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(54) **METHOD FOR PRODUCING A BIOMOLECULE ASSAY CHIP**

VERFAHREN ZUR HERSTELLUNG EINES BIOMOLEKÜLTEST-CHIPS

PROCEDE POUR LA PRODUCTION D'UNE PUCE POUR L'ANALYSE DE BIOMOLECULES

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## Description

Technical Field

**[0001]** The present invention relates to a method of producing a chip on which biomolecules are immobilized in an aligned state.

Background Art

**[0002]** A DNA chip, a protein chip, and the like are currently used as useful tools for comprehensively analyzing biomolecules. In the case of these chips, DNAs or proteins are immobilized on a substrate in an array form, and they are then subjected to an appropriate reaction system that depends on purposes, so as to comprehensively identify molecules that are likely to exhibit functions of interest.

**[0003]** With regard to such DNA chip, at present, the method of Affymetrix, Inc., which comprises synthesizing DNA on a chip using an optical lithography technique and then matching location information and sequence information, and the method of Stanford University, which comprises attaching DNA whose sequence has been known to a chip, are widely used. In order to examine a large number of sequences at once, the Affymetrix method is advantageous. In order to examine a limited number of DNAs whose sequences have been known, the Stanford method is convenient. Thus, the two types of methods are used, depending on purposes.

**[0004]** On the other hand, with regard to production of a protein chip, Phylos, Inc. has attempted to hybridize the mRNA region of IVV (*in vitro* virus) with an Affymetrix-type DNA substrate, so as to assign numbers to proteins on a chip and to non-covalently immobilize them thereon (Non-Patent Document 1). However, a majority of protein chips are produced by the Stanford method of producing such protein chips by spotting a limited number of specific proteins. Hence, under the present circumstances, it is extremely difficult to produce protein chips from an enormous library comprising genes having unknown sequences. In this respect, the method of Phylos, Inc. is most advantageous. However, since hybridization is used in immobilization of proteins in the method of Phylos, Inc., this method is not considered as a stable immobilization method, when an enzyme assay is carried out under various buffer conditions. Thus, this method has not yet been practically used. In addition, since this method comprises a process of introducing a region to be hybridized as a tag, it is not necessarily said that it is efficient from the viewpoint of practical utility.

**[0005]** Biyani Manish et al. (Nucleic Acids Research, Vol. 34, No. 20, 1 January 2006, pages 140-1, XP002479343) relates to solid-phase translation and RNA-protein fusion considering an approach for folding quality control and direct immobilization of proteins using anchored mRNA. Mable H A et al. (Biotechnology Progress, American Institute of Chemical Engineers, US, Vo. 11, 1 January 1995, pages 393-396, XP002940195) relates to RNA transcription from immobilized DNA templates.

**[0006]** The present inventors have provided various techniques for identification and development of useful proteins using the IVV (*in vitro* virus) method (Patent Document 1). The IVV method has attracted attention as a method of selecting a peptide molecule of interest from large quantities of peptides having random sequences or a peptide library comprising such peptides, which is advantageous in terms of evolutionary engineering. The IVV method is capable of matching nucleic acid sequence information and the activity or function of the corresponding protein on a one-to-one basis. Thus, if this method could be used in a comprehensive analysis, proteins with desired activity and information regarding nucleic acid sequences encoding the same can be obtained simultaneously in a large volume. However, at the present stage, techniques of applying such method to comprehensive analyses have not yet been reported.

**[0007]**

[Non-Patent Document 1] Weng et al., Proteomics. 2: 48-57, 2002

[Patent Document 1] International Publication WO2006/041194

Disclosure of the Invention

Problems to be Solved by the Invention

**[0008]** Under the aforementioned circumstances, the present inventors have conducted intensive studies directed towards developing a method of individually immobilizing each mutant proteins on a chip from a DNA library comprising an enormous number of mutants, without a step of cloning them. As a result, the inventors have discovered that an mRNA chip and a protein chip that correspond to a DNA chip in which desired DNAs are aligned in an array form can be produced in a series of steps, by using a μTAS technique, thereby completing the present invention.

**[0009]** Thus, it is an object of the present invention to provide a method for producing a biomolecule assay chip using a microreactor chip, and a chip produced by the aforementioned method.

**[0010]** Moreover, it is another object of the present invention to provide a method for synthesizing another biomolecule from a biomolecule immobilized on the aforementioned chip without changing the position of the biomolecule immobilized, and a chip produced by the aforementioned method.

Means for Solving the Problems

**[0011]** Specifically, the present invention relates to the following (1) to (15):

(1) A first aspect of the present invention relates to a method for producing a chip on which protein-nucleic acid complexes are immobilized in an aligned state, which comprises the following steps (a) to (e):

(a) a step of producing a substrate 1 on which a plurality of DNA are immobilized in an aligned state;
(b) a step of filling microreactors on a microreactor chip comprising the microreactors at positions overlapping with the sequence positions of DNA immobilized on the substrate 1 produced in the step (a), with reaction reagents for synthesizing mRNA;
(c) a step of closely attaching the microreactor chip to the substrate 1 so that the reaction reagents for synthesizing mRNA are allowed to come into contact with DNA, and then carrying out a reaction of synthesizing mRNA in the microreactors;
(d) superposing the microreactor chip on a substrate 2 so that the reaction solutions contained in the microreactors on the microreactor chip are allowed to come into contact with the substrate 2 after completion of the step (c), so as to immobilize mRNA on the substrate 2; and
(e)immersing the chip with aligned mRNA on substrate 2 in a solution containing a cell-free translation system for synthesizing the protein-nucleic acid complex.

(2) A second aspect of the present invention relates to the method according to claim 1, wherein the step of producing "a substrate 1 on which a plurality of DNA are immobilized in an aligned state" described in the step (a) according to claim 1 further comprises the following steps (a) to (c):

(a) a step of diluting mixed solutions of DNA and reaction reagents for amplifying DNA, and then filling the microreactors on the microreactor chip with the diluted solutions, so that a single molecule or less of DNA can be present therein in a probability distribution manner;
(b) a step of carrying out a reaction of amplifying DNA; and
(c) a step of superposing the microreactor chip on the substrate 1 so that the reaction solutions contained in the microreactors on the microreactor chip are allowed to come into contact with the substrate 1, so as to immobilize the amplified DNA on the substrate

(3) A third aspect of the present invention relates to the method according to (2) above, wherein the reaction of amplifying DNA is a polymerase chain reaction.
(4) A fourth aspect of the present invention relates to the method according (1) to (3) above, wherein the DNA are those to which linker DNA ligates.
(5) A fifth aspect of the present invention relates to "the method according to (4) above, wherein puromycin binds to the linker DNA.
(6) A sixth aspect of the present invention relates to "the method according (1) to (5) above, wherein it comprises immobilizing avidin on the substrate 1 and thereby biotinylating DNA.
(7) A seventh aspect of the present invention relates to the method according to any one of (1) to (6) above, wherein the mRNA is immobilized on the substrate 2 via the puromycin-binding linker DNA.

Effects of the Invention

**[0012]** According to the present invention, chips on which DNAs, RNAs, and proteins are immobilized to the corresponding positions can be produced. Thus, it becomes possible to monitor the results by matching nucleic acid sequence information on the DNA and the function of the protein on a one-to-one basis.

**[0013]** Using the method of the present invention and chip produced therefrom, a molecule (DNA or a protein) which has been modified to achieve desired functions can be identified in a relatively short time.

**[0014]** Using the method of the present invention and chip produced therefrom, gene functions (particularly associated with enzyme) obtained by genome analysis or the like can be identified in a relatively short time.

**[0015]** Using the method of the present invention and chip produced therefrom, several gene combinations are produced in order to analyze a metabolic pathway, and they are then assayed, so as to rapidly identify a pathway from a substrate to a metabolite.

**[0016]** When the method of the present invention and chip produced therefrom are used, an excellent inhibitor candidate can be selected by adding a single type of enzyme to each well, also adding a substrate and an inhibitor candidate thereto, and observing a change in the concentration of the substrate due to the addition of the substrate and the inhibitor candidate.

Brief Description of the Drawings

**[0017]**

Figure 1 is a schematic view showing a process of producing the chip of the present invention.
Figure 2 is a view schematically showing a step of constructing a GFP mutant library.
Figure 3 shows the results of electrophoresis of a PCR product obtained by diluting a GFP cDNA library and then performing PCR using it as a template.
Figure 4 shows the results obtained by confirming a single molecule of PCR product immobilized on a chip by a method of detecting the fluorescence of

Cy3. Neg.: a negative control containing no DNA.

Figure 5 shows the results obtained by allowing a chip on which a PCR product is immobilized to come into contact with a microplate filled with T7 transcriptase and a reaction solution thereof so as to carry out a transcription reaction, and then by confirming RNA existing in each well on the microplate via electrophoresis.

Figure 6 shows the measurement results of enzyme reactions (aldehyde-degrading enzyme) in microreactors.

Best Mode for Carrying Out the Invention

**[0018]** The present invention provides: a method for producing a chip, which comprises repeating a step of superposing a microreactor chip comprising microreactors on a substrate to synthesize other biomolecules from biomolecules immobilized in an aligned state without changing the positions of the biomolecules immobilized, and finally producing a chip on which biomolecules different from the initially immobilized biomolecules are immobilized. More specifically, there is provided, for example, a method for producing a chip on which proteins are immobilized from a chip on which DNAs are immobilized, via consecutive steps, without changing the corresponding positions of DNAs-proteins on the chips.

**[0019]** According to the present invention, from a DNA chip on which DNAs of a single type from various types of DNAs selected from a DNA library or the like are immobilized in an aligned state, a protein chip on which proteins encoded by the individual DNAs are immobilized in positions corresponding to the positions of the DNAs immobilized can be produced.

**[0020]** Moreover, if the proteins immobilized on the aforementioned protein chip are immobilized as complexes with mRNAs, it is possible to carry out a reverse transcription reaction on the protein chip, and thereby to produce a chip on which DNAs encoding the proteins are immobilized. Herein, with regard to mRNA, a DNA chip is superposed on a microreactor chip, and a transcription reaction is carried out in each microreactor, so that the corresponding mRNA can be obtained in the microreactor.

**[0021]** A complex of mRNA and a protein can be produced, for example, via linker DNA to which a compound specifically binding to a protein, such as puromycin, binds. That is, if linker DNA having a sequence complementary to the 3'-terminus of mRNA, to which puromycin binds, is used, the linker binds to the mRNA via a complementary portion. Thus, in the generated complex, puromycin binds to the mRNA via the linker DNA. It is to be noted that the binding of linker DNA to puromycin can be carried out according to a known method.

**[0022]** Furthermore, using a microreactor chip comprising microreactors corresponding to the positions of proteins immobilized on the chip, the activity and the like of all the proteins immobilized on the chip can be measured, after production of the chip. For example, a microreactor chip in which reaction solutions for measuring the activity of proteins immobilized on the chip are added to individual microreactors is superposed on a substrate, so that the positions of the microreactors correspond to the positions of the proteins immobilized, and so that each protein is allowed to sufficiently come into contact with the reaction solution in each microreactor. Thereafter, an activity-measuring reaction is carried out. Thus, it becomes possible to simultaneously analyze the activity of all the proteins immobilized on the chip.

**[0023]** The present method is useful for selection of a mutant having the excellent activity of a protein of interest, etc. For instance, when a chip on which a DNA library in which mutations have been systematically introduced into specific proteins is immobilized is used as an initial DNA chip, production of a protein chip corresponding to the DNA chip, the measurement of the activity of proteins on the protein chip, and evaluation of such activity can be carried out as a single step. If a desired protein can be identified based on the evaluation results, DNA encoding the protein can be easily obtained by reverse transcription of mRNA. Thus, the primary sequence information of the protein can also be easily obtained. Based on the obtained primary sequence information, other proteins can be further searched.

**[0024]** The main embodiments of the present invention will be described below.

**[0025]** An embodiment of the present invention relates to a method for producing a chip on which protein-nucleic acid complexes are immobilized in an aligned state, which comprises the following steps (a) to (e):

(a) a step of producing a substrate 1 on which a plurality of DNA are immobilized in an aligned state;
(b) a step of filling microreactors on a microreactor chip comprising the microreactors at positions overlapping with the sequence positions of DNA immobilized on the substrate 1 produced in the step (a), with reaction reagents for synthesizing mRNA;
(c) a step of closely attaching the microreactor chip to the substrate 1 so that the reaction reagents for synthesizing mRNA are allowed to come into contact with DNA, and then carrying out a reaction of synthesizing mRNA in the microreactors;
(d) superposing the microreactor chip on a substrate 2 so that the reaction solutions contained in the microreactors on the microreactor chip are allowed to come into contact with the substrate 2 after completion of the step (c), so as to immobilize mRNA on the substrate 2; and
(e)immersing the chip with aligned mRNA on substrate 2 in a solution containing a cell-free translation system for synthesizing the protein-nucleic acid complex.

**[0026]** Herein, the "DNA" can preferably be converted to another biomolecule *in vitro*. The "DNA" should be im-

mobilized on a substrate in an aligned state. A method using an avidin-biotin bond, a method of modifying DNA with a functional group such as an amino group, an aldehyde group or an SH group, and then subjecting a chip to a surface treatment with a silane coupling agent having an amino group, an aldehyde group, an epoxy group or the like, and other methods can be used for immobilization of the DNA. Of these, a method using an avidin-biotin bond is particularly preferable. In this case, it is preferable that avidin be immobilized on a substrate and that biotin bind to DNA.

**[0027]** The term "microreactor chip" is used herein to mean an ultrafine reacting device produced by forming fine flow passages or fine hollows on a substrate such as a polymer, glass or silicon, in which a chemical reaction can be carried out in the flow passages or hollows (microreactors). In order to suppress adsorption of biomolecules, the surface of the microreactor chip is preferably modified with PEG and the like.

**[0028]** In the present embodiment, it is necessary to allow the biomolecule immobilized on a substrate to come into contact with a reaction reagent in each microreactor, so that they can be reacted with each other. Herein, the surfaces of substrate 1 and substrate 2 are not necessarily flat. For example, in order to increase the surface area on which biomolecules are immobilized, asperity may be formed thereon. However, when the substrate is superposed on the microreactor chip, in order to prevent the leakage of reagents and the like from all the microreactors on the microreactor chip, the surface of a portion of the substrate, with which the microreactor chip is allowed to come into contact, should be flat.

**[0029]** Moreover, in order to carry out the aforementioned reaction under optimal conditions, other apparatuses for controlling the temperature of the microreactor, a pH condition in the microreactor, etc. may be combined with the microreactor chip. When the biomolecule 1 is DNA and the biomolecule 2 is RNA, for example, the term "reaction reagent" is used herein to mean a reagent necessary for transcription. As such reaction reagent, either a commercially available reaction reagent or an originally prepared reaction reagent may be used.

**[0030]** The substrate 1 used in the step (a) of the present embodiment can also be produced by the following method. A mixture of several types of DNAs (e.g. a DNA library, etc.) is first mixed with a DNA amplification reagent. The obtained mixture is diluted with a suitable buffer, and it is then dispensed into microreactors. Herein, the mixture is diluted with the buffer, and the thus diluted solution is then dispensed in each microreactor, so that a single molecule of DNA can be contained in each microreactor. The timing of dilution of the DNA and the amplification reagent is not particularly limited. After completion of the pouring, the conditions of the microreactor chip are determined, so that the conditions are suitable for amplification of the DNA. Thereafter, the reaction is carried out. As a result, DNAs of different types can be amplified in individual microreactors. Subsequently, the microreactor chip comprising the amplified DNAs is closely attached to the substrate 1, so that the chip is allowed to come into contact with the substrate, and the DNA molecule in the microreactor is thereby immobilized on the substrate 1. When DNA is amplified, a PCR reaction is preferably used. For the PCR reaction, commercially available products such as a reaction solution necessary for the reaction can be used. Moreover, when the DNA in the microreactor is immobilized on the substrate, an amplification reaction is performed so that biotin is incorporated into the DNA, whereas the substrate is coated with avidin. Thus, the DNA can be easily immobilized on the substrate via an avidin-biotin bond. As a method of incorporating biotin into DNA, a method using PCR primers labeled with biotin and the like can be applied.

**[0031]** Furthermore, DNA serving as a linker may be ligated to the nucleic acid, and puromycin may be bound to the terminus thereof.

**[0032]** In the present embodiment, the mRNA synthesized in the microreactors are immobilized on another substrate (substrate 2), so as to produce a chip on which the mRNA are immobilized in an aligned state. Herein, as a method of immobilizing the mRNA on a substrate, a linker having a sequence complementary to a portion of the RNA, for example, has previously been immobilized on the substrate, so that the RNA in the microreactor can be easily immobilized on the substrate. The linker used herein preferably has a sequence complementary to sequences shared by all different types of RNAs existing in different microreactors. As the "sequences shared by all different types of RNAs," the sequences of PCR primers used in amplification of DNA used as a template of RNA and the like may be used. Further, puromycin may also bind to the terminus of a linker. It is desired to establish a hydrophilic flexible structure such as polyethylene glycol (PEG) between such linker DNA and puromycin. Moreover, it is preferable to establish a sequence portion consisting of one or more nucleotides that does not hybridize with mRNA, so that it can be easily ligated via T4 RNA ligase after completion of the hybridization with the mRNA.

**[0033]** This disclosure relates to: a method for synthesizing proteins from the mRNA immobilized on a chip produced in the aforementioned embodiment by allowing the produced chip to come into contact with various types of reaction solutions; and a chip produced by the aforementioned method. A solution containing a cell-free translation system is allowed to come into contact with a chip on which the mRNA is immobilized, so as to react them using the mRNA as a template to synthesize a protein. In this case, if a puromycin-binding linker is used in the aforementioned first embodiment, the synthesized protein binds to the linker via the puromycin, and thus a chip on which the porteins are immobilized can be produced. The cell-free translation systems that can be used herein include *Escherichia coli*, rabbit reticulocyte, and wheat germ-derived cell-free system. Commercially available products may also be used. The thus synthe-

sized protein is a complex with mRNA, liker DNA, and a protein. Further, a complex of the protein with DNA, linker DNA, and a protein, corresponding to the DNA, can also be produced by performing a reverse transcription reaction from mRNA to DNA in a reaction solution that contains reverse transcriptase.

**[0034]** By measuring the activity or the like of the protein immobilized on the chip produced in the aforementioned other embodiment, a protein retaining desired activity can be easily identified on the chip. Furthermore, by determining the sequence of DNA corresponding to the identified protein, the sequence information of the DNA corresponding to the protein having desired activity can be easily obtained. Herein, when the activity of a protein is measured, there is prepared a microreactor chip comprising microreactors corresponding to the positions of the immobilized on the chip produced in the aforementioned other embodiment. Thereafter, each microreactor on the microreactor chip has previously been filled with a solution necessary for the measurement of the protein activity, and the aforementioned chip is superposed on the microreactor chip for the reaction, thereby measuring the activity of the protein immobilized on the chip.

**[0035]** Examples will be given below. However, these examples are not intended to limit the scope of the present invention.

Examples

**[0036]** A method for producing a protein chip from DNA is as follows (Figure 1).

Step 1: Construction of a library (cDNA)
Step 2: Dilution of a library to result in a concentration of a single molecule on a chip and single-molecule PCR
Step 3: Immobilization of a PCR product on the chip
Step 4: Transcription on the chip
Step 5: Transfer of a transcription product to another chip and a reaction of ligating it to linker DNA on the surface thereof
Step 6: Addition of cell-free translation system to the chip of step 5, and immobilization of DNA and a protein on the chip by a reaction of ligating RNA to the protein and a reverse transcription reaction

1. Construction of library

**[0037]** GFP (Green Fluorescent Protein) was selected as a model protein, and random mutation was introduced into the threonine at position 65 that is important for fluorescence activity so as to create a library. In this library, a DNA construct having a T7 promoter and 5' UTR (TMV omega) at the 5'-terminal side and a spacer at the 3'-terminal side was constructed by the method as shown in Figure 2. The following primer 1 and primer 2 were used to amplify a DNA fragment comprising the T7 promoter and 5'UTR region of the first step. In addition, the following primer 3 and primer 4 were used to perform PCR on a region containing GFP.

(Primer 1)
5'-GATCCCGCGAAATTAATACGACTCACTATAGGG-3' (SEQ ID NO: 1)
(Primer 2)
5'-CAGAGTAGTGACAAGTGTTGGCCATGG-3' (SEQ ID NO: 2)
(Primer 3)
5'-TTTCCCCGCCGCCCCCTTATTATTATTTGTAGAGCTCATCCATGC-3' (SEQ ID NO: 3)
(Primer 4)
5'-GGCCAACACTTGTCACTACTCTGNNNTATGGTGTTCAATGCTTTTCCCG-3' (SEQ ID NO: 4)

2. Dilution to result in concentration of single molecule DNA and single-molecule PCR

**[0038]** A fragment (853 bp) amplified by PCR was purified using the DNA purification kit of QIAGEN, resulting in a concentration of 7.04 nM. This cDNA was diluted with nuclease-free purified water to have concentrations of $4 \times 10^8$, $4 \times 10^7$, $4 \times 10^6$, $4 \times 10^5$, $4 \times 10^4$, $4 \times 10^3$, $4 \times 10^2$, 40, 4, and 0.4 molecules per μl. Using these as templates, a PCR reaction was carried out in a 1 × ExTaq PCR buffer (Takara), with dNTP of a final concentration of 0.2 mM, a 2-μM forward primer (5'-Cy3GATCCCGCGAAATTAATACGACTCACTATAGGG-3' (SEQ ID NO: 5); the 5'-terminus labeled with Cy3), and a reverse primer (5'-BiotinTTTCCCCGCCGCCCCCTTATTATTATTTGTAGAGC (SEQ ID NO: 6); the 5'-terminus labeled with biotin), also using 0.5 U ExTaq DNA polymerase (Takara). The PCR reaction conditions consisted of 50 cycles of 95°C for 2 minutes and (94°C (30 seconds), 64°C (30 seconds) and 72°C (60 seconds)), and finally an elongation reaction at 72°C for 10 minutes. 2 μl of aliquot was obtained from the obtained reaction product, and it was then analyzed by 8 M urea denatured polyacrylamide gel electrophoresis (Figure 3).

3. Immobilization of PCR product on chip

**[0039]** The amplified single-molecule PCR product was purified with the purification column of QIAGEN, and the concentration thereof was then measured with Nanodrop. The PCR solution containing biotinylated DNA was mixed with an equal volume of 2 × binding buffer (20 mM Tris-HCl (pH 8.0), 2 mM EDTA, 2 M NaCl, and 0.2% TritonX-100), and the obtained mixture was then added to a 384-well microplate. Subsequently, a 10-μl-scale reaction product was transferred onto a streptavidin-coated slide glass (ArrayIt), and it was then incubated for 15 minutes. Thereafter, the surface of the glass was washed with 1 × binding buffer. The amplified DNA emitted the fluorescence of Cy3. Thus, the slide glass was

examined using a fluoroimager (Typhoon) (Figure 4).

4. Transcription on chip

**[0040]** The cDNA on the aforementioned slide glass was further superposed on a 384-well microplate filled with T7 reverse transcriptase and 20 μl of the reaction solution (RiboMAX; Promega), so that they were reacted at 37°C for 3 hours. Thereafter, the DNA template was decomposed by DNase, the reaction solution was then collected from each well, and it was then purified using RNA RNeasy Kit (QIAGEN). It was analyzed by 8 M urea denatured polyacrylamide gel electrophoresis (Figure 5).

5. Measurement of biomolecular activity in microreactor

**[0041]** In order to measure the activity of biomolecules immobilized on a chip produced by the present invention, the activity of a model protein was measured. As a measurement model system, aldehyde reductase (AKR) was used. A trace enzyme reaction was measured on a microreactor array chip comprising 10,000 microreactors with a diameter of 6 μm and a height of 6 μm (0.15 pl) produced from PDMS (Polydimethylsiloxane) produced by the aforementioned method. That is, 2 μM (10,000/reactor) enzyme AKR, 20 mM glucuronate as a substrate, and 0.2 mM NADPH as a coenzyme were enclosed in a reactor. For an aldehyde reductase reaction (aldehyde + NADPH → alcohol + NADP+), using the i ray (365 nm) of a mercury lamp as an excitation light, the self-fluorescence intensity of NADPH with a wavelength of 400 nm or greater was detected using a high-sensitive cold CCD camera. Based on a change in the fluorescence intensity of NADPH, the progression of the aldehyde reductase reaction was measured. From the measurement results (Figure 6), the enzyme activity was calculated to be 1-10 (NADPH oxidized/min/AKR). Thus, it became clear that the activity of enzyme contained in each microreactor can be evaluated by optical measurement. In addition, such enzyme reaction can be evaluated not only by such optical measurement but also by electrochemical measurement. It is also possible to realize parallel measurement by accumulating microelectrodes in such a microreactor.

Industrial Applicability

**[0042]** The chip of the present invention is useful for the high throughput processing of an assay for pharmaceutical candidate molecules in the development of pharmaceutical products. In addition, the present chip can also be used as a diagnostic chip in the field of diagnostic agents, diagnostic apparatuses, and the like. Moreover, it can also be used in the analyses of metabolizing systems such as bioethanol synthase. Furthermore, the method of the present invention for producing the aforementioned chip can contribute as an evolutional engineering method to the discovery of functional molecules useful for environmental and energetic fields in the future.

**Claims**

1. A method for producing a chip on which protein-nucleic acid complexes are immobilized in an aligned state, which comprises the following steps (a) to (e):

   (a) a step of producing a substrate 1 on which a plurality of DNA are immobilized in an aligned state;
   (b) a step of filling microreactors on a microreactor chip comprising the microreactors at positions overlapping with the sequence positions of DNA immobilized on the substrate 1 produced in the step (a), with reaction reagents for synthesizingmRNA;
   (c) a step of closely attaching the microreactor chip to the substrate 1 so that the reaction reagents for synthesizing mRNA are allowed to come into contact with DNA, and then carrying out a reaction of synthesizing mRNA in the microreactors;
   (d) superposing the microreactor chip on a substrate 2 so that the reaction solutions contained in the microreactors on the microreactor chip are allowed to come into contact with the substrate 2 after completion of the step (c), so as to immobilize mRNA on the substrate 2; and
   (e)immersing the chip with aligned mRNA on substrate 2 in a solution containing a cell-free translation system for synthesizing the protein-nucleic acid complex.

2. The method according to claim 1, wherein the step of producing "a substrate 1 on which a plurality of DNA are immobilized in an aligned state" described in the step (a) according to claim 1 further comprises the following steps (a) to (c):

   (a) a step of diluting mixed solutions of DNA and reaction reagents for amplifying DNA, and then filling the microreactors on the microreactor chip with the diluted solutions, so that a single molecule or less of DNA can be present therein in a probability distribution manner;
   (b) a step of carrying out a reaction of amplifying DNA; and
   (c) a step of superposing the microreactor chip on the substrate 1 so that the reaction solutions contained in the microreactors on the microreactor chip are allowed to come into contact with the substrate 1, so as to immobilize the amplified DNA on the substrate.

3. The method according to claim 2, wherein the reaction of amplifying DNA is a polymerase chain reac-

tion.

4. The method according to any one of claims 1 to 3, wherein the DNA are those to which linker DNA ligates.

5. The method according to claim 4, wherein puromycin binds to the linker DNA.

6. The method according to any one of claims 1 to 5, wherein it comprises immobilizing avidin on the substrate 1 and thereby biotinylating DNA.

7. The method according to any one of claims 1 to 6, wherein the mRNA is immobilized on the substrate 2 via the puromycin-binding linker DNA.

**Patentansprüche**

1. Verfahren zur Herstellung eines Chips, an dem Protein-Nukleinsäure-Komplexe in einem alignierten Zustand immobilisiert sind, welches die folgenden Schritte (a) bis (e) umfasst:

   (a) einen Schritt zur Herstellung eines Substrats 1, an dem eine Vielzahl von DNA in einem alignierten Zustand immobilisiert ist;
   (b) einen Schritt zum Befüllen von Mikroreaktoren an einem Mikroreaktor-Chip, umfassend die Mikroreaktoren an Positionen, die sich mit den Sequenzpositionen von an dem Substrat 1 immobilisierter DNA überlappen, das in dem Schritt (a) hergestellt wird, mit Reaktionsreagenzien für die Synthese von mRNA;
   (c) einen Schritt zum engen Anlagern des Mikroreaktor-Chips an dem Substrat 1, sodass die Reaktionsreagenzien für die Synthese von mRNA mit DNA in Kontakt treten können und anschließend das Ausführen einer Reaktion zur Synthese von mRNA in den Mikroreaktoren;
   (d) Auflagern des Mikroreaktor-Chips auf ein Substrat 2, sodass die in den Mikroreaktoren an dem Mikroreaktor-Chip enthaltenen Reaktionslösungen mit dem Substrat 2 nach Abschluss des Schrittes (c) in Kontakt treten können, um mRNA an dem Substrat 2 zu immobilisieren; und
   (e) Eintauchen des Chips mit alignierter mRNA an Substrat 2 in eine Lösung, die ein zellfreies Translationssystem für die Synthese des Protein-Nukleinsäure-Komplexes enthält.

2. Verfahren nach Anspruch 1, wobei der in dem Schritt (a) beschriebene Schritt zur Herstellung "eines Substrats 1, an dem eine Vielzahl von DNA in einem alignierten Zustand immobilisiert ist" nach Anspruch 1 ferner die folgenden Schritte (a) bis (c) umfasst:

   (a) einen Schritt zum Verdünnen von Mischlösungen von DNA und Reaktionsreagenzien zur Amplifikation von DNA und anschließend das Befüllen der Mikroreaktoren an dem Mikroreaktor-Chip mit den verdünnten Lösungen, sodass ein einzelnes Molekül oder weniger von DNA darin mit einer Wahrscheinlichkeitsverteilung vorliegen kann;
   (b) einen Schritt zur Durchführung einer Reaktion zur Amplifikation von DNA; und
   (c) einen Schritt zum Auflagern des Mikroreaktor-Chips auf das Substrat 1, sodass die in den Mikroreaktoren an dem Mikroreaktor-Chip enthaltenen Reaktionslösungen mit dem Substrat 1 in Kontakt treten können, um die amplifizierte DNA an dem Substrat zu immobilisieren.

3. Verfahren nach Anspruch 2, wobei die Reaktion zur Amplifikation von DNA eine Polymerase-Kettenreaktion ist.

4. Verfahren nach einem der Ansprüche 1 bis 3, wobei die DNA diejenige ist, an die sich Linker-DNA bindet.

5. Verfahren nach Anspruch 4, wobei sich Puromycin an die Linker-DNA bindet.

6. Verfahren nach einem der Ansprüche 1 bis 5, wobei es die Immobilisierung von Avidin an dem Substrat 1 und somit die Biotinylierung von DNA umfasst.

7. Verfahren nach einem der Ansprüche 1 bis 6, wobei die mRNA an dem Substrat 2 über die Puromycin bindende Linker-DNA immobilisiert ist.

**Revendications**

1. Procédé de construction d'une puce sur laquelle des complexes protéine-acide nucléique sont immobilisés à l'état aligné, qui comprend les étapes (a) à (e) suivantes :

   (a) une étape consistant à préparer un substrat 1 sur lequel une pluralité d'ADN sont immobilisés à l'état aligné ;
   (b) une étape consistant à remplir des microréacteurs sur une puce à microréacteurs comprenant les microréacteurs au niveau de positions chevauchant les positions séquentielles des ADN immobilisés sur le substrat 1 préparé dans l'étape (a), avec des réactifs pour synthétiser l'ARNm ;
   (c) une étape consistant à fixer étroitement la puce à microréacteurs au substrat 1 de sorte que les réactifs pour synthétiser l'ARNm soient amenés à venir en contact avec l'ADN, et ensuite à effectuer une réaction de synthèse

d'ARNm dans les microréacteurs ;
(d) une étape consistant à superposer la puce à microréacteurs sur un substrat 2 de sorte que les solutions réactionnelles contenues dans les microréacteurs sur la puce à microréacteurs soient amenées à venir en contact avec le substrat 2 au terme de l'étape (c), de façon à immobiliser l'ARNm sur le substrat 2 ; et
(e) une étape consistant à immerger la puce avec les ARN alignés sur le substrat 2 dans une solution contenant un système de traduction sans cellule pour synthétiser le complexe protéine-acide nucléique.

**2.** Procédé selon la revendication 1, ladite étape de préparation «d'un substrat 1 sur lequel une pluralité d'ADN sont immobilisés à l'état aligné » décrite dans l'étape (a) selon la revendication 1 comprend en outre les étapes (a) à (c) suivantes :

(a) une étape consistant à diluer des solutions mixtes d'ADN et de réactifs pour amplifier l'ADN, et ensuite à remplir les microréacteurs sur la puce à microréacteurs avec les solutions diluées, de sorte qu'une seule molécule d'ADN ou moins puisse y être présente selon une distribution de probabilités ;
(b) une étape consistant à effectuer une réaction d'amplification d'ADN ; et
(c) une étape consistant à superposer la puce à microréacteurs sur le substrat 1 de sorte que les solutions réactionnelles contenues dans les microréacteurs sur la puce à microréacteurs soient amenées à venir en contact avec le substrat 1, de façon à immobiliser l'ADN amplifié sur le substrat.

**3.** Procédé selon la revendication 2, ladite réaction d'amplification d'ADN étant une amplification en chaîne par polymérase.

**4.** Procédé selon l'une quelconque des revendications 1 à 3 ; lesdits ADN étant ceux auquel un ADN lieur se ligature.

**5.** Procédé selon la revendication 4, la puromycine se liant à l'ADN lieur.

**6.** Procédé selon l'une quelconque des revendications 1 à 5, celui-ci comprenant l'immobilisation d'avidine sur le substrat 1 et ainsi la biotinylation de l'ADN.

**7.** Procédé selon l'une quelconque des revendications 1 à 6, ledit ARNm étant immobilisé sur le substrat 2 par le biais de l'ADN lieur se liant à la puromycine.

Figure 1

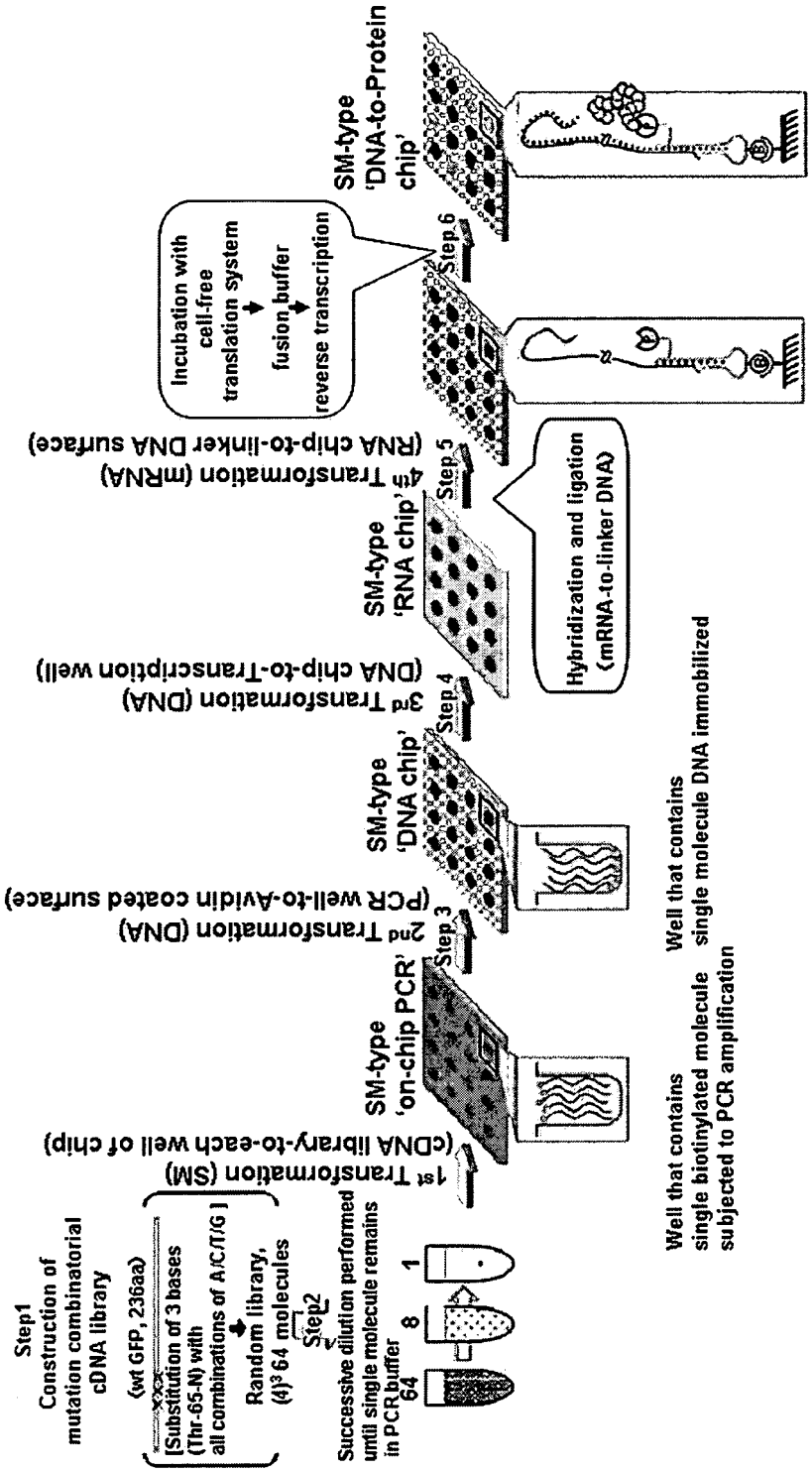

Step1
Construction of mutation combinatorial cDNA library
(wt GFP, 236aa)
[Substitution of 3 bases (Thr-65-N) with all combinations of A/C/T/G ]
Random library, (4)3 64 molecules
Step2
Successive dilution performed until single molecule remains in PCR buffer
64
8
1
1st Transformation (SM)
(cDNA library-to-each well of chip)
SM-type 'on-chip PCR'
Step 3
2nd Transformation (DNA)
(PCR well-to-Avidin coated surface)
SM-type 'DNA chip'
Step 4
3rd Transformation (DNA)
(DNA chip-to-Transcription well)
SM-type 'RNA chip'
Step 5
4th Transformation (mRNA)
(RNA chip-to-linker DNA surface)
Hybridization and ligation
(mRNA-to-linker DNA)
Incubation with
cell-free
translation system
fusion buffer
reverse transcription
Step 6
SM-type 'DNA-to-Protein chip'
Well that contains single biotinylated molecule
Well that contains single molecule DNA immobilized subjected to PCR amplification

Figure 2

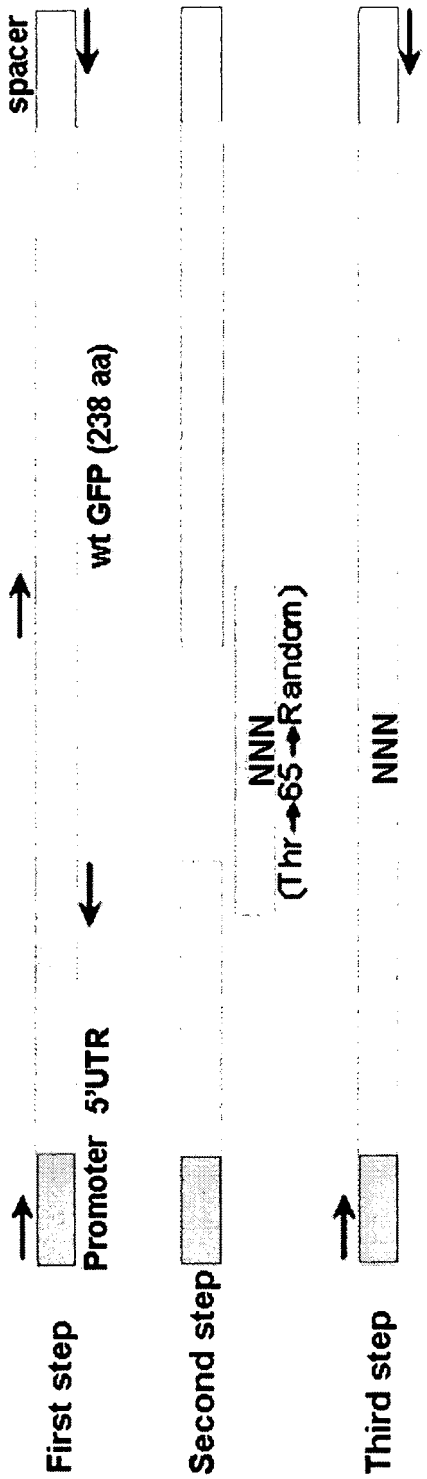
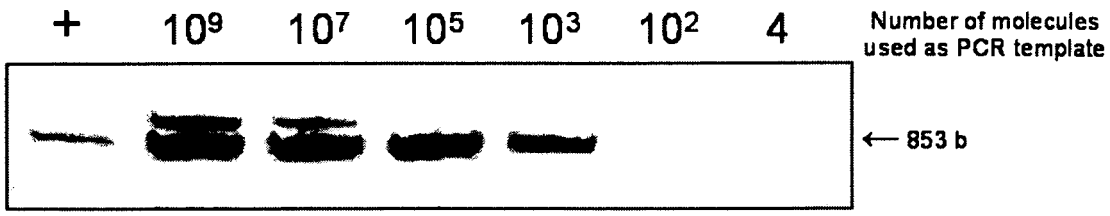

spacer
wt GFP (238 aa)
NNN
(Thr→65→Random)
NNN
Promoter
5'UTR
First step
Second step
Third step

Figure 3




(*PCR was performed for 30 cycles)

Figure 4

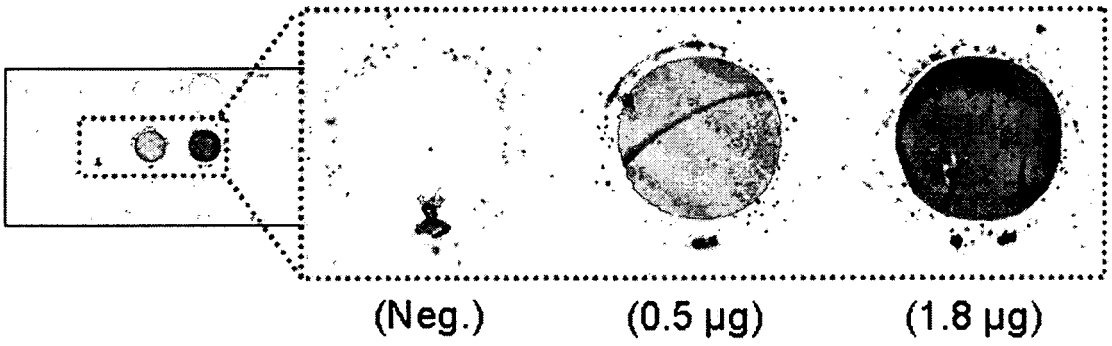


Figure 5

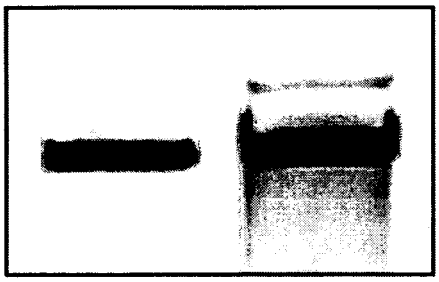
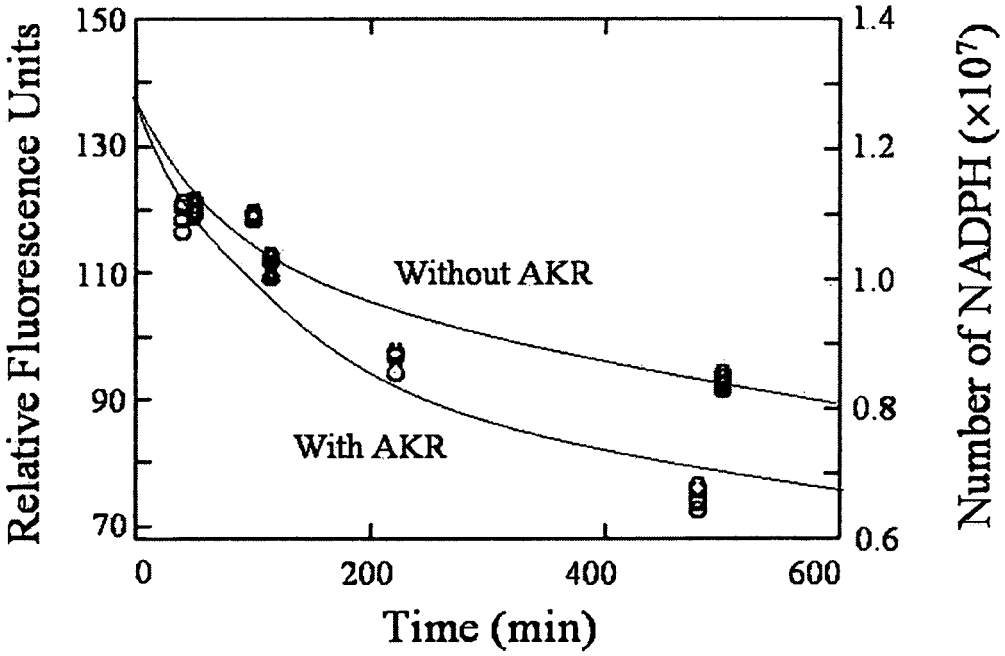



Figure 6



## REFERENCES CITED IN THE DESCRIPTION

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- **BIYANI MANISH et al.** *Nucleic Acids Research,* 01 January 2006, vol. 34 (20), 140-1 **[0005]**
- **MABLE H A et al.** *Biotechnology Progress, American Institute of Chemical Engineers,* 01 January 1995, vol. 11, 393-396 **[0005]**
- **WENG et al.** *Proteomics,* 2002, vol. 2, 48-57 **[0007]**