**Title:** DISCRIMINATION METHOD OF HYBRIDIZATION BETWEEN PROBES AND NUCLEOTIDES IN SAMPLE ON THE BIO CHIP USING ENZYME REACTION

![Diagram of the method](image)

**Abstract:** The present invention relates to a discrimination method of hybridization between probes and nucleotides in a sample on the bio chip using enzyme reaction. According to the present invention, the hybridization between probes and nucleotides in the sample is discriminated by using an enzyme which can recognize and cleave either of the probe hybridized or unhybridized with the nucleotides in the sample to remove either one on the bio chip, followed by detection. Therefore, by using the discrimination method according to the present invention, it is possible to obtain a more precise result by removing false-positive reactions, as compared to the conventional methods, to solve problems involved in the pretreatment of a sample and to permit the SNP analysis which is important in the genetic diversity studies. Thus, the present invention can be usefully used in studies using a bio chip and their applied fields.
DISCRIMINATION METHOD OF HYBRIDIZATION
BETWEEN PROBES AND NUCLEOTIDES
IN SAMPLE ON THE BIO CHIP USING ENZYME REACTION

Background of Invention

Technical Field

The present invention relates to a method for discriminating hybridization between probes and nucleotides in a sample from a reaction using a bio chip.

Background Art

From the 1900's, as bioinformatics and DNA sequencing technologies has made rapid progress, research on genomes of living things including a human being has been actively conducted. Studies of functions of genes constituting a genome and relation therebetween may provide information which promotes great development in various fields such as drug development, disease diagnosis and crops breeding. Therefore, there is an increased demand for a method for effectively analyzing an enormous amount of genetic information.

However, conventionally used methods for analyzing genes require much time, effort, cost and intensive skills and also have a limit in the number of samples, whereby it is impossible to analyze a large amount of samples in a short period of time.

Therefore, research and studies have been conducted to find a new analysis system which can overcome defects of the conventional gene analysis methods and the bio chip technology which can analyze thousands of or ten thousands of genetic information materials by combining mechanical automation and electronic control
technologies with molecular biological knowledge attracts public attentions to meet the need for the new analysis system.

The bio chip is a biological micro chip which may be formed by microarraying and attaching hundreds to hundreds of thousands, at most, of nucleotides including, for example, DNA, DNA fragment, cDNA, oligonucleotide, RNA or RNA fragment, of which sequences are known, at regular intervals on a small solid substrate made of glass, silicone or nylon so as to analyze the expression pattern, distribution mode and mutation of genes.

Nucleotides which can work as probes to detect a specific genetic information material contained in a sample are fixed on a bio chip. When this bio chip is treated with the sample to be assayed, nucleotides contained in the sample bind to probes fixed on the surface of the bio chip at different binding degrees according to their base sequence complementarity to give a hybridization state. By detection and analysis of the hybridization state, it is possible to obtain information of the whole nucleotides contained in the sample at the same time. In this way, the bio chip allows to attain huge information in a short time and thus attracts attentions as a technology to bring a revolutionary change in various fields such as scientific technology research and new drug development process, clinical diagnosis, agriculture, food, environmental field, and the like.

The core technology in connection with the bio chip includes a technology for attachment and fixation of probes, a technology for signal detection and a technology for information processing.

Firstly, the technology for attachment of probes to fix a large amount of nucleotides on a bio chip at a time has been rapidly developed by applying various theories. Hyseq and Incyte possess a technology of transferring cDNA onto a glass
plate treated with poly-L-lysine using a pin, followed by integration. As an improvement of this technology, Incyte has developed a new technology by incorporating the principle of a cartridge used in an ink jet printer instead of a pin. Also, Affymetrix has developed a method for synthesizing tens of thousands of different base sequences by applying photolithography which is used in the semiconductor process and Nanogen has developed a method for electrically fixing genes using electrical properties of DNA which has negative charge.

Also, the information processing technology which allows to establish a database with information gathered from a bio chip and renders it to be utilized in research has already consolidated its firm foundation owing to the highly advanced information and communication technology.

However, as compared to these technologies, the signal detection technology, that is a method for detecting an interaction between probes and nucleotides in a sample to discriminate hybridized or un-hybridized probes with the sample nucleotides makes a relatively slow progress. Signal detection methods which are used at present include, for example, laser-induced fluorescence detection, electrochemical detection and mass detection.

The laser-induced fluorescent detection is a method for optically discriminating hybridization between probes and nucleotides, in which a fluorescent material is bonded to the nucleotides in a sample and the hybridization is detected by a fluorescence detector and is currently the most commonly used method. However, this method requires pretreatment reaction to bond a fluorescent material to a sample prior to hybridization, causing loss or contamination of the sample (Conner, B. Proc. Natl. Acad. Sci. U.S.A., 80:278, 1983).

The electrochemical detection is a method for detecting hybridization of
probes using an electrochemical reaction of other chemicals, that is oxidation-reduction reaction, on an electrode where the probes couple with a sample. This method is not yet developed as a general detection method. Since it has a recognition level inferior to the fluorescence detection method, it cannot provide a satisfactory result in terms of accuracy. Thus, it is hard to discriminate hybridization of probes only by the electrochemical detection and a separate method for electrochemically discriminating hybridization of probes is always required.

The mass detection is a detection method by electrically signalizing the interaction between probes and nucleotides in a sample. Its representative example is Electrochemical Quartz Crystal Microbalance (QCM) detection, in which the change in frequency according to the mass of probes immobilized on a quartz plate vibrating at a high frequency is measured.

In addition, as a newly developed optical method, there is known surface plasmon resonance (SPR) detection, in which the difference of signals by the change in mass of probes is detected.

These methods recently attract public attentions for their utility since they can measure bonding affinity of DNA by the difference of mass without, for example, fluorescent labeling of nucleotides in a sample. However, they still need much research for their utilization in the information interpretation of a bio chip.

As described above, though there has been proposed various methods for detecting hybridization between proves and nucleotides in a sample, it is still required to develop a method for precisely detecting hybridization by perfectly removing a false-positive reaction.

However, the coupling of probes with nucleotides in a sample can accomplished when their sequences are complementary to each other for the most
part but not all. In other words, they can bond to each other even when their sequences are not complementary to each other for a base or a part of the sequences, that is, they are not perfectly complementary to each other. Like this, the nucleotides with a base or a part uncomplementary to probes can hardly be discriminated from the nucleotides having perfect complementarity to probes. It is not ready to assay SNP (single nucleotide polymorphism) having a difference of about a base by the existing methods.

Accordingly, it is impossible to obtain satisfactory results by the existing detection methods in technologies of recognizing interactions between molecules in a reaction using a bio chip. Thus, it is desired to develop a method for precisely discriminating hybridization between probes and nucleotides in a sample for the advance in the bio chip field, of which demand is increased from day to day.

**Disclosure of Invention**

Therefore, the present invention has been made in view of the above problems, and it is an object of the present invention to provide an effective method for discriminating hybridization between probes and nucleotides in a sample from a reaction using a bio chip.

The present invention is directed to a method for discriminating hybridization between probes and nucleotides in a sample from a reaction using a bio chip by an enzyme reaction.

According to the present invention, discrimination of hybridization between probes and nucleotides in a sample from a reaction using a bio chip is carried out by hybridizing probes with nucleotides in a sample, followed by treatment with an enzyme which can recognize and cleave any one of the probes hybridized with the
nucleotides and the probes unhybridized with the nucleotides between proves and nucleotides in a sample hybridization.

According to the present invention, the probe refers to a substance which can bond to a nucleic acid having complementarity with itself to be hybridized, that is, DNA, DNA fragment, cDNA, oligonucleotide, RNA, RNA fragment, etc.

According to the present invention, the nucleotides refers to a substance which can complementarily bond to a specific probe immobilized on a substrate, that is, DNA, DNA fragment, cDNA, oligonucleotide, RNA, RNA fragment, etc.

According to the present invention, the enzyme reaction refers to a reaction using an enzyme which can recognize and cleave probes unhybridized with the nucleotides after hybridization. Since the unhybridized probes are present in the form of a single strand after hybridization, S1 nuclease, mung bean nuclease, RNase A and RNase H which can recognize and cleave only a single-stranded nucleic acid can be used.

The S1 nuclease and mung bean nuclease are usefully used in a reaction using a DNA chip, since they can recognize and cleave only single-stranded DNA.

RNase A is usefully used in a reaction using a RNA chip, since it can recognize and cleave only single-stranded RAN.

RNase H is usefully used in an analysis of a DNA sample using RNA as a probe, since it can selectively cleave single-stranded RNA in the hybridization of RNA and DNA.

According to the present invention, the enzyme reaction refers to a reaction using an enzyme which can recognize and cleave probes hybridized with the nucleotides after hybridization. Since the hybridized probes are present in the form of a double strand after hybridization, DNase which can recognize and cleave only a
double-stranded nucleic acid can be used.

After the discrimination of hybridization according to the present invention, a method for detecting interactions between the probes and the nucleotides can be carried out by a conventional method such as laser-induced fluorescence detection, electrochemical detection and mass detection.

**Brief Description of Drawings**

The above and other objects, features and advantages of the present invention will be more clearly understood from the following detailed description taken in conjunction with the accompanying drawings, in which:

Fig. 1 is a schematic view showing the procedures of the method for detecting hybridization between probes and nucleotides in a sample using the discrimination method according to the present invention;

Fig. 2 is a schematic view showing the procedures of the method for optically detecting hybridization between probes and nucleotides in a sample using the discrimination method according to the present invention;

Fig. 3 is a schematic view showing the procedures of the method for electrochemically detecting hybridization between probes and nucleotides in a sample using the discrimination method according to the present invention;

Fig. 4 shows the results of the optical detection of unhybridized probes using the discrimination method according to the present invention; and

Fig. 5 shows the results of the electrochemical detection of probes unhybridized or hybridized with nucleotides in a sample using the discrimination method according to the present invention.
Best Mode for Carrying Out the Invention

Now, the present invention will be explained in detail with reference to Fig. 1.

As shown in Fig. 1a, probes 1, 2 are immobilized onto a substrate 3. When a nucleic acid which can complementarily bond to the probe 1 is applied, the probe 1 is hybridized with the nucleic acid to form a double-stranded nucleic acid 11, as shown in Fig. 1b, while the probe 2 which is not hybridized with the nucleic acid remains in the form of a single strand.

After hybridization, the substrate 3 is treated with an enzyme which can recognize and cleave only the single-stranded nucleic acids, as shown in Fig. 1c, followed by washing. As a result, the unhybridized probe 2 is removed remaining a part 22 attached to the substrate 3, as shown in Fig. 1d. Therefore, after the treatment with an enzyme, only the probe 1 which is hybridized with the nucleic acid is present on the substrate 3. By detecting the presence of the probe according to a known detection method, it is possible to precisely discriminate the probes hybridized with the nucleic acid from the probes unhybridized with the nucleic acid.

Meanwhile, when an enzyme which has an activity opposite to that of the enzyme used in the foregoing method, that is, an enzyme which can recognize and cleave only double-stranded nucleic acids is applied on the substrate, followed by washing, the hybridized probe is removed on the contrary to Fig. 1d. Therefore, there remains the probes unhybridized with the nucleic acid on the substrate and it is thus possible to precisely discriminate the probes hybridized with the nucleic acid from the probes unhybridized with the nucleic acid.

The production process of the bio chip used in the present invention varies according to a detection method of interaction between the probe and the nucleic acid in the sample after the enzyme reaction.
An instant case where laser-induced fluorescence detection is used as a detection method of interaction between the probe and the nucleic acid after the discrimination according to the present invention is explained in detail with reference to Fig. 2.

According to the present invention, probes 5, 6 with fluorescent materials 4 of one type or different types attached are immobilized on a substrate 7 upon preparation of a chip, as shown in Fig. 2a, unlike the conventional fluorescence detection methods in which a fluorescent material is attached to a sample.

After washing the substrate with the probes immobilized, a sample containing nucleic acids is applied to the probes. The probe 5 is hybridized with the nucleic acid to form a double-stranded nucleic acid 55 while the probe 6 remains in the form of a single strand without hybridization with the nucleic acid, as shown in Fig. 2b.

After hybridization, the substrate is treated with an enzyme which can recognize and cleave only single-stranded nucleic acids. As a result, the unhybridized probes 66 are removed and there remain only the hybridized probes on the substrate, as shown in Fig. 2c. The substrate can be subjected to fluorescence detection.

Therefore, by performing the laser-induced fluorescence detection after the discrimination method according to the present invention, it is possible to reduce the pretreatment process and thereby, to conveniently and precisely conduct an experiment. Also, since only the fluorescence attached on the hybridized probes is detected, it is possible to obtain a more precise result and thereby to solve some problems involved in the conventional laser-induced detection methods.

An instant case where electrochemical detection is used as a detection
method of interaction between the probe and the nucleic acid after the discrimination according to the present invention is explained in detail with reference to Fig. 3.

In preparation of a chip, probes 9, 10 with thiol group 8 attached on one end so as to form a chemical bond with the gold surface are immobilized on a gold electrode 11, as shown in Fig. 3a. The thiol group has properties of being readily attached to the gold surface and thus, is widely used to attach chemicals including DNA to a gold surface.

After washing the electrode with the probes immobilized, probes are hybridized with the nucleotides in the sample. As a result, the probes hybridized with the nucleotides form double strands and the probes unhybridized with the nucleotides remain single strands, as shown in Fig. 3b.

After hybridization, the electrode is treated with an enzyme which can recognize and cleave single-stranded nucleic acids. As a result, the unhybridized probes are removed, leaving a part which is attached to the electrode, as shown in Fig. 3c. The electrode with only the hybridized probed attached was subject to a cyclic voltammetry.

The cyclic voltammetry is a method to measure an electric current generated as a voltage is applied after an electrode is dipped in an electrolyte. When a gold electrode is dipped in a Fe(CN)₆³⁻ solution and a voltage is applied, an electric current flows by the electrochemical reaction of Fe(CN)₆³⁻. Here, when the electrode has hybridized probes attached on its surface, negatively charged Fe(CN)₆³⁻ cannot approach to the electrode due to the negative charges of the probes, whereby the electrochemical reaction hardly occurs (Fig. 3b). On the other hand, when the electrode have the probes removed, Fe(CN)₆³⁻ can freely react to generate Fe(CN)₆⁴⁻ (Fig. 3c). Therefore, the cyclic voltamogram (CV), obtained by measuring the
electric current, shows a significant difference in the two cases.

Therefore, by performing the electrochemical detection after the discrimination method according to the present invention, it is possible to significantly increase recognition level over the conventional electrochemical detection since there is added a step to remove the probes unhybridized with the nucleotides in a sample in advance prior to detecting hybridization.

Thus, the discrimination method according to the present invention can effectively remove false-positive reactions which may occur due to the co-presence of the hybridized probes and the unhybridized probes to the final result interpretation by removing either one of the probes hybridized or unhybridized with the nucleotides in the sample.

With respect to a group of nucleotides in a sample and probes, the discrimination method according to the present invention can provide a more precise result by making various chips and put the results therefrom together according to how to detect the interaction between the probes and nucleotides in the sample after the enzyme reaction.

The discrimination method according to the present invention can be usefully used in a SNP analysis. When nucleotides to be detected have complementarity to the sequence of a probe for the most part except for a base or a part, they can bond to the probe as a whole while leaving a part where the sequence is not complementary as a single strand. At this time, an enzyme which can degrade the single stranded nucleic acid is applied according to the present invention, the unhybridized part is cleaved and thus, it is possible to precisely detect a base or a part which is not complementary.

The discrimination method according to the present invention can be applied
to gene function studies including SNP analysis, gene recombination organism studies, cancer and disease-related gene diagnosis, plant and animal inspection, ecological studies according to environmental changes, food safety examination, new drug development, gene mutation search, mutant pedigree, organ transplantability test, pathogenic microorganism identification, medical jurisprudence and DNA archaeology.

EXAMPLE

Now, the discrimination method of the present invention will be explained in detail by the following examples. However, the present invention is not limited thereto.

Example 1: Discrimination of probes hybridized or unhybridized with nucleotides in a sample according to the present invention and optical detection thereof

Using the discrimination method of the present invention, probes hybridized or unhybridized with nucleotides in a sample were discriminated and optically detected.

With respect to a part of M13mp18 DNA sequence, oligonucleotides of 15 nucleotides were synthesized using a DNA synthesizer and were used as probes. One end of each probe was labeled with Cy3, a fluorescent material. 1 μl of 1 μM solution of the fluorescent labeled probes was arranged in two groups of 4 lines on a slide glass coated with ODS(octadecyl silane) and incubated at 24 °C overnight to immobilize the probes on the substrate.

The substrate with the probes immobilized was washed with 5 mM Tris
buffer (pH 7.4) containing 10 mM sodium chloride and 2 μl of 10 U/ml S1 nuclease solution was applied over the lower four lines. In order to prevent the applied solution from being dried, a gold electrode is placed in a vessel with water scattered at the bottom. The container is then sealed and incubated at 35 °C overnight to immobilize probes on the electrode. The substrate was washed with 5 mM Tris buffer containing 10 mM sodium chloride and detected for fluorescence using ScanArray Lite, supplied by Packard BioScience. The results are shown in Fig. 4.

As shown in Fig. 4, at the upper part where S1 nuclease was not applied and hence, the probes remained, fluorescence was observed (Fig. 4a), whereas at the lower part where S1 nuclease was applied and hence, the probes were removed, no fluorescence was observed (Fig. 4b). There was a significant difference of the detected fluorescence between the two parts.

Therefore, it was proved that the detection of hybridization via an optical assay after an enzyme reaction can be more readily and precisely performed by effectively removing single-stranded probes which were not hybridized with a sample using an enzyme capable of recognizing and cleaving only single-stranded nucleic acids.

Example 2: Discrimination of probes hybridized or unhybridized with nucleotides in a sample according to the present invention and electrochemical detection thereof

Using the discrimination method of the present invention, probes hybridized or unhybridized with nucleotides in a sample were discriminated and electrochemically detected.
Probes were prepared as oligonucleotides as in Example 1. One end of each probe was labeled with a thiol group instead of Cy3. Gold wire with a diameter of 1 mm was used as a gold electrode and was wrapped with Teflon except for the section so as to avoid contacting with a solution. The electrode was dipped in a piranha solution for 15 minutes to wash the surface and rinsed with deionized water and then 1 M potassium phosphate buffer (pH 7.0). The electrode with nothing immobilized thereon was subjected to CV using 100B, supplied by BAS (Fig. 5a).

1 μl of 1.0 μM probes dissolved in 1 M potassium phosphate buffer was applied on the rinsed gold electrode. In order to prevent the applied solution from being dried, the gold electrode is placed in a vessel with water scattered at the bottom. The container is then sealed and incubated at 35 °C overnight to immobilize probes on the electrode. The electrode with the probes immobilized thereon was washed with 5 mM Tris buffer containing 10 mM sodium chloride.

15 For hybridization, a sample solution of 5.0 μM oligonucleotide having a base sequence complementary to the probes dissolved in 10 mM Tris buffer containing 0.1 M sodium chloride and 1 mM EDTA was applied on the electrode with the probes immobilized thereon and incubated 35 °C for 2 hours. The electrode was washed with 5 mM Tris buffer containing 10 mM sodium chloride and subjected to CV (Fig. 5c). An electrode with the probes immobilized thereon as described above, without hybridization, was also subjected to CV (Fig. 5b).

2 μl of 10 U/ml S1 nuclease solution was applied on the electrodes with hybridization and without hybridization and incubated at 35 °C for 2 hours. The substrates were washed with 5 mM Tris buffer containing 10 mM sodium chloride and subjected to CV (Figs. 5d and 5e).
As shown in Fig. 5, before the enzyme reaction, the electrodes without hybridization (Fig. 5b) and with hybridization (Fig. 5c) showed CV shapes similar to each other but significantly different from that of the electrode with nothing immobilized thereon (Fig. 5a).

It is because the electric current intensity during the electrochemical reaction varies according to whether or not nucleic acids are present on the surface of the electrode. Since the electrode without hybridization have single-stranded nucleic acids on its surface and the electrode with hybridization have single-stranded nucleic acids along with double-stranded nucleic acids on its surface, both electrodes generated electric currents at a similar level by negative charges of the nucleic acids.

However, after the enzyme reaction, the two electrodes showed entirely different CV results.

After the enzyme reaction, the electrode without hybridization showed CV similar to that of the electrode with nothing immobilized thereon (Figs. 5a and 5d). Since the probes on the electrode without hybridization had been removed by S1 nuclease, the electrode became the same with the empty electrode. Therefore, the electrochemical reaction occurs freely, regardless of negative charges of the nucleotides.

On the other hand, the electrode with hybridization showed the same CV before and after the enzyme reaction (Fig. 5e). It is because S1 nuclease could not remove the nucleotides which had become the double-stranded form by hybridization, whereby there were nucleotides on the electrode even after the enzyme reaction.

Therefore, it was proved that the detection of hybridization via an electrochemical assay after an enzyme reaction can be more readily and precisely performed by effectively removing single-stranded probes which were not hybridized
with a sample using an enzyme capable of recognizing and cleaving only single-stranded nucleic acids.

**Industrial Applicability**

By the discrimination method according to the present invention, there is no need of a pretreatment of a sample to discriminate hybridization between probes and nucleotides in a sample in an experiment using a bio chip. Also, since the probes hybridized or unhybridized with the nucleotides in a sample are removed during an experimental process prior to detection, it is possible to considerably reduce errors and false-positive reactions during interpretation of results which may occur in the whole treatment process of the sample.

Therefore, the method according to the present invention can be usefully applied to studies using a bio chip and their applied fields, including SNP analysis which is important in the studies of genetic diversity.
What is claimed is:

1. A method for discriminating hybridization between probes and nucleotides in a sample prior to detection of interaction between the probes and the nucleotides in the sample comprising hybridizing the probes and nucleotides on a bio chip, treating the bio chip with an enzyme capable of recognizing and cleaving any one of probes hybridized with the nucleotides in the sample and probes unhybridized with the nucleotides in the sample.

2. The method according to claim 1, wherein the probes are any one selected from DNA, DNA fragment, cDNA, oligonucleotide, RNA and RNA fragment.

3. The method according to claim 1, wherein the sample nucleotides are any one selected from DNA, DNA fragment, cDNA, oligonucleotide, RNA, RNA fragment.

4. The method according to any one of claims 1 to 3, wherein the enzyme is an enzyme that recognizes and cleaves only single stranded nucleic acids.

5. The method according to claim 4, wherein the enzyme is any one selected from S1 nuclease, mung bean nuclease, RNase A and RNase H.

6. The method according to any one of claims 1 to 3, wherein the enzyme is an enzyme that recognizes and cleaves only double-stranded nucleic acids.
7. The method according to claim 6, wherein the enzyme is DNase.

8. The method according to any one of claims 1 to 3, wherein the detection of interaction between probes and nucleotides in the sample is carried out by any one selected from laser-induced fluorescent detection, electrochemical detection and mass detection.
[DRAWING]

[FIG. 1]

a 1 2 b 11 2
react of probes with sample

3

 enzyme
treatment

d 11 22 c 11 2
washing

3

[FIG. 2]

a 4 5 6 7 4
b 55 6 7 55 6

c 55 66 7
A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12Q 1/68

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)

IPC7 C12Q 1/68

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

Korean Patents and applications for inventions since 1975

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

eKIPASS, Delphion

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X See patent family annex.

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Date of the actual completion of the international search

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