(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
3 December 2009 (03.12.2009)

PCT

(10) International Publication Number
WO 2009/145355 A1

(51) International Patent Classification:
C12Q 1/68 (2006.01)

(21) International Application Number:
PCT/JP2009/060208

(22) International Filing Date:
28 May 2009 (28.05.2009)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
2008-139638 28 May 2008 (28.05.2008) JP

(71) Applicants *(for all designated States except US)*: **FUJIFILM Corporation** [JP/JP]; 26-30, Nishiazabu 2-chome, Minato-ku, Tokyo, 1060031 (JP). **National University Corporation Tokyo Medical and Dental University** [JP/JP]; 5-45, Yushima 1-chome, Bunkyo-ku, Tokyo, 1138510 (JP).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **KANEHARA, Hideyuki. INAZAWA, Johji. IMOTO, Issei.**

(74) Agents: **TAKAMATSU, Takeshi** et al.; Koh-Ei Patent Firm, Kawabe Bldg., 7-9, Shimbashi 3-chome, Minato-ku, Tokyo, 1050004 (JP).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

WO 2009/145355 A1

(54) **Title**: METHOD FOR MEASURING NUCLEIC ACID USING NUCLEIC ACID MICROARRAY

FIG. 1A

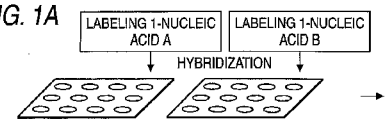


FIG. 1B

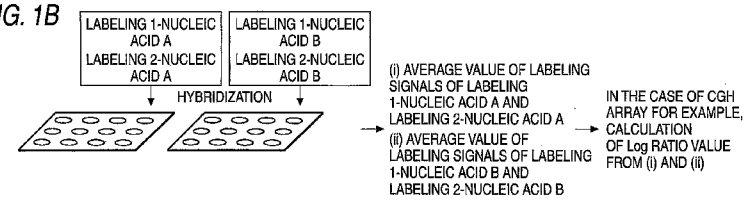
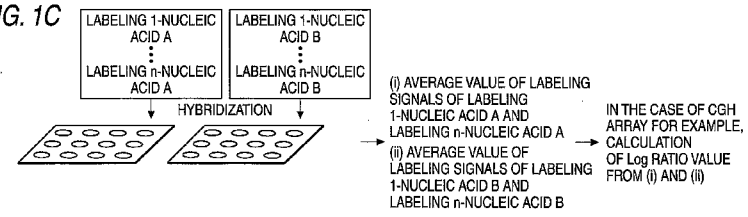


FIG. 1C



(57) **Abstract**: A method for measuring a target nucleic acid includes: labeling one target nucleic acid with at least two labeling reagents to prepare a labeled target nucleic acid; allowing the labeled target nucleic acid to hybridize with a probe nucleic acid on a nucleic acid microarray; reading labeled amounts obtained from the respective at least two labeling reagents of the hybridized nucleic acid; and averaging the labeled amounts to obtain an average value of the labeled amounts.

DESCRIPTION

METHOD FOR MEASURING NUCLEIC ACID USING NUCLEIC ACID MICROARRAY

Technical Field

This invention relates to a method for measuring a target nucleic acid by allowing a labeled target nucleic acid, prepared by labeling substantially the same target nucleic acid obtained from substantially the same target cell with at least two or more labeling reagents, to hybridize with a probe nucleic acid on a nucleic acid microarray, reading off their hybridized amounts, and averaging the labeled amounts obtained from respective labeling reagents or numerical values obtained by processing the same.

Background Art

Nucleic acid microarray was invented by Stephen P. A. Fodor *et al.* in 1991, which rendered possible analysis of from hundreds to tens of thousands of genes at the same time (S. P. A. Fodor *et al.*, Science, 251, 767 – 773 (1991)). Thereafter, various types of nucleic acid microarrays have been developed (Newest Techniques and Applications of Bio-chips (written in Japanese), edited by T. Matsunaga, CMC Publication (2004)).

Chromosome analyzing techniques are methods in which a chromosome is stained using a specific staining liquid and deletion or excess of a gene is detected from the staining pattern using a microscope, and there are G staining method, Q staining method and the like various methods.

Thereafter, FISH (Fluorescence In Situ Hybridization) method and CGH (Comparative Genomic Hybridization) method and SKY (Spectral Karyotyping) method based on the FISH method have been developed.

The CGH method is a method for detecting excess, deletion, amplification and the like abnormal copy numbers of a genomic DNA within a short period of time using a chromosome as the object. This is a technique developed by Joe Gray, Dan Pinkel, O-P Kallioniemi *et al.*, and the result has been reported in Science in 1992 (A. Kallioniemi *et al.*, Science, 258, 818 – 821 (1992)). However, there are various problems regarding its detection accuracy, such as a problem in that a chromosomal aberrancy cannot be detected when it is not occurring within a large region generally covering from 5 to 10 Mb (J. Inazawa and M. Minaguchi, Rinsho Kensa (Clinical Inspections), 49, 497 – 502 (2005)).

An array CGH method has been newly proposed as a tool which can detect a genomic structural aberrancy occurred at a level of from several 10 kb to several Mb between the base level resolution of sequencers and the resolution of 5 to 10 Mb by which chromosomal analysis can be handled (D. Pinkel *et al.*, Nat. Genet., 20, 207 – 211 (1998) and I. Imoto and J. Inazawa, Saibo Kogaku (Cell Engineering), 23, 355 – 361 (2004)). Different from the arrays which are used for inclusive analysis such as expression analysis and the like, the array CGH method uses an array in which genomic DNA fragments, such as BAC (Bacterial Artificial Chromosomes) clones and YAC (Yeast Artificial Chromosomes) clones, and oligonucleotide probes are spotted on a substrate, thereby arranging these nucleic acids on a solid substrate. This is a method for detecting the number of copies of genes by extracting DNA samples from an abnormal cell collected from a patient or the like and a normal cell collected from a healthy person, staining them with respectively different fluorescent

materials, mixing both of them, and then allowing them to undergo interaction with a probe on the nucleic acid microarray via hybridization, reading off fluorescence signals generated from the interacted nucleic acids using a scanner or the like, and comparing intensity ratio of the signals from the normal cell and abnormal cell.

When a nucleic acid microarray is carried out, a variation of data occurs caused by various factors. As a result of generating variation of data, accuracy and determinability of the nucleic acid microarray are reduced, so that various problems occur such as a wrong diagnosis when the nucleic acid microarray is used in diagnosis. As the method for correcting variations, a method in which the same probe nucleic acid is spotted at several points on a slide glass and an average value thereof is obtained, and the like have been reported (Report on the result, 2005th Yasukichi Mori Memorial Foundation for Research Promotion, Regulation of sulfur metabolic pathway in yeast using transcriptome and metabolome data, by J. Imoto (all written in Japanese)).

Summary of Invention

The problem to be solved by the invention is to provide a method for reducing variation of data, which frequently occurs in the nucleic acid microarray. In the case of CGH array for example, difference in the number of copies becomes clear and its diagnosing accuracy and the like can be improved by reducing variation of data.

The invention is a method for reducing variation of data occurring in the nucleic acid microarray, effected by simultaneously or individually carrying out labeling of substantially the same target nucleic acid obtained from substantially the same target cell with at least two or more labeling reagents, to hybridize with a probe nucleic acid on a nucleic acid microarray, and then calculating average of the labeled

amounts obtained from respective labeling reagents or of the numerical values obtained by processing the same.

That is, the invention consists of the following constructions.

(1) A method for measuring a target nucleic acid, the method comprising:

labeling one target nucleic acid with at least two labeling reagents to prepare a labeled target nucleic acid;

allowing the labeled target nucleic acid to hybridize with a probe nucleic acid on a nucleic acid microarray;

reading labeled amounts obtained from the respective at least two labeling reagents of the hybridized nucleic acid; and

averaging the labeled amounts to obtain an average value of the labeled amounts.

(2) The method as described in (1) above,

wherein at least one of the at least two labeling reagents is a fluorescent material.

(3) The method as described in (1) or (2) above,

wherein a solution containing the labeled target nucleic acid is subjected to a purification process.

(4) The method as described in (1) or (2) above,

wherein a solution containing the labeled target nucleic acid is not subjected to a purification process.

(5) The method as described in any one of (1) to (4) above,

wherein a labeled correcting nucleic acid obtained by linking a labeling reagent to a correcting nucleic acid is used together with the labeled target nucleic acid, the labeled correcting nucleic acid being capable of hybridizing with the probe

nucleic acid on the nucleic acid microarray.

(6) The method as described in any one of (1) to (5) above,

wherein the at least two labeling reagents include a combination of Cy3 and Cy5.

Brief Description of Drawings

Figs. 1A to 1C are theories on the case of applying the invention to the mono color method, wherein Fig. 1A is a theory of the conventional mono color method and Fig. 1B is a theory on the case of applying the invention to the mono color method. A nucleic acid A is labeled with labeling reagents 1 and 2 and hybridized on one plate of a nucleic acid microarray. Also, a nucleic acid B is labeled with the labeling reagents 1 and 2 and hybridized on another one plate of nucleic acid microarray. Average of labeled values obtained from the labeling reagent of respective labeling reagents is obtained. Thereafter, in the case of CGH array for example, the number of copies is computed by calculating Log Ratio. Fig. 1C is a theory of a case in which the invention was further generalized by applying it to the mono color method. The nucleic acid A is labeled with n species of labeling reagents and hybridized on one plate of a nucleic acid microarray. Also, the nucleic acid B is labeled with n specie of labeling reagents and hybridized on another one plate of nucleic acid microarray. Average of labeled values obtained from the labeling reagent of respective labeling reagents is obtained. Thereafter, in the case of CGH array for example, the number of copies is computed by calculating Log Ratio;

Figs. 2A to 2C are theories on the case of applying the invention to the two color method, wherein Fig. 2A is a theory of the conventional two color method and Fig. 2B is a theory on the case of applying the invention to the two color method. A

nucleic acid A is labeled with labeling reagents 1 and 2 and a nucleic acid B is labeled with the labeling reagents (1) and (2). A mixture thereof is hybridized on one plate of nucleic acid microarray. Average of labeled values obtained from the labeling reagent of respective labeling reagents is obtained. Thereafter, in the case of CGH array for example, the number of copies is computed by calculating Log Ratio. Fig. 2C is a theory of a case in which the invention was further generalized by applying it to the two color method. The nucleic acid A is labeled with n species of labeling reagents and the nucleic acid B is labeled with n specie of labeling reagents. A mixture thereof is hybridized on one plate of nucleic acid microarray. Average of labeled values obtained from the labeling reagent of respective labeling reagents is obtained. Thereafter, in the case of CGH array for example, the number of copies is computed by calculating Log Ratio;

Fig. 3 is a result of CGH array in the case of using one species of labeling reagent by the conventional mono color method;

Fig. 4 is an exemplary example of the case of using two species of labeling reagents by the mono color method by the invention. The Female was labeled using Cy3 and Cy5 and average value of their fluorescence values was calculated, and the Male was labeled using Cy3 and Cy5 and average value of their fluorescence values was calculated. The Log Ratio was calculated from these two fluorescence values;

Fig. 5 is a result of plotting a difference between the average value of Log Ratio values of the region corresponding to the X-chromosome (corresponds to the closed circles in Fig. 3) and the average value of Log Ratio values of the region corresponding to the autosome (corresponds to the open circles in Fig. 3), calculated from Fig. 3, and a difference between the average value of Log Ratio values of the region corresponding to the X-chromosome (corresponds to the closed circles in Fig.

4) and the average value of Log Ratio values of the region corresponding to the autosome (corresponds to the open circles in Fig. 4), calculated from Fig. 4. In comparison with the conventional method, namely a method which uses one species of labeling reagent, the method of the invention, namely a method in which two species of labeling reagents are used and the value calculated from their average value is used, a performance as the CGH array, namely difference between a mutation nucleic acid (in this case, the number of copies of Female X-chromosome as a model) and an autosome (nucleic acid not undergoing mutation), is large, and these results show that diagnoses can be carried out more easily; and

Fig. 6 is a result of plotting degree of dispersion of Log Ratio value of the region corresponding to the autosome (corresponds to the open circles in Fig. 3) calculated from Fig. 3 and degree of dispersion of Log Ratio value of the region corresponding to the autosome (corresponds to the open circles in Fig. 4) calculated from Fig. 4. In comparison with the conventional method, namely a method which uses one species of labeling reagent, the method of the invention, namely a method in which two species of labeling reagents are used and the value calculated from their average value is used, degree of dispersion is small and a performance as the CGH array, namely variation of data, is small, so that a difference between a mutation nucleic acid (in this case, the number of copies of Female X-chromosome as a model) and an autosome (nucleic acid not undergoing mutation) can be easily distinguished, and these results show that diagnoses can be carried out more easily.

Description of Embodiments

· Nucleic acid microarray

The nucleic acid microarray as used herein means an array in which a probe

nucleic acid is bonded to the surface of a solid material at a high density. The solid material is not particularly limited, with the proviso that it is a material to which a probe nucleic acid can be bonded, such as a generally used slide glass or the like glass material, as well as a plastic material. In addition, those in which the probe nucleic acid is enclosed in a gel substance can also be used. Shape of the solid material, such as plane, peak of a projection, granular, inside of a capillary or the like is not particularly limited too. A DNA microarray produced using a spotter and a DNA chip produced by a semiconductor technique are also included in the nucleic acid microarray of the invention.

· Multiple sample microarray

According to the invention, use of a multiple sample microarray is also possible. The multiple sample microarray is a nucleic acid microarray which has two or more hybridization regions on one plate of microarray. It is necessary to prepare the regions where hybridization is carried out in such a manner that these are not mutually mixed with a hybridization solution which contains a labeled nucleic acid and the like. For example, distance between the hybridization regions can be separated. Also, it is possible to change polarity of the hybridization regions from that of the other regions. For example, the hybridization regions are made into hydrophilic nature, and the other regions into hydrophobic nature. In addition, heights of the hybridization regions and other regions can also be changed. For example, height of the non-hybridization regions can be increased by lowering height of the material of hybridization regions in comparison with that of the regions other than the hybridization regions, or by applying a seal or the like to at least a part of the regions having no relation to the hybridization regions, so that the hybridization solution which contains a labeled nucleic acid and the like is not mixed between the

hybridization regions. The number of hybridization regions is not particularly limited in the invention, and it may be two or more in the case of the multiple sample microarray. In addition, it is desirable that kind, concentration, the number of spots and position of the probe to be fixed to the hybridization regions are identical, but since there is a case in that that these can be corrected by data treatment, these parameters may be different.

For example, in citing an exemplary example of the use of the multiple sample microarray, in the case of the mono color method CGH array as a kind of mono color method multiple sample microarray, it becomes possible to reduce variation of data when hybridization is carrying out, on one slide glass, at a hybridization region where a normal cell-derived labeled target nucleic acid prepared by labeling a target nucleic acid with Cy3 and Cy5 hybridizes and at another hybridization region where an abnormal cell-derived labeled target nucleic acid labeled with Cy3 and Cy5 hybridizes, and then Log Ratio is calculated from the average value of fluorescence values calculated from fluorescence values of the normal cell-derived Cy3-target nucleic acid and Cy5-target nucleic acid and the average value of fluorescence values calculated from fluorescence values of the abnormal cell-derived Cy3-target nucleic acid and Cy5-target nucleic acid.

· Hybridization region

The hybridization region according to the invention is a term used for the multiple sample microarray, and for example, when two hybridization regions are arranged on one plate of multiple sample array using a normal cell-derived labeled target nucleic acid and an abnormal cell-derived labeled target nucleic acid as the samples in the case of hybridization by mono color method CGH array, an effect similar to the case of carrying out an array test twice can be obtained by allowing a

sample containing the normal cell-derived labeled target nucleic acid to hybridize with one of the hybridization regions and allowing a sample containing the abnormal cell-derived labeled target nucleic acid to hybridize with the other hybridization region. In this manner, a region where one sample hybridizes is called hybridization region in the invention. In the case of one hybridization region, it is called nucleic acid microarray. Regarding the nucleic acid probe spotted on the hybridization regions, it is desirable that kind, position, concentration and the number of the probe are substantially the same for the hybridization regions, but since these can be corrected in the later step, these are not always be the same.

· Mono color method, two color method, multiple color method

According to the invention, a mono color method, two color method or multiple color method can be used.

The mono color method of the invention is a method called one color method as a byname which is a method in which a labeled target nucleic acid prepared by labeling one species of target nucleic acid is used for one plate of nucleic acid microarray or one hybridization region. For example, so far as one species of target nucleic acid is used, it may be labeled with several labeling reagents. In addition, in the case of the simultaneous use of a labeled correcting nucleic acid, even when the labeling reagent of this correcting nucleic acid is different from that of the target nucleic acid, this is called mono color method in this invention so far as one species of target nucleic acid is used.

The two color method is a method in which two different species of labeled target nucleic acids are used for one plate of nucleic acid microarray or one hybridization region. For example, so far as two species of different target nucleic acids are used, it may be labeled with several labeling reagents. In addition, in the

case of the simultaneous use of a labeled correcting nucleic acid, even when the labeling reagent of this correcting nucleic acid is different from those of the target nucleic acids, this is called two color method in this invention so far as two species of target nucleic acids are used.

The multiple color method is a method in which various different species of target nucleic acids are used for one plate of nucleic acid microarray or one hybridization region. For example, so far as many species of target nucleic acids are used, these may be labeled with more species of labeling reagents than the number of the species of target nucleic acids. In addition, in the case of the simultaneous use of a labeled correcting nucleic acid, even when the labeling reagent of this correcting nucleic acid is different from those of the target nucleic acids, this is called multiple color method in this invention so far as various species of target nucleic acids are used.

· Calculation of labeled amount average value

The invention is a method which reduced variations in comparison with the case of using a single labeling reagent, by the use of the average of labeled values respectively obtained from a labeled nucleic acid prepared by labeling one species of target nucleic acid with two or more different labeling reagents.

A theory of the nucleic acid measuring method of the invention is shown in Figs. 1A to 1C. In this connection, Figs. 1A to 1C are theories on the case of the mono color method. Fig. 1A is a theory of the conventional mono color method. Figs. 1B and 1C are theories of the nucleic acid measuring method of the invention. Fig. 1B is a theory in the case of the use of two different labeling reagents. A nucleic acid A is labeled with labeling reagents 1 and 2 and hybridized on one plate of nucleic acid microarray or a hybridization region. Also, a nucleic acid B is labeled

with labeling reagents 1 and 2 and hybridized on another plate of nucleic acid microarray or another hybridization region. Variation of labeled values is reduced by calculating average of the labeled values obtained from the labeling 1-nucleic acid A and labeling 2-nucleic acid A. In addition, average value of the labeled values obtained from the labeling 1-nucleic acid B and labeling 2-nucleic acid B is also calculated. For example, in the case of CGH array, Log Ratio is calculated using average nucleic acid labeled value of the nucleic acid A and average nucleic acid labeled value of the nucleic acid B. In that case, since averaged, namely variation-reduced, labeled values are used, variation of the Log Ratio value is also reduced so that accuracy of the CGH array is increased in comparison with the case of not using average labeled values. Thereafter, in the case of CGH array for example, the number of copies is computed by calculating Log Ratio. Fig. 1C is a theory in the case of using n species of different labeling reagents.

According to the invention, respective labeled values obtained from a target nucleic acid prepared by labeling substantially the same target nucleic acid obtained from substantially the same target cell with different labeling reagents may be subjected to various processing and then such values may be averaged. For example, fluorescence values corrected using a correcting nucleic acid may be averaged. Also, Log Ratio values may be once calculated from the labeled values and then the Log Ratio values may be averaged. Thus, according to the invention, the labeled values directly read off after hybridization may be averaged as such and the averaged value may be used in the subsequent calculations, or the labeled values may be corrected using a correcting nucleic acid or corrected by calculating Log Ratio values and then subsequent calculations may be carried out, that is, average values may be calculated after processing the labeled values and used in the subsequent calculations.

In addition, there is a method for suppressing variation of date by arranging a large number of spots of substantially the same probe nucleic acid on a nucleic acid array and using average of the labeled values obtained therefrom or of their processed values, and a combination with this method can also be made in the invention. For example, by carrying out averaging of respective labeled values obtained from a target nucleic acid prepared by labeling substantially the same target nucleic acid obtained from substantially the same target cell with different labeling reagents, and by further calculating average values of the average values calculated by the method of the invention from the spots prepared by spotting substantially the same probe nucleic acid on the nucleic acid microarray, the resulting values may be used. By these, variation of the data of nucleic acid microarray is further reduced. For example, in the case of CGH array, variation of its Log Ratio is reduced so that the number of copies can be judged precisely, and in addition, diagnostic accuracy of a cancer and the like is improved.

· Labeling

Labeling is an act to allow a detectable substance to bind to a target nucleic acid, and any substance can be incorporated into the invention with the proviso that it is detectable. For example, a fluorescent material, an inorganic compound, an enzyme, a radioisotope, FRET (fluorescence resonance energy transfer) and the like can be included.

· Fluorescent material

As the fluorescent material, though its use is not particularly limited, fluorescein isothiocyanate (FITC), Cy3, Cy5, Cy7, green fluorescent protein (GFP), blue fluorescent protein (BFP), yellow fluorescent protein (YFP), red fluorescent protein (RFP), Alexa, acridine, DAPI, ethidium bromide, SYBR Green, Texas Red, a

rare metal fluorescence labeling agent (4,4'-bis(1",1",1",2",2",3",3"-heptafluoro-4",6"-hexanedion-6"-yl)-chlorosulfo-o-terphenyl (BHHCT)), ethidium bromide, Acridine Orange, TAMRA, ROX and the like can be used.

As the inorganic compound, though its raw material to be used is not particularly limited, for example, a quantum dot made of a semiconductor inorganic material can be cited. As its example, silica, CdTe, ZnSe, CdSe nanoparticulate can be cited. This particulate can change its generating fluorescence wavelength by changing its particle diameter, and it becomes blue at a diameter of 2 nm, green at a diameter of 3 nm, yellow at a diameter of 4 nm and red at a diameter of 5 nm. Accordingly, its fluorescence may be detected, or the presence of its particle may be detected. For example, as a measure for detecting the presence of particle, AFM (atomic force microscope) can be used.

Further, digoxigenin (DIG), biotin and the like can also be used. As an example of the application of biotin, it can be used in the detection of purple color development which can be seen when avidin is allowed to bind to biotin that has been linked to a probe, biotin-linked alkaline phosphatase is allowed to bind thereto, and then nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate are added thereto as substrates of alkaline phosphatase.

In addition, it can be labeled non-enzymatically. For example, ULS arrayCGH Labeling Kit (mfd. by Kreatech Biotechnology BV) and the like can also be used.

The invention can use all of the above-mentioned fluorescent materials with the proviso that the labeling reagents linked to a nucleic acid are substantially distinguishable using a detector, but as a case which can be suitably used among

them, for example, when a target nucleic acid is labeled with two species of labeling reagents, a combination of Cy3 and Cy5 can be used.

· Direct labeling method, indirect labeling method

As the labeling method, either a direct labeling method or an indirect labeling method may be used in the invention. The direct labeling method is a method in which, when Cy-dye is used for example, a target nucleic acid is converted into single strand, a short chain nucleic acid is allowed to hybridize therewith and a Cy-dye-linked nucleotide derivative is mixed therewith together with nucleotides, all in advance, and then a labeled target nucleic acid is synthesized at one step. Though there is a problem in that the uptake efficiency when an enzyme uptakes a non-natural type nucleotide derivative is lower than that of a natural type nucleotide, the process is convenient and it can be suitably used in the invention. The indirect labeling method is a method in which, when Cy-dye is used for example, a target nucleic acid is converted into single strand, a short chain nucleic acid is allowed to hybridize therewith and a nucleotide derivative having a substituent group to which Cy-dye can be linked, such as a nucleotide derivative having aminoallyl group, is mixed therewith together with natural type nucleotides, all in advance, and a labeled target nucleic acid is obtained by firstly synthesizing a target nucleic acid having this substituent group and then Cy-dye is linked via aminoallyl group. Though the process of this method is complex, three-dimensional structure of the nucleotide derivative to which aminoallyl group was bonded is structurally closer to the natural type nucleotide than the Cy-dye-bonded nucleotide derivative to be used in the direct labeling method and its efficiency to uptake labeling reagent is high, so that this can also be suitably used in the invention.

According to the invention, random primer method (primer extension

method), nick translation method, PCR (polymerase chain reaction) method, terminal labeling method and the like can be used as the method for introducing a labeling reagent into a target nucleic acid. The random primer method can be suitably used particularly in the invention.

The random primer method is a method in which a random primer nucleic acid of from several bp (base pair) to a little over ten bp is allowed to hybridize with a target nucleic acid and a labeled target nucleic acid is synthesized by simultaneously carrying out amplification and labeling using a polymerase. The nick translation method is a method in which DNA is degraded by allowing a DNA polymerase to act upon a double-stranded nucleic acid nicked, for example, with DNase I, and a labeled target nucleic acid is synthesized at the same time by the DNA polymerase activity. The PCR method is a method in which a labeled target nucleic acid is obtained by preparing two species of primers and by simultaneously carrying out amplification and labeling through a PCR reaction using the primers. The terminal labeling method is, in the case of labeling the 5' end, a method in which a labeling reagent is incorporated into the 5' end of a target nucleic acid dephosphorylated with alkaline phosphatase, effected by the phosphorylation reaction of T4 polynucleotide kinase. The method for labeling 3' end is a method in which a labeling reagent is added to the 3' end of a target nucleic acid by a terminal transferase.

The number of labeling reagents which can be used in the invention may be two species or more based on one species of target nucleic acid but is not particularly limited with the proviso that a difference between different labeling reagents can be distinguished.

According to the invention, a labeled target nucleic acid prepared by allowing

at least two species of labeling reagents to react with substantially the same target nucleic acid obtained from substantially the same target cell can be obtained by one labeling reaction, by allowing a mixture of at least two species of labeling reagents to react with substantially the same target nucleic acid obtained from substantially the same target cell. For example, by carrying out labeling of a target nucleic acid obtained from a normal cell using a mixture of Cy3 and Cy5, a mixture of a normal cell-derived target nucleic acid labeled with Cy3 and a normal cell-derived target nucleic acid labeled with Cy5 can be obtained by one labeling reaction.

According to the invention, a labeled target nucleic acid prepared by allowing at least two species of labeling reagents to react with substantially the same target nucleic acid obtained from substantially the same target cell can also be obtained by mixing two or more of those in which one species of labeling reagent is allowed to react with substantially the same target nucleic acid obtained from substantially the same target cell by one labeling reaction. For example, a normal cell-derived target nucleic acid labeled with Cy3 and Cy5 can be obtained by mixing a normal cell-derived target nucleic acid labeled with Cy3 obtained by carrying out labeling of a target nucleic acid obtained from a normal cell using Cy3 and a normal cell-derived target nucleic acid labeled with Cy5 obtained by carrying out its labeling using Cy5.

· Probe nucleic acid

The probe nucleic acid means a nucleic acid which can hybridize with a labeled nucleic acid, which may be completely hybridize with a sample, or may be partially hybridize therewith or hybridize with at least 50% or more thereof as the number of bases of the sample nucleic acid.

As the probe nucleic acid, a chemically synthesized nucleic acid, a cDNA (complementary DNA), BAC (bacterial artificial chromosomes), YAC (yeast

artificial chromosomes) and the like can be used. Also, a nucleic acid prepared by chemically modifying a DNA, RNA or the like natural type nucleic acid may be used as a probe nucleic acid. For example, a PNA (peptide nucleic acid), a BNA (bridged nucleic acid), a methyl phosphonate type DNA, a phosphorothioate type DNA, a phosphoroamidate type DNA, a boranophosphate type DNA and the like can be used. In addition, a chimeric nucleic acid can also be used. For example, those in which a DNA moiety and an RNA moiety are mixed in a nucleic acid and a natural type nucleic acid and a modified type nucleic acid are mixed therein can be used.

Regarding the method for binding a probe nucleic acid onto a solid substrate, a method in which nucleotides are chemically synthesized one by one using an amidite monomer to which a photodesorption group typified by GeneChip is bonded and using a mask, a method in which a desired sequence is chemically synthesized by spotting an amidite monomer on a solid substrate by an ink jet method, a method in which a nucleic acid is synthesized on a substrate by changing pH of a solution on an electrode and releasing a protecting group making use of the change in pH, a method in which a nucleic acid chemically synthesized in advance is purified and spotted on a substrate, a method in which a cDNA is spotted using a spotter, and the like can be used. Regarding the spotting method, an ink jet method, a pin array method and the like can be used.

· Target cell

The target cell according to the invention is a cell to be analyzed or a cell to be evaluated such as a diagnosis, which contains a nucleic acid for use in the measurement of the expressed amount, presenting amount, the number of copies and the like of the nucleic acid, for example, using a nucleic acid microarray, and is a cell that contains a nucleic acid to be hybridized by the probe nucleic acid bonded to the

nucleic acid microarray.

The target cell of the invention may be substantially the same target cell group. For example, when cancer cells are extracted from a cancer tissue as abnormal cells, these generally become a mixture of cancer cells and normal cells. Since it is very difficult to collect the cancer cells alone from the mixture thereof, even when partially contaminated with different cells, it is considered that substantially the same target cell group is used in the invention. In addition, a cell obtained by picking up a specific cell alone using LCM (laser capture microdissection) can also be used in the invention.

A comparing cell and a cell to be compared can also be included in the target cell. For example, the comparing cell can be regarded as a normal cell, and the cell to be compared as an abnormal cell. The abnormal cell is a cell which is different from the normal cell, such as a cell having a gene that underwent a certain mutation in the gene as the object of interest, and the normal cell is a cell which does not substantially underwent the mutation. For example, based on the normal cell obtained from a patient who is not causing a cancer, a cell obtained from a patient who is suffering from a cancer can be used as an abnormal cell. In addition, a cell in which a gene inside the cell was changed caused by the addition of a drug or the like reagent can also be called an abnormal cell, and a cell to which the drug was not added a normal cell.

· Target nucleic acid

The target nucleic acid according to the invention is a nucleic acid extracted from a cell to be analyzed for the measurement of the existing amount, the number of copies and the like of the nucleic acid, for example, using a nucleic acid microarray, which is the nucleic acid to be hybridized by the probe bonded to the nucleic acid

microarray. For example, in the case of CGH array, a nucleic acid extracted from a normal cell, a nucleic acid extracted from an abnormal cell such as a cancer cell, or the like is called a target nucleic acid.

· Purification

The term purification according to the invention is used synonymously with extraction, separation, fractionation and the like. Also, as a measure for that, a method which uses a cartridge that carries a nucleic acid adsorbent membrane of silica, a cellulose derivative or the like, ethanol precipitation or isopropanol precipitation, phenol-chloroform extraction, a solid phase extraction cartridge which uses an ion exchange resin, a silica carrier to which octadecyl group or the like hydrophobic substituent group, a resin having a size excluding effect or the like and a method by a chromatography or the like can be included. In addition, according to the invention, solvent substitution is also called purification in a broad sense.

· Purification of target nucleic acid

According to the invention, it is necessary to carry out purification when a target nucleic acid is prepared from a target cell. In the case of not carrying out purification, various side reactions occur during the fluorescence labeling and at the same time, protein, lipid and the like cell lysates exert great influence upon background noises, so that performance of the nucleic acid microarray is considerably lowered when purification is not carried out.

· Labeled target nucleic acid

The labeled target nucleic acid means a target nucleic acid after linking of a labeling reagent to the target nucleic acid, and it is called labeled target nucleic acid in the invention regardless of whether or not it was purified.

· Purified solution containing labeled target nucleic acid

The purified solution containing labeled target nucleic acid means a nucleic acid solution obtained by carrying out a purification process for a solution containing a labeled target nucleic acid.

· Un-purified solution containing labeled target nucleic acid

The un-purified solution containing labeled target nucleic acid means a solution containing a labeled target nucleic acid, obtained by carrying out a labeling process for a solution containing a target nucleic acid extracted from a target cell and then carrying out absolutely no purification process. In this case, the purification process means a process for removing unreacted labeling reagent, enzyme and the like after carrying out the labeling treatment, and it does not include a purification process for preparing the target nucleic acid solution. Since purification is not carried out, this is a solution of the labeled target nucleic acid coexisting with various unreacted compounds added by the labeling process, such as the enzyme, nucleotides, ions contained in the buffer and the like. In addition to the un-purified solution containing labeled target nucleic acid, a nucleic acid solution after carrying out heating, EDTA addition and the like treatment for the purpose of inactivating the coexisting enzyme is also called un-purified solution containing labeled target nucleic acid according to the invention.

<Correcting nucleic acid>

The correcting nucleic acid means a nucleic acid which is used for correcting spotting amount between spots of objects. Accordingly, the correcting nucleic acid must be a nucleic acid which can hybridize with the probe nucleic acid on the nucleic acid microarray. This correcting nucleic acid may have any sequence or chain length with the proviso that it can perform hybridization. For example, in the case of CGH array, Cot-1 DNA can be suitably used. In general, Cot-1 DNA is a nucleic acid used

for blocking a repeating sequence, and in the case of a CGH array which uses a BAC probe, the repeating sequence is contained in substantially all of the spots of the probe. In addition, it is also suitable to use a normal cell-derived target nucleic acid as the correcting nucleic acid. These correcting nucleic acids may be substantially the same between the nucleic acid microarrays mutually used in comparison, but are more preferably of the same concentration and the same lot.

· Labeled correcting nucleic acid

The labeled correcting nucleic acid means a correcting nucleic acid in which a labeling reagent is linked to the correcting nucleic acid, which can be used in the invention after purification or without purification, and these are also called labeled correcting nucleic acid in the invention.

<Hybridization buffer>

The hybridization buffer according to the invention, in the case of CGH array for example, is a buffer consisting of dextran sulfate having the action to increase nucleic acid concentration, formamide having the action to lower melting temperature of nucleic acid, a buffer solution for keeping pH of the solution at a constant level, and the like.

According to the invention, it is desirable to use a hybridization buffer consisting of a combination of at least two species among a compound having an excluded volume, a compound which lowers melting temperature, a compound that keeps the solution at a constant pH and a nucleic acid having the action to improve noise and the like of nucleic acid microarray, as the hybridization buffer.

As the substance having excluded volume effect, polyethylene glycol (PEG, JP-A-2000-325099) and dextran sulfate are known. By the addition of these substances, nucleic acid hydrating water molecules are surrounded due to the

excluded volume effect so that concentration of the nucleic acid is increased, and as a result, rate of the hybridization becomes quick. Accordingly, these substances can also be added to the hybridization buffer.

As the compound which lowers melting temperature, formamide, glycerol, formaldehyde, DMSO, DMF, GuSCN, iodine and the like can be added to the hybridization buffer. Melting point (Tm) of nucleic acid can be lowered by a factor of 0.6°C as the formamide concentration increases 1%.

As the buffer solution, any buffer solution can be used with the proviso that it can keep pH at a constant level, but those which are generally used for biochemistry are desirable. For example, it is desirable to use SSC or a good buffer solution. As the good buffer solution, Bis-Tris (N,N-bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane), MES (2-morpholinoethanesulfonic acid), HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid), PIPES (piperazine-1,4-bis(2-ethanesulfonic acid)), ACES (N-(2-acetamino)-2-aminoethanesulfonic acid), CAPS (N-cyclohexyl-3-aminopropanesulfonic acid), TES (N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid) and the like can be exemplified.

<Hybridization solution>

The hybridization solution according to the invention, in the case of CGH array for example, is a solution prepared by adding a nucleic acid which blocks a repeating sequence, such as Cot-1 DNA or the like, a labeled correcting nucleic acid, a tRNA having the action to reduce background noise of the nucleic acid microarray, a surfactant and other reagents to the hybridization buffer.

This is a solution having a property to assist hybridization of the labeled target nucleic acid with the probe nucleic acid on the nucleic acid microarray, or a

solution which accelerates a hybridization having high coinciding degree of sequences of the probe nucleic acid and labeled target nucleic acid and, on the other hand, suppresses a hybridization having low coinciding degree of sequences. For example, as a general composition of hybridization solution for nucleic acid microarray, a hybridization buffer consisting of 6 x SSC, 5 d Denhardt solution, 0.2% SDS, human Cot-1 DNA, poly(A) and yeast tRNA is used (Cell Technology, Supplement, Genomic Science Series 1, DNA Microarray and Latest PCR Method, edited by M. Matsumura and H. Nawa; all written in Japanese). For example, in the case of CGH array, a hybridization solution consisting of formamide, dextran sulfate, 20 x SSC, yeast tRNA and 20% SDS is used (I. Imoto and J. Inazawa, Saibo Kogaku, (Cell Technology), Vol. 23, p. 355, 2004). Since dextran sulfate has the effect to increase concentration of labeled target nucleic acid due to its excluded volume effect and formamide lowers melting point (Tm) of double strand, these are prepared in such a manner that the labeled target nucleic acid having high coinciding degree of sequences becomes high in hybridization efficiency and the labeled target nucleic acid having low coinciding degree of sequences becomes low in hybridization efficiency.

As the nucleic acid having the action to improve noise and the like of the nucleic acid microarray, denatured salmon sperm DNA, poly(A), poly(dA), tRNA, human Cot-1 DNA, mouse Cot-1 DNA and the like nucleic acid components prevent nonspecific hybridization, hybridization of probe DNA and adhesion of the probe DNA to membranes and substrates, and can inhibit background as a result thereof. These nucleic acids can be used as a component of the hybridization solution.

In addition to this, as describe in JP-A-2005-087109, a phospholipid can also be used as one of the composition of hybridization solution of the invention.

Further, Denhardt solution (a solution containing Ficoll, polyvinyl pyrrolidone and bovine serum albumin as the main components) can also be used as a component of the hybridization solution. This solution can reduce background noise of the array.

Further, a quaternary ammonium salt, betaine, can be added to the hybridization solution. Betaine is concerned in the hydrogen bond of nucleic acid and its action to inhibit nonspecific hybridization has been reported (Biochemistry, 32, 137 – 144 (1993)).

Further, TMAC (tetramethyl ammonium chloride) can also be added to the hybridization solution (Proc. Natl. Acad. Sci. USA, 82, 1585 – 1588 (1985)).

In addition, a commercially available hybridization solution can also be used. For example, ExpressHyb (Clontech), PerfectHyb (TOYOBO), ULTRAhyb (Ambion) and the like can be used.

As the surfactant, a cationic surfactant, an anionic surfactant, and a nonionic surfactant can be used. As the anionic surfactant, it is desirable to use sodium dodecyl sulfate. Sodium dodecyl sulfate can efficiently suppress background. In addition to this, N-lauryl sarcoside, lithium lauryl sulfate and the like are used. As the nonionic surfactant, Tween (registered trademark), Triton X (registered trademark) and the like are known. These surfactants can be used in the invention.

· Hybridization solution containing labeled target nucleic acid

The hybridization solution containing labeled target nucleic acid as used in the invention is a solution prepared by mixing the hybridization solution with at least one labeled target nucleic acid. According to the invention, the labeled target nucleic acid may be either purified or not purified.

· Block nucleic acid

The block nucleic acid is a nucleic acid having the action to cover, namely block, the repeating sequence presenting in the probe nucleic acid on the nucleic acid microarray, via hybridization, and in the case of CGH array for example, human Cot-1 DNA, mouse Cot-1 DNA and the like are used. These nucleic acids can also be used in the invention. For example, in the case of the use of a BAC clone as the probe nucleic acid on CGH array, the fluorescent value of hybridization read off from the CGH array becomes high but difference in the copy numbers becomes small when these block nucleic acids are not used, so that diagnostic accuracy and the like performance as the CGH array is lowered. In addition, according to the invention, the block nucleic acid can also be used as one of correcting nucleic acids.

· Method for reading off hybridization

A method which uses a fluorescence scanner is the method most suitably used in the invention as the method for reading off hybridization on the nucleic acid array. This is suitably used when a fluorescent material is used as the labeling reagent of labeled target nucleic acid. As the fluorescence scanner, for example, there are FLA-8000 (Fuji Photo Film), GenePix 4000B (Axon Instruments) and the like. Also, when a particulate is used as the labeling reagent, AFM (atomic force microscope) and the like can also be used. In addition, when chemiluminescence is used as the labeling reagent, it can be directly observed with the naked eye. These methods can be used also in the invention.

· Automation

Regarding the method of the invention, all steps can be carried out by manual labor, some steps can be carried out using automated machines or all steps can be carried out using automated machines. By carrying out automation, accuracy of each step, such as pipetting accuracy, reaction time and reaction temperature, can be

precisely controlled in comparison with manual labor, so that accuracy of nucleic acid microarray is improved and, at the same time, it becomes unnecessary to carry out troublesome operations.

Examples

[Example 1]

The following describes the invention further in detail based on examples, but the invention is not limited thereto.

Comparative Example: Mono color method CGH array using one labeling reagent

<Preparation of un-purified labeled target nucleic acid solution>

A 1.7 ml capacity micro tube (platinum tube, mfd. by BM Equipment) was charged with 3 μl (0.375 μg) of a female DNA, 8 μ l of water (distilled water, DNase- and RNase-free, mfd. by GIBCO) and 20 μ l of 2.5 x Random Primers Solution (mfd. by Invitrogen) and heat treatment was carried out at 95°C for 5 minutes on BLOCK INCUBATOR BI-535A (mfd. by ASTEC). Five minutes thereafter, the micro tube was taken out to carry out 10 minutes of rapid cooling treatment on ice. A 5 μl portion of 10 x dCTP Nucleotide Mix (mfd. by Invitrogen), 3 μl of 250 nmol Cy3-dCTP Bulk Pack (mfd. by GE Healthcare Bio-Science) and 1 μl of Exo-Klenow Fragment (mfd. by Invitrogen) were added thereto and amplification reaction and labeling reaction were carried out at 37°C for 2 hours on the BLOCK INCUBATOR BI-535A. Two hours thereafter, the micro tube was taken out from the incubator and subjected to a heating treatment on the BLOCK INCUBATOR BI-535A which had been set to 65°C, thereby inactivating the Exo-Klenow Fragment contained in the reaction solution and obtaining un-purified Cy3-

Female.

By carrying out the above-mentioned operation also on a male DNA, un-purified Cy3-Male was obtained.

<Preparation of MM #1 solution containing Cot-1 DNA>

A 65 μl portion of Cot-1 DNA was put into a 1.7 ml capacity micro tube and 6.5 μl of 3 M sodium acetate (pH 5.2) and 167 μl of ethanol at -20°C were added thereto and mixed therewith, and then the mixture was allowed to stand at -80°C for 10 minutes. Thereafter, centrifugation was carried out at 4°C and at 15000 rpm for 30 minutes using a centrifuge MX-300 manufactured by TOMY SEIKO. After the centrifugation, since the precipitate accumulated on the bottom of the centrifugation tube, the supernatant was discarded while taking care in not sucking the precipitate. Next, the remaining ethanol was removed by allowing the centrifugation tube to stand for 10 minutes while opening its cap. Ten minutes thereafter, 18 μl of 20% SDS was added thereto and allowed to stand for 30 minutes. After 30 minutes, 20 μl of MM #1 (prepared by mixing and dissolving 1 g of dextran sulfate (mfd. by SIGMA), 5 ml of formamide and 1 ml of 20 x SSC and then adjusting the liquid volume to 7 ml) was added thereto and thoroughly mixed by stirring.

It is desirable that this process is not carried out by a user but a kit producer prepares in advance.

<Preparation of hybridization solution>

A 20 μl portion of the un-purified Cy3-Female prepared in the above-mentioned <Preparation of un-purified labeled target nucleic acid solution> and 38 μl of the MM #1 solution containing Cot-1 DNA prepare in <Preparation of MM #1 solution containing Cot-1 DNA> were put into a 1.7 ml capacity micro tube and stirred by sufficiently carrying out Vortex treatment.

In addition, a hybridization solution containing un-purified Cy3-Male was prepared from the un-purified Cy3-Male in the same manner as described in the above.

<Pretreatment of CGH array>

As the CGH array, a microarray of 64 spots consisting of 4 spots of each of the 16 genes prepared from BAC clones, wherein these genes were spotted at NGK INSULATORS, was used.

About 200 ml of a blocking solution (mfd. by Matsunami Glass) was put into a glass container, the CGH array was soaked therein, and blocking reaction was carried out for 20 minutes using SLIDE WASHER SW-4 (mfd. by JUJI FIELD) while moving the slide glass up and down. After 20 minutes of the reaction, the CGH array was taken off from the blocking solution and put into a container filled with 200 ml of water. This was washed for 3 minutes using the SLIDE WASHER and, 3 minutes thereafter, again put into a container filled with 200 ml of fresh water and washed using the SLIDE WASHER. Three minutes thereafter, this was put into a container filled with 200 ml of ethanol and again washed using the SLIDE WASHER. Three minutes thereafter, the CGH array was taken off and its centrifugation was carried out using a table top centrifuge Spin Dryer mini 2350 (mfd. by TOMY SEIKO) to dry the CGH array. After blocking, the CGH array was soaked in boiling water for 2 minutes, subsequently soaked in 70% ethanol of -20°C, 85% ethanol of -20°C and 100% ethanol of -20°C, and the CGH array was dried by carrying out 1 minute of centrifugation with the spin drier. In this connection, two plates of already blocked CGH array were prepared.

<Hybridization>

Heating treatment of the two sample solutions prepared in <Preparation of

hybridization solution> was carried out at 75°C for 16 minutes. Thereafter, pre-incubation was carried out at 42°C for 30 minutes or more. Each of the solutions was added dropwise to one plate of CGH array, and a gap cover glass (24 x 50 mm) (mfd. by Matsunami Glass) was put thereon.

These two slide glasses were put into Tight Box No. 1 (mfd. by CHOPLA), in which kimtowel was set up for the purpose of preventing drying of the slide glasses and 4 ml of 50% formamide/2 x SSC solution was added to the top thereof, and the box was put into a constant temperature oven (HYBRIDIZATION INCUBATOR, HB-80, mfd. by TAITEC) to carry out hybridization at 37°C for 16 hours.

<Washing of CGH array>

After carrying out the hybridization, each of them was put into a SUMILON tube filled with 45 ml of a solution of 2 x SSC and soaked therein until the cover glass peeled off spontaneously from the slide glass. Next, the slide glass from which the cover glass came off was put into a 50 ml capacity SUMILON tube (mfd. by Sumitomo Bakelite) filled with 45 ml of 50% formamide/2 x SSC solution, and the tube was put into the HYBRIDIZATION INCUBATOR to carry out 15 minutes of washing at 50°C by shaking the stage at a speed of 30 revolutions per minute. Next, this was put into a 50 ml capacity SUMILON tube filled with 45 ml of 2 x SSC/0.1% SDS solution, and the tube was put into the constant temperature incubator to carry out 30 minutes of washing at 50°C by shaking the stage at a speed of 30 revolutions per minute. Next, this was put into a 50 ml capacity SUMILON tube filled with 45 ml of 2 x SSC solution and 5 minutes of washing was carried out at room temperature by shaking the stage at a speed of 30 revolutions per minute. After the washing, the microarray was dried by carrying out 1 minute of centrifugation using Spin Dryer-Mini MODEL 2350.

<Data read off>

The thus dried two microarray plates were subjected to scanning using GenePix 4000B (mfd. by Axon Instruments).

<Data treatment>

By reading off fluorescence values from the respective slide glasses, Log Ratio value was calculated from the Cy3-Female-derived fluorescence value and the Cy3-Male-derived fluorescence value.

<Results>

A graph of the Log Ratio is shown in Fig. 3.

Inventive Example: Mono color method CGH array using two species of labeling reagents

<Preparation of un-purified labeled target nucleic acid solution>

The following un-purified labeled target nucleic acid solutions were prepared by the same method of Comparative Example.

1. Un-purified Cy3-Female
2. Un-purified Cy5-Female
3. Un-purified Cy3-Male
4. Un-purified Cy5-Male

<Preparation of MM #1 solution containing Cot-1 DNA>

Preparation of MM #1 solution containing Cot-1 DNA was carried out by the same method of Comparative Example.

<Preparation of hybridization solution>

A 20 μl portion of the un-purified Cy3-Female, 20 μl of the un-purified Cy5-Female and 38 μl of the MM #1 solution containing Cot-1 DNA prepare in <Preparation of MM #1 solution containing Cot-1 DNA> were put into a 1.7 ml

capacity micro tube and stirred by sufficiently carrying out Vortex treatment.

In addition, 20 μl of the un-purified Cy3-Male, 20 μl of the un-purified Cy5-Male and 38 μl of the MM #1 solution containing Cot-1 DNA prepare in <Preparation of MM #1 solution containing Cot-1 DNA> were put into a 1.7 ml capacity micro tube and stirred by sufficiently carrying out Vortex treatment.

<Pretreatment of CGH array>

Pretreatment of CGH array was carried out in the same manner as in Comparative Example.

<Hybridization>

Heating treatment of the two sample solutions prepared in <Preparation of hybridization solution> (a hybridization solution consisting of un-purified Cy3-Female and un-purified Cy5-Female and a hybridization solution consisting of un-purified Cy3-Male and un-purified Cy5-Male) was carried out at 75°C for 16 minutes. Thereafter, pre-incubation was carried out at 42°C for 30 minutes or more. Each of the solutions was added dropwise to one plate of CGH array, and a gap cover glass (24 x 50 mm) (mfd. by Matsunami Glass) was put thereon.

These two slide glasses were put into Tight Box No. 1 (mfd. by CHOPLA), in which kimtowel was set up for the purpose of preventing drying of the slide glasses and 4 ml of 50% formamide/2 x SSC solution was added to the top thereof, and the box was put into a constant temperature oven (HYBRIDIZATION INCUBATOR, HB-80, mfd. by TAITEC) to carry out hybridization at 37°C for 16 hours.

<Washing of CGH array>

Washing of CGH array was carried out by the same method of Comparative Example.

<Data read off>

The thus dried two microarray plates were subjected to scanning using GenePix 4000B (mfd. by Axon Instruments).

<Data treatment>

Fluorescence values were read off from the respective slide glasses. In this case, the Log Ratio value was calculated using the average value of the Cy3-Female-derived fluorescence value and the Cy5-Female-derived fluorescence value and the average value of the Cy3-Male-derived fluorescence value and the Cy5-Male-derived fluorescence value.

<Results>

A graph of the Log Ratio is shown in Fig. 4.

When Fig. 3 and Fig. 4 are compared, Fig. 4 in which labeling was carried out using two labeling reagents and the Log Ratio values were calculated using their average values shows good results as CGH array because variation of data is little.

Fig. 5 is a result of plotting a difference between the average value of X-chromosome-derived Log Ratio values and the average value of autosome-derived Log Ratio values. That is, the legend described as "one species" is a result of plotting a difference between the average value of Log Ratio values of the moiety corresponding to the X-chromosome of closed circles of Fig. 3 and the average value of Log Ratio values of the moiety corresponding to the autosome of open circles of Fig. 3, and the "two species" is a result of plotting a difference between the average value of Log Ratio values of the moiety corresponding to the X-chromosome of closed circles of Fig. 4 and the average value of Log Ratio values of the moiety corresponding to the autosome of open circles of Fig. 4. When two species of labeling reagents were used and their average value was calculated, difference in the Log Ratio of autosome and X-chromosome became large in comparison with the case

of the use of one species of labeling reagent. Performance as the CGH array becomes superior when the difference in Log Ratio becomes large, and for example, its diagnostic difference becomes small when used for diagnosis.

Fig. 6 shows the degree of dispersion when STDEV (degree of dispersion) which represents variation of Log Ratio of the autosomal moiety was calculated using one species of labeling reagent (calculated from the autosome of open circle in Fig. 3) and two species of labeling reagents (calculated from the autosome of open circle in Fig. 4). That is, it is better that the degree of dispersion is small, showing that when two species of labeling reagents are used and their average value is calculated, the degree of dispersion becomes small, namely variation of data becomes small, in comparison with the case of the use of one species of labeling reagent. Small degree of dispersion, namely small variation of data, means that when the CGH array is used for diagnosis for example, its diagnostic accuracy becomes high.

These results show that when two species of labeling reagents are used and the average value is used as the labeling value, performance as the nucleic acid microarray becomes superior in comparison with the case of the use of one species of labeling reagent. In addition, these results show that when more many species of labeling reagents are used and their average value is used, it becomes a nucleic acid microarray method having further large Log Ratio difference and small degree of dispersion and more high accuracy.

Industrial Applicability

By the use of the method of the invention, it becomes possible to reduce variations formed by various causes, by only a minimum change of protocol without greatly adding or changing the protocol. Also, since it is possible to apply this

method to mono color method, two color method or other methods, it becomes possible to reduce the variations conveniently and markedly effectively. Also, it is possible to use an unpurified labeled target nucleic acid solution, and in that case, the protocol can be markedly simplified. In addition, automation is also easy.

This application is based on Japanese patent application JP 2008-139638, filed on May 28, 2008, the entire content of which is hereby incorporated by reference, the same as if set forth at length.

CLAIMS

1. A method for measuring a target nucleic acid, the method comprising:

labeling one target nucleic acid with at least two labeling reagents to prepare a labeled target nucleic acid;

allowing the labeled target nucleic acid to hybridize with a probe nucleic acid on a nucleic acid microarray;

reading labeled amounts obtained from the respective at least two labeling reagents of the hybridized nucleic acid; and

averaging the labeled amounts to obtain an average value of the labeled amounts.

2. The method according to claim 1,

wherein at least one of the at least two labeling reagents is a fluorescent material.

3. The method according to claim 1 or 2,

wherein a solution containing the labeled target nucleic acid is subjected to a purification process.

4. The method according to claim 1 or 2,

wherein a solution containing the labeled target nucleic acid is not subjected to a purification process.

5. The method according to any one of claims 1 to 4,

wherein a labeled correcting nucleic acid obtained by linking a labeling reagent to a correcting nucleic acid is used together with the labeled target nucleic acid, the labeled correcting nucleic acid being capable of hybridizing with the probe nucleic acid on the nucleic acid microarray.

6. The method according to any one of claims 1 to 5,

wherein the at least two labeling reagents include a combination of Cy3 and Cy5.

FIG. 1A

FIG. 1B

FIG. 1C

FIG. 2A

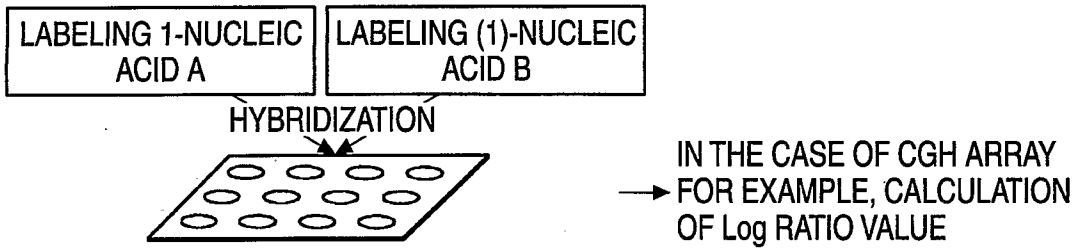


FIG. 2B

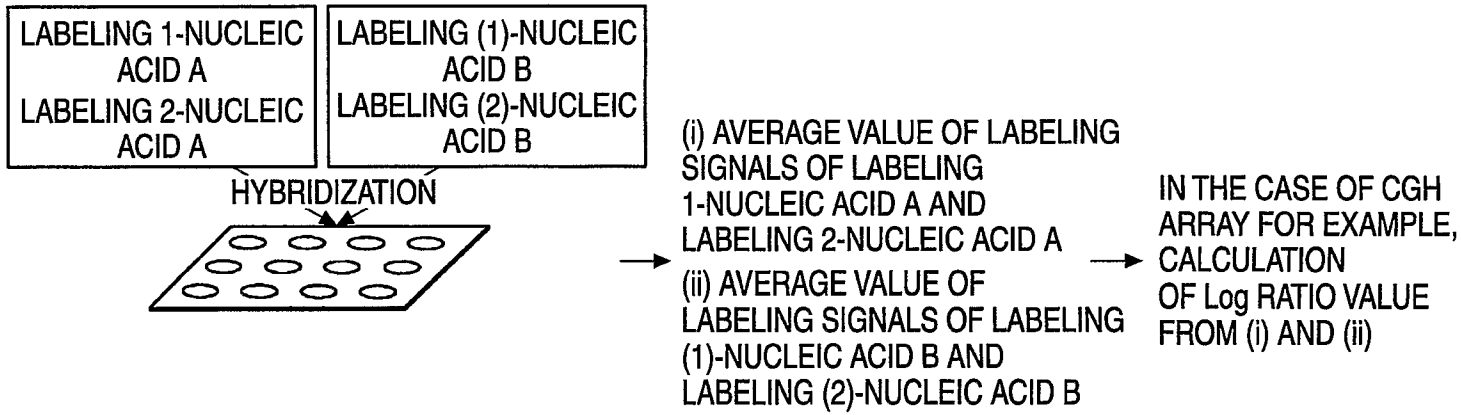


FIG. 2C

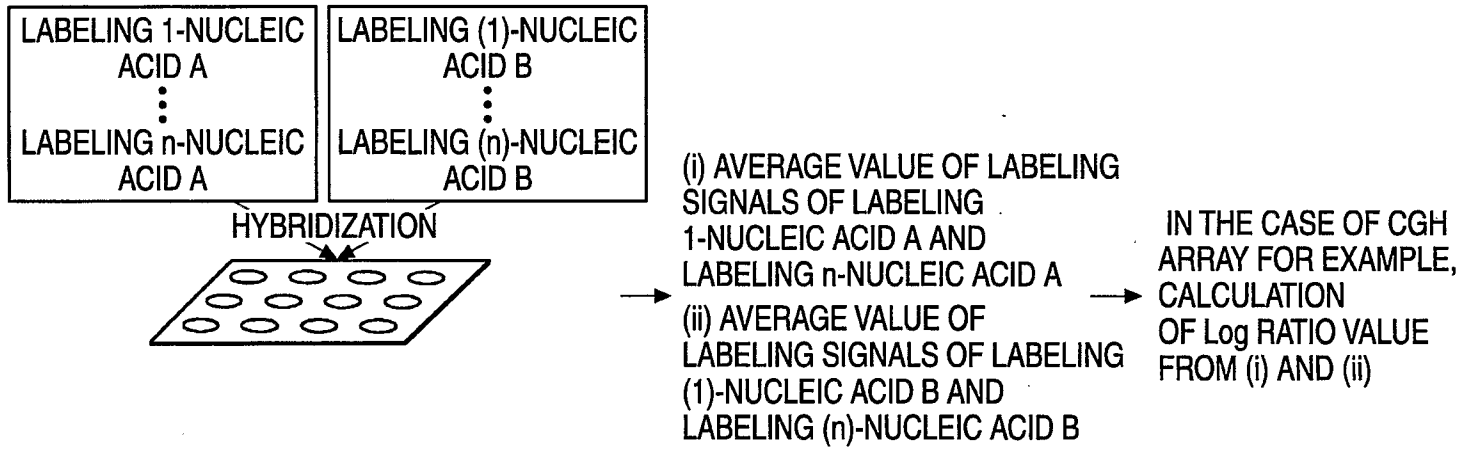


FIG. 3

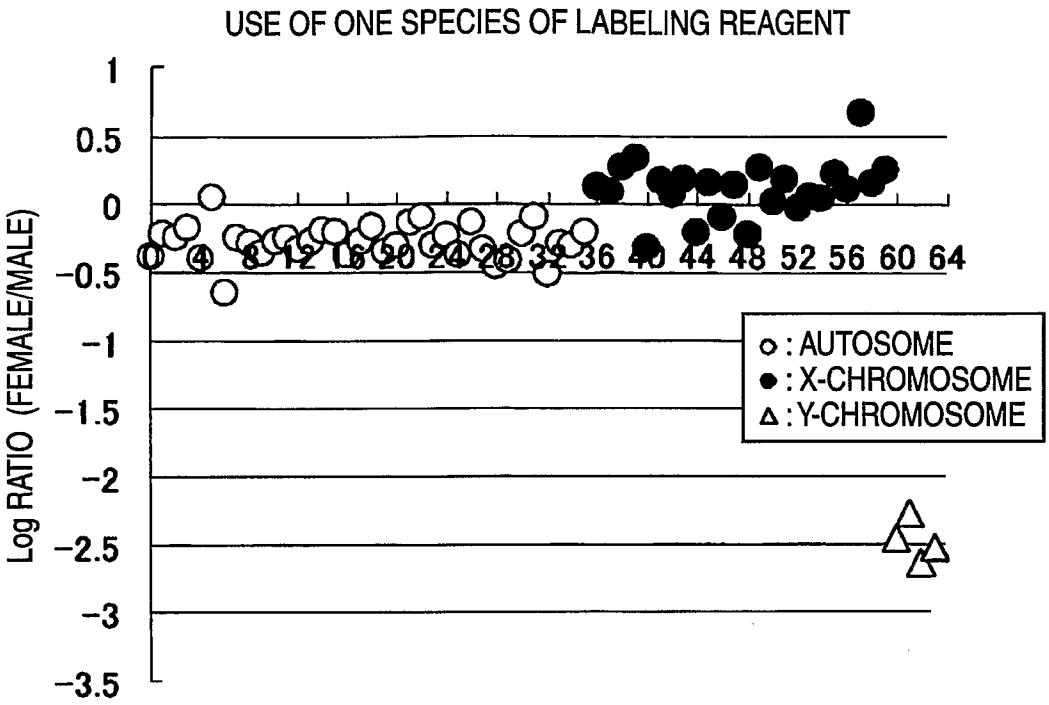

USE OF ONE SPECIES OF LABELING REAGENT
Log RATIO (FEMALE/MALE)
1
0.5
0
-0.5
-1
-1.5
-2
-2.5
-3
-3.5
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60 64
○ : AUTOSOME
● : X-CHROMOSOME
△ : Y-CHROMOSOME

FIG. 4

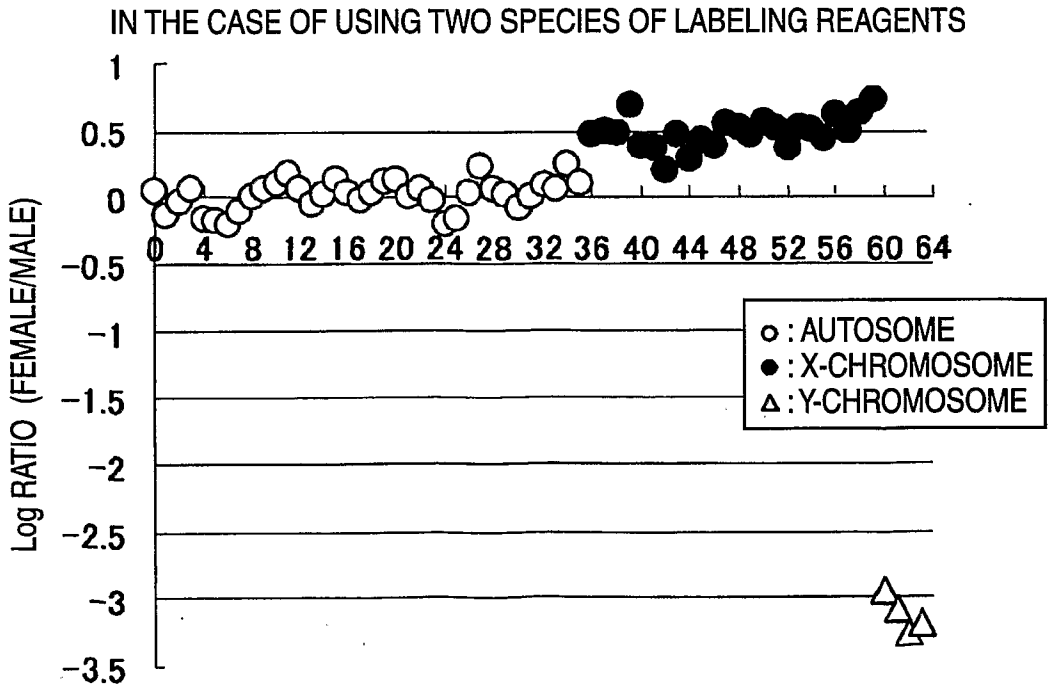

IN THE CASE OF USING TWO SPECIES OF LABELING REAGENTS
Log RATIO (FEMALE/MALE)
1
0.5
0
-0.5
-1
-1.5
-2
-2.5
-3
-3.5
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60 64
○ : AUTOSOME
● : X-CHROMOSOME
△ : Y-CHROMOSOME

FIG. 5

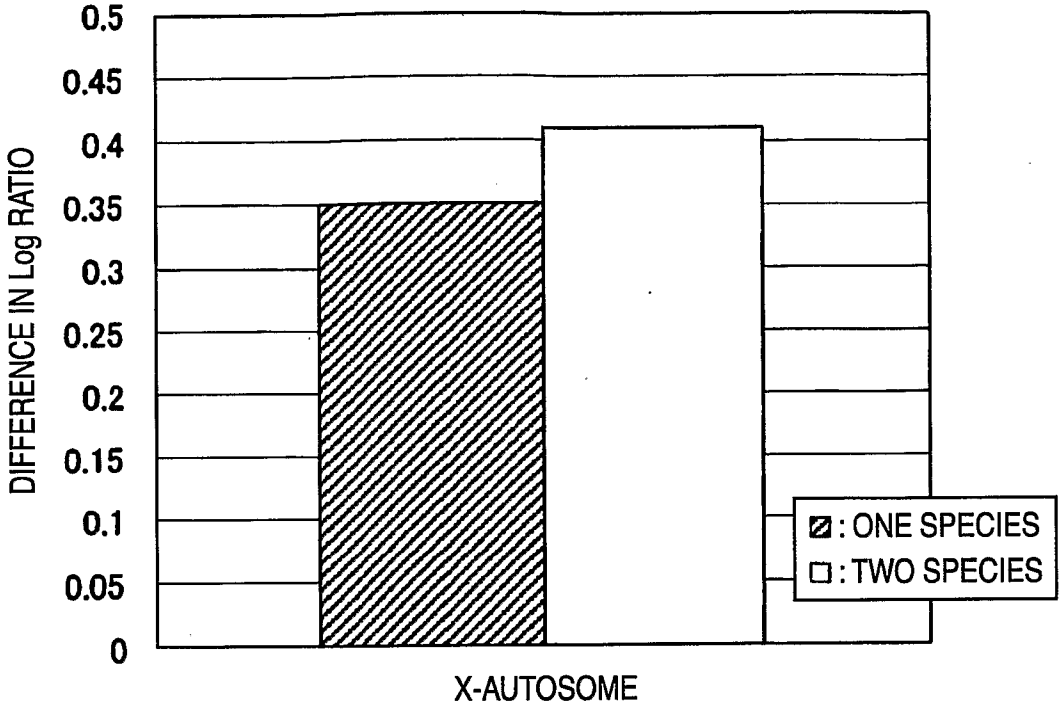
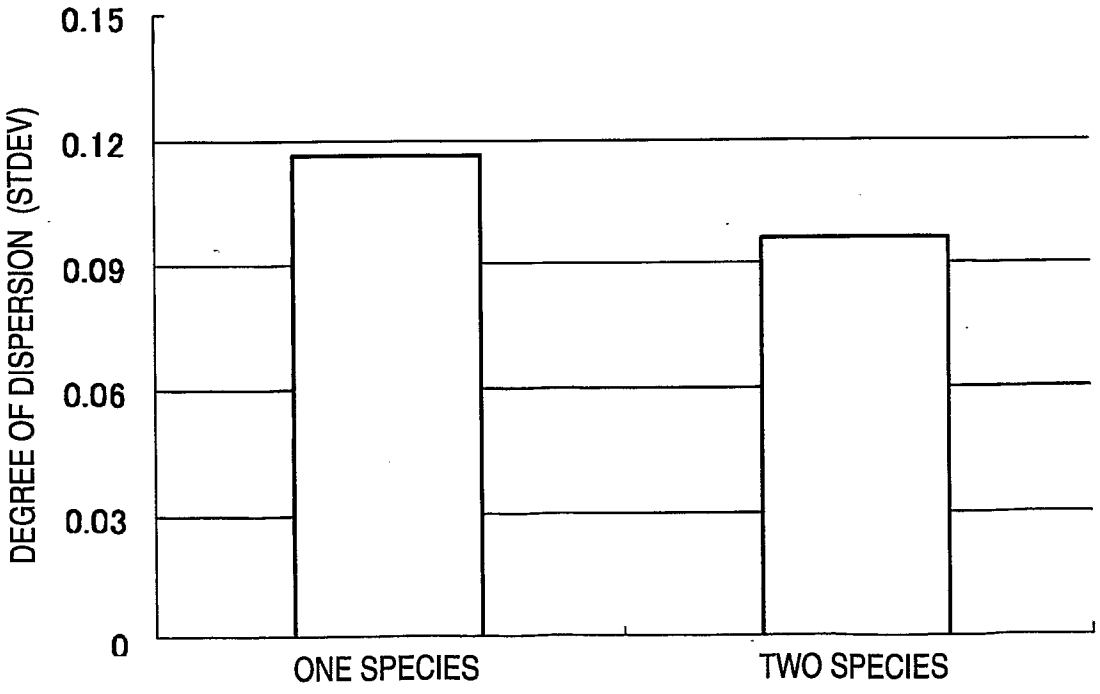

0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
DIFFERENCE IN Log RATIO
: ONE SPECIES
: TWO SPECIES
X-AUTOSOME

FIG. 6


0.15
0.12
0.09
0.06
0.03
0
DEGREE OF DISPERSION (STDEV)
ONE SPECIES
TWO SPECIES

INTERNATIONAL SEARCH REPORT

International application No
PCT/JP2009/060208

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68

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)
C12Q

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, EMBASE, BIOSIS, MEDLINE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	S. SOKOL: BIO PLOT USER'S GUIDE, [Online] 6 September 2007 (2007-09-06), XP002537227 Retrieved from the Internet: URL:http://biopuce.insa-toulouse.fr/ExperimentExplorer/doc/BioPlot/> [retrieved on 2009-07-14] the whole document -----	1-6
X	DABNEY ALAN R ET AL: "A new approach to intensity-dependent normalization of two-channel microarrays." BIOSTATISTICS (OXFORD, ENGLAND) JAN 2007, vol. 8, no. 1, January 2007 (2007-01), pages 128-139, XP002537228 ISSN: 1465-4644 page 130 -----	1-6
	-/--	

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 July 2009	31/07/2009

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Fax: (+31–70) 340–3016	Reuter, Uwe

INTERNATIONAL SEARCH REPORT

International application No
PCT/JP2009/060208

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GOMES L I ET AL: "Comparative analysis of amplified and nonamplified RNA for hybridization in cDNA microarray" ANALYTICAL BIOCHEMISTRY, ACADEMIC PRESS INC, NEW YORK, vol. 321, no. 2, 15 October 2003 (2003-10-15), pages 244-251, XP004457163 ISSN: 0003-2697 page 246	1-6
X	WESSENDORF SWEN ET AL: "Automated screening for genomic imbalances using matrix-based comparative genomic hybridization." LABORATORY INVESTIGATION; A JOURNAL OF TECHNICAL METHODS AND PATHOLOGY JAN 2002, vol. 82, no. 1, January 2002 (2002-01), pages 47-60, XP002537229 ISSN: 0023-6837 page 57	1-6
A	MARTIN-MAGNIETTE MARIE-LAURE ET AL: "Normalization for triple-target microarray experiments" BMC BIOINFORMATICS, BIOMED CENTRAL, LONDON, GB, vol. 9, no. 1, 28 April 2008 (2008-04-28), page 216, XP021031791 ISSN: 1471-2105 page L, paragraph 2	