone
BF Burkina Faso	GM Gambia	MC Monaco	SN Senegal
BG Bulgaria	GN Guinea	MD Republic of Moldova	SZ Swaziland
BJ Benin	GW Guinea-Bissau		TD Chad
BR Brazil	GR Greece	MG Madagascar	TG Togo
BY Belarus	HU Hungary	MK The former Yugoslav Republic of Macedonia	TJ Tajikistan
CA Canada	ID Indonesia		TM Turkmenistan
CF Central African Republic	IE Ireland		TR Turkey
	IL Israel	ML Mali	TT Trinidad and Tobago
CG Congo	IS Iceland	MN Mongolia	
CH Switzerland	IT Italy	MR Mauritania	UA Ukraine
CI Côte d'Ivoire	JP Japan	MW Malawi	UG Uganda
CM Cameroon	KE Kenya	MX Mexico	US United States of America
CN China	KG Kyrgyzstan	NE Niger	
CU Cuba	KP Democratic People's Republic of Korea	NL Netherlands	UZ Uzbekistan
CY Cyprus		NO Norway	VN Viet Nam
CZ Czech Republic		NZ New Zealand	YU Yugoslavia
DE Germany	KR Republic of Korea	PL Poland	ZW Zimbabwe

DOCKET ALARM
Find authenticated court documents without watermarks at docketalarm.com.

Specification

Method for Determining Nucleic Acid Base Sequences and Apparatus for Determining Nucleic Acid Base Sequences

Technical Field

This invention relates to an apparatus for analyzing DNA and RNA and the like, and more particularly to an apparatus that is effective for determining DNA and RNA base sequences or, alternatively, analyzing limited enzyme fragments or specific fragments.

Technical Background

Techniques for analyzing DNA, RNA, and the like are important in the fields of medicine and biology, inclusive of gene analysis and genetic diagnosis. The determination or DNA and RNA base sequences, and the analysis of limited enzyme fragments and specific fragments, are both based on separation by molecular weight by electrophoresis. A fragment or fragment group is subjected beforehand to radioactive labeling or fluorescent labeling, and, after conducting electrophoresis, or during electrophoresis, analysis is effected by measuring molecular weight-separation development patterns. Recently, in connection with genome analysis, the demand for DNA base sequence determining apparatuses, in particular, has grown, and the development of such devices is on-going. DNA base sequence determination using fluorescent labeling is now described. Prior to electrophoretic separation, a dideoxy reaction is carried out by the Sanger method. A nucleotide having a length of approximately 20 bases that is complementary to a known portion of the base sequence of the sample DNA to be analyzed is synthesized and *labeled with a fluorophore* [lit. *a fluorophore is labeled*]. This oligonucleotide is complementary-chain bound to approximately 10^{-12} mol of the sample DNA as a primer, and a complementary chain elongation reaction is conducted using polymerase. At this time, four deoxynucleotide triphosphates are added as substrates, namely deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), deoxythymidine (dTTP), and, in addition thereto, dideoxyadenosie triphosphate (ddATP). When ddATP is incorporated by complementary-chain elongation, because the complementary chain is not elongated beyond that, fragments of various lengths and terminated by adenine (A) are prepared. Reactions are conducted independently wherein, instead of ddATP in the reaction noted above, dideoxycytidine triphosphate (ddCTP), dideoxyguanosine triphosphate (ddGTP), and dideoxyguanosine triphosphate (ddTTP), respectively, are added. However, while the primers used in each reaction have the same base sequence, four types of fluorophores, which can be mutually distinguished by separating fluorescence, are *used for labeling* [lit. *are labeled*].

When the four types of reactant noted above are mixed, fragments complementary to the sample DNA, having a length of up to approximately 1000 bases, and with lengths differing one base at a time, are prepared, with four types of fluorophores being *used in labeling* [lit. *labeled*], according to the terminal base type. The number of fragments of each base length, respectively, is approximately 10^{-15} mol. Next, the samples prepared are

DOCKET ALARM Find authenticated court documents without watermarks at docketalarm.com.

separated with a resolving power of 1 base by electrophoresis. In electrophoresis, wide use is made of a slab gel wherein acrylamide is polymerized between two glass plates separated by an interval of approximately 0.3 mm. When the sample is injected at the upper end of the slab gel, and an electric field is applied to the upper and lower ends of the slab gel, the sample migrates toward the lower end while separating. When a position approximately 30 cm from the upper end is irradiated by a laser while conducting electrophoresis, separated fluorescently labeled fragments are excited as they pass the position of laser irradiation, in order from the shorter to the longer. When the emitted fluorescence is measured while being spectrally separated with the use of multiple filters, the terminal base types of all of the fragments can be determined, in order, from the shorter fragments to the longer, based on the change over time in the fluorescent intensity of the four types of fluorophore. With the order of these base types being in a complementary relationship with the sample DNA, the base sequence of the sample DNA can be determined.

A number of new DNA base sequence determination methods have been proposed which do not employ electrophoresis. With a first prior art, when a complementary-chain elongation reaction is conducted by polymerase using the sample DNA as a template, four types of base are added, in order, one at a time, and the base quantity incorporated into the complementary chain at each step is quantified by photoabsorption or fluorescence, whereupon the base sequence of the sample DNA is determined (TOKKAI [Unexamined Patent Application] No. H4-505251/1992, gazette). With a second prior art, using the sample DNA as a template, and employing four types of base labeled mutually differently, a complementary-chain elongation reaction is conducted by polymerase, after which one base at a time is released from the 3′ end of the complementary chain synthesized by exonuclease, and the labels of the released bases are measured, in order, to determine the base sequence of the sample DNA (Journal of Biomolecular Structure & Dynamics 7, 301 – 309 (1989)). With a third prior art, the base sequence of sample DNA is determined by repeating a cycle of steps, namely a step for conducting a DNA polymerase reaction using four types of dNTP derivative (MdNTP) having labels that can be detected, and that, incorporated into a template DNA as DNA polymerase substrate, can stop a DNA chain elongation reaction by the presence of a protective group, a step for then detecting the MdNTP incorporated, and a step for returning the MdNTP to an elongatable state. With this prior art, DNA chain elongation is stopped at the point in time when one base is elongated, oxygen and the substrate are removed from the system (solution) wherein the template, primer, and MdNTP are present, the MdNTP incorporated is detected, the protective group (and label) of the MdNTP incorporated into the template are released, and a condition is induced wherein DNA chain elongation is possible (Pat. Applic. No. H2-57978/1990). However, these proposals are at present in the idea stage, and there are no reports of them having been made practicable.

Disclosure of Invention

Currently, insofar as practical methods of determining DNA base sequences are concerned, separation by molecular weight is conducted by electrophoresis. For DNA base sequence determination, a resolving power of 1 base length is required for separation by

DOCKET ALARM Find authenticated court documents without watermarks at docketalarm.com.

molecular weight. The resolving power of electrophoresis ordinarily declines as the lengths of the bases to be separated become longer. That is, even though one base length can be separated between a base length of 50 bases and one of 51 bases, that does not mean that one base length can be separated between a base length of 500 bases and one of 501 bases. How long of a base length can be resolved by 1 base length, that is, the limit of base length separation, is determined by the electrophoresis conditions, that is, the composition of the separation medium, the electrophoresis path length, and the electric field intensity. In order to increase the limit of base length separation, various optimizations have been implemented to date, but there are no reports of a limit exceeding a length of 1000 bases (Electrophoresis 13, 495 – 499 (1992), Electrophoresis 13, 616 – 619 (1992)). The maximum of 1000 bases for the limit of base length separation has been theoretically explained (Electrophoresis 13, 574 – 582 (1992)). That is, insofar as electrophoresis is employed, the maximum length at which the base sequence can be determined from one sample DNA is a length of 1000 bases. With large-scale base sequence determination typified by genome analysis, on the other hand, the limit of base length separation holds the key to analytical efficiency (Science 254, 59 – 67 (1991)). The YAC clone, which is a typical large clone, for example, has a base length of approximately 1M bases, wherefore, a minimum of 1000 samples must be analyzed in order to analyze each base length of 1000 bases. However, it is not possible to prepare fragments one by one having a base length of 1000 bases, in order, from the end of a long DNA, without either excess or insufficiency. In practice, random fragments are prepared, these are randomly analyzed, and the base sequence of the original long DNA is reconfigured using overlaps in the base sequences of the fragments. This method, called the shotgun method, is the most widely used method today in genome analysis. However, in order to impart overlaps to random fragments so as to enable reconfiguration, it is necessary to repeatedly analyze the same base sequence, over and over, which is a problem.

The degree of this overlapping, which is called redundancy, increases as the base length wherewith one-time base sequence determination is possible becomes smaller, that is, as the limit of base length separation in electrophoresis becomes smaller. For a limit of base length separation of 1000 bases, a redundancy of approximately 10 is required. That is, it is necessary to conduct base sequence determination for a length that is 10 times that of the DNA for which the base sequence is to be determined. As a consequence, one must analyze approximately 10,000 samples in order to determine the base sequence for a base length of 1M bases. The number of samples that can be analyzed in one day by one DNA base sequence determination device is 100 at most. To determine the base sequence of the entire base length of 1M bases with one apparatus would require 100 days. In order to practically conduct genome analysis, or the genetic diagnostics the importance whereof will increase in future, requires art whereby base sequences 1M bases long can be determined in a few days.

Another problem with the prior art is the necessity therewith of having many DNA samples in order to determine base sequences. In order to conduct the dideoxy reactions with the Sanger method, a DNA sample of approximately 10^{-12} mol is ordinarily needed for one sample. For that reason, it is necessary to refine and amplify sample DNA beforehand, using techniques such as cloning or PCR. Such procedures require time and effort, and

DOCKET ALARM Find authenticated court documents without watermarks at docketalarm.com.

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.