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(54) **METHOD FOR MULTIPLEX NUCLEIC ACID ANALYSIS**

VERFAHREN ZUR MULTIPLEX-NUKLEINSÄUREANALYSE

PROCÉDÉ D'ANALYSE D'ACIDE NUCLÉIQUE MULTIPLEXE

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EP 2 893 034 B9

Description**FIELD OF INVENTION**

5 **[0001]** The present invention relates to the general fields of nucleic acid analysis in a biological sample, particularly the determination of genetic aberrations using a multiplex ligation-dependent method.

BACKGROUND

10 **[0002]** Nucleic acid analysis in a sample has many applications in both basic research and clinical settings. For example, nucleic acid analysis may be used to identify genetic aberrations in a patient blood sample. Genetic aberrations account for a large number of pathological conditions, including syndromic disorders (e.g., Down's syndrome) and diseases (e.g., breast cancer). Genetic aberrations may be, but is not limited to, single nucleotide polymorphisms (SNPs), gene copy number variants (CNVs), chromosomal rearrangements (e.g., insertions, deletions and duplications), gene mutations (e.g., single nucleotide changes, insertions, and deletions), nucleic acid modifications (e.g., methylation, acetylation and phosphorylations), gene over-expression (e.g., an oncogenes such as RAS), and gene under-expression (e.g., a tumor suppressor gene such as p53). In addition, nucleic acid analysis may be used to identify pathogens and transgenic organisms.

15 **[0003]** Many techniques have been developed for nucleic acid analysis. For one example, techniques such as oligo-nucleotide ligation assay (OLA) and ligation chain reaction (LCR) have been used to detect SNPs. See, e.g., Abravaya, et al., 1995, Detection of point mutations with a modified ligase chain reaction (Gap-LCR), *Nucleic Acids Res.* 23:675-82; Landegren et al., 1988, A ligase-mediated gene detection technique, *Science*. 241:1077-80; Schwartz et al., 2009, Identification of cystic fibrosis variants by polymerase chain reaction/oligonucleotide ligation assay, *J Mol Diagn.* 11:211-5. WO2010114599 A1. For another example, microarrays and high throughput DNA sequencing may be used to detect chromosome rearrangements and gene copy numbers. See, e.g., Agilent Human Genome CGH Microarray (Agilent Technologies, Inc., Santa Clara, CA), and the Illumina HiSeq DNA Sequencing Assays (Illumina, Inc., San Diego, CA). However, the nucleic acid analysis using these known techniques are either not easily multiplexed (e.g., the OLA and LCR methods), or time-consuming, expensive and/or inaccurate (e.g., microarrays and high throughput DNA sequencing).

25 **[0004]** Therefore, it is desirable to have a new technique that makes the nucleic acid analysis easily multiplexed and efficient. An object of the present invention is to provide methods and kits for multiplexed and efficient nucleic acid analysis in a sample. The methods and kits based on the present invention may be suitable for adaptation and incorporation into a compact device or instrument for use in a laboratory or a clinical setting, or in the field.

30 **[0005]** No reference cited in this background section is to be construed as an "admission" of prior art. Applicant expressly reserves the right to demonstrate, where appropriate, that the references cited herein do not constitute prior art under the applicable statutory provisions.

SUMMARY

40 **[0006]** The present invention is defined by the appended claims.

45 **[0007]** In one aspect, the present invention provides methods for multiplex nucleic acids analysis in a sample. In one embodiment, nucleic acids in a sample are assayed by adding a set of probes into the sample to form a mixture; denaturing nucleic acids in the mixture; hybridizing the set of probes to the complementary regions of target nucleic acids; performing a ligation reaction on the hybridized probes to form a third probe; amplifying the third probe with a set of primers to obtain an amplification product; and assaying the presence, absence or quantity of the target nucleic acids in the sample by determining the presence, absence or quantity of the third probe in the amplification product.

50 **[0008]** In each set of probes, there are at least a first probe having a first portion at least partially complementary to a first region of the target nucleic acid in the sample and a second portion forming a first primer binding site (herein also referred to as primer binding sequence); and a second probe having a first portion at least partially complementary to a second region of the target nucleic acid in the sample and a second portion forming a second primer binding site. The 5' end of the first probe is essentially adjacent to the 3' end of the second probe when both probes are hybridized to the target nucleic acid.

55 **[0009]** The set of primers used to amplify the ligation product comprises a first primer at least partially complementary to the first primer binding site; and a second primer at least partially complementary to the second primer binding site.

60 **[0010]** In some embodiments, the method is used to assay multiple target nucleic acids in a multiplexed manner. For example, the presence, absence or quantity of more than about 48, 96, 192, 384 or more target nucleic acids in the sample may be assayed in a multiplexed manner. The probes corresponding to all the target nucleic acids may be added to the sample and a single ligation reaction is performed to obtain ligation products for all the target nucleic acids.

[0011] The sample to be assayed may be a sample of a bodily fluid, a biopsy tissue, or a paraffin-embedded tissue from an animal. The bodily fluid may be blood, plasma, serum, urine, sputum, spinal fluid, cerebrospinal fluid, pleural fluid, nipple aspirates, lymph fluid, fluid of the respiratory, intestinal, or genitourinary tracts, tear fluid, saliva, breast milk, fluid from the lymphatic system, semen, cerebrospinal fluid, intra-organ system fluid, ascitic fluid, tumor cyst fluid, amniotic fluid, or combinations thereof from an animal, e.g., a human.

[0012] In some embodiments, nucleic acids are extracted from the sample before forming a mixture with the set of probes. The target nucleic acid may be DNA, RNA, or cDNA. The RNA may be reverse transcribed into cDNA before forming a mixture with the set of probes.

[0013] In some embodiments, at least one primer of the set of primers is labeled with a detectable moiety, e.g., an oligonucleotide tag or a fluorescent dye. The fluorescent dye may be FAM (5-or 6-carboxyfluorescein), VIC, NED, PET, Fluorescein, FITC, IRD-700/800, CY3, CY5, CY3.5, CY5.5, HEX, TET, TAMRA, JOE, ROX, BODIPY TMR, Oregon Green, Rhodamine Green, Rhodamine Red, Texas Red, or Yakima Yellow. In other embodiments, at least one primer of the set of primers includes a stuffer sequence. The stuffer sequence in some primers may have about 10 to about 500 nucleotides. The stuffer sequence in other primers may be about 10 to about 60 nucleotides. In some preferred embodiments, no primer has more than about 125 nucleotides, preferably about 75 nucleotides. In still other embodiments, at least one primer of the set of primers includes an oligonucleotide comprising a sequence GTTCTT or a functional equivalent variant of the oligonucleotide comprising a sequence GTTCTT.

[0014] In further embodiments, at least one probe of the set of probes includes a stuffer sequence. In some instances, the stuffer sequence may have about 1 to about 200 nucleotides. In other instances, the stuffer sequence has about 1 to about 55 nucleotides. In some preferred embodiments, the third probe has no more than about 250 nucleotides. In other preferred embodiments, the third probe has no more than about 140 nucleotides. In still some preferred embodiments, no probe has more than about 125 nucleotides. In still some other preferred embodiments, no probe has more than about 70 nucleotides. In some further preferred embodiments, no probe has more than about 60 nucleotides.

[0015] In some embodiments, the multiplexity of nucleic acid analysis may result from the modifications that at least one primer of the set of primers is labeled with a detectable moiety and that at least one primer of the set of primers includes a stuffer sequence. In other embodiments, the multiplexity of nucleic acid analysis may result from the modifications that at least one primer of the set of primers is labeled with a detectable moiety and that at least one probe of the set of probes includes a stuffer sequence. In still other embodiments, the multiplexity of nucleic acid analysis may result from the modifications that at least one primer of the set of primers includes a stuffer sequence and that at least one probe of the set of probes includes a stuffer sequence. In some preferred embodiments, the multiplexity of nucleic acid analysis may result from the modifications that at least one primer of the set of primers is labeled with a detectable moiety, that at least one primer of the set of primers includes a stuffer sequence, and that at least one probe of the set of probes includes a stuffer sequence. In these embodiments, the determination of the presence, absence or quantity of the third probe in the amplification product may be carried out by measuring the presence, absence or quantity of the third probe in the amplification product on the basis of detectable moieties, fragment sizes, or both. The measurement may be carried out using capillary electrophoresis.

[0016] The method according to the present invention may be applied in many nucleic acid assays. For one example, single nucleotide polymorphisms in target nucleic acids may be detected using the method. For this purpose, an allele-specific probe corresponding to the single nucleotide polymorphism may be designed. In addition, a mismatch may be introduced at one or more positions, e.g., the second, third, or fourth nucleotide away from the polymorphic nucleotide in the allele-specific probe so that the specificity of the probe binding is enhanced.

[0017] For a second example, copy number variants in target nucleic acids may be detected using the method. In one instance, a deletion of one or more exons in the dystrophin gene may be determined using one or more probe pairs selected from SEQ ID NOs: 158-541.

[0018] For a third example, the method may be used to screen for an unknown point mutation, insertion, or deletion of nucleotides. In one instance, a point mutation in the dystrophin gene may be screened using one or more probe pairs selected from SEQ ID NOs: 158-541.

[0019] For a fourth example, the method may be used to measure the presence, absence or relative amount of messenger RNA, methylated DNA, a pathogen or a transgenic organism. A pathogen may be a virus or a bacterium. A transgenic organism may be a transgenic plant such as transgenic corn, transgenic rice, transgenic soybean and transgenic cotton, or a transgenic animal such as a transgenic cow, a transgenic pig, a transgenic sheep and a transgenic dog.

[0020] In some embodiments, the steps of denaturing, hybridization and ligation may be repeated about 1 to about 100 times. The denaturing step may be carried at about 90°C to about 99°C for about 5 seconds to about 30 minutes, and the hybridization and the ligation steps may be carried out simultaneously at about 4°C to about 70°C for about 1 minute to about 48 hours. In a preferred embodiment, the denaturing step is carried at about 95°C for about 30 seconds, and the hybridization and the ligation steps are carried out simultaneously at about 58°C for about 4 hours, and the steps of denaturing, hybridization and ligation are repeated 4 times.

[0021] In another aspect of the present invention, a method is provided for detecting small copy number changes. In

one embodiment, for purpose of detecting small copy number changes of a target nucleic acid, two or more sets of probes are used to hybridize to two or more target sites in the same target nucleic acid, with each set of probes hybridizing to a different target site. The small copy number changes may be, for example, a quantitative variation of the target nucleic acid between two samples. In some instances, the quantitative variation of the target nucleic acid is about 0.1% to about 30%.

[0022] In some embodiments, for each set of probes hybridizing to each target site in the same target nucleic acid, one or more sets of reference probes are used to hybridize to one or more reference target sites, with each set of reference probes hybridizing to a different reference target site. For example, about 1 to about 100 sets of reference probes are used for an individual target site. In some instances, about 6 sets of reference probes are used for an individual target site.

[0023] In some embodiments, the same set of primers is used to amplify a group of ligation products (also referred to as the third probes). The ligation products may be formed from one or more sets of probes hybridizing to one or more target sites and from one or more sets of reference probes hybridizing to one or more reference target sites. For example, in one group of ligation products, there may be ligation products formed from about 1 to about 100 sets of probes hybridizing to gene target sites of interest and from about 1 to about 100 sets of reference probes hybridizing to reference target sites.

[0024] In other embodiments, about 50 to about 500 sets of probes hybridizing to about 50 to about 500 target sites on a target nucleic acid are used to detect small copy number variation of the target nucleic acid in a sample. In this case, multiple groups of ligation products formed from a plurality of probes for targets site and probes for reference sites may be obtained. For each group of ligation products, the same primer pair may be used to amplify the ligation products in that group.

[0025] In some preferred embodiments, the target nucleic acids are from human chromosome 21, human chromosome 18, human chromosome 13, human chromosome region 22q11.2, or the pseudoautosomal regions of human chromosomes X or Y. As such, the method according to the present invention may be used to detect fetal aneuploidy for chromosomes 21, 18, 13, X, Y and 22q11.2 in a maternal blood or urine sample. In one instance, the method is used to detect fetal Down's syndrome in a maternal blood or urine sample using one or more probe pairs selected from SEQ ID NOs: 559-942.

[0026] In a further aspect of the present invention, a kit for assaying nucleic acids in a sample is provided. In one embodiment, the kit includes one or more sets of probes corresponding to a target nucleic acid; one or more sets of primers for amplifying the third probe; optionally reagents including a ligase, a buffer for a ligation reaction, a DNA polymerase, a buffer for polymerase chain reaction or a combination thereof; and optionally a brochure containing instructions of using the kit.

[0027] In some embodiments, the set of probes includes a first probe having a first portion at least partially complementary to a first region of a target nucleic acid in the sample and a second portion forming a first primer binding site; and a second probe having a first portion at least partially complementary to a second region of the target nucleic acid in the sample and a second portion forming a second primer binding site. The 5' end of the first probe may be essentially adjacent to the 3' end of the second probe when both probes are hybridized to the target nucleic acid and the first and the second probes may be ligated to form a third probe. In some instances, at least one probe of the set of probes includes a stuffer sequence. The stuffer sequence may have about 1 to about 200 nucleotides.

[0028] In other embodiments, the set of primers includes a first primer at least partially complementary to the first primer binding site; and a second primer at least partially complementary to the second primer binding site. In some instances, at least one primer of the set of primers is labeled with a detectable moiety, e.g., an oligonucleotide tag or a fluorescent dye. The fluorescent dye may be FAM (5-or 6-carboxyfluorescein), VIC, NED, PET, Fluorescein, FITC, IRD-700/800, CY3, CY5, CY3.5, CY5.5, HEX, TET, TAMRA, JOE, ROX, BODIPY TMR, Oregon Green, Rhodamine Green, Rhodamine Red, Texas Red, or Yakima Yellow. In still other embodiments, at least one primer of the set of primers includes a stuffer sequence. The stuffer sequence has about 10 to about 500 nucleotides.

[0029] In some embodiments, the kit is for detecting Duchenne muscular dystrophy and includes one or more sets of probes comprising probe pairs selected from SEQ ID NOs: 158-541. In other embodiments, the kit is for detecting fetal Down's syndrome in a maternal blood sample and includes one or more sets of probes comprising probe pairs selected from SEQ ID NOs: 559-942.

BRIEF DESCRIPTION OF THE DRAWINGS

[0030]

Figure 1 is a schematic flowchart depicting the method of increasing the multiplexity by employing fluorescent dye labeled primers for amplifying ligation products.

Figure 2 is a schematic flowchart depicting the method of increasing multiplexity by employing primers with stuffer

sequences for amplifying ligation products.

Figure 3 is a schematic flowchart depicting the method of increasing the fold of multiplexing by employing both fluorescent dye labeled primers and primers with stuffer sequences for amplifying ligation products.

Figure 4 is a schematic flowchart depicting an exemplary analysis of 48 SNPs in a multiplex assay by employing both fluorescent dye labeled primers and primers with stuffer sequences for amplifying ligation products. X and Y refer to primer binding sites on the probes. LSHS refers to locus-specific hybridization sequences. Stuffer A refers to an allelic-specific stuffer sequence of 2 nucleotides in length. Stuffer L1 and stuffer L2 refer to stuffer sequences used for adjusting the size of the ligated probe. ASHS refers to allele-specific hybridization sequences. F1, F2, F3 and F4 refer to forward primers labeled with blue, green, yellow and red fluorescent dyes, respectively. R1 and R2 refer to reverse primers with different stuffer sequences. F1/R1(6) refers to 12 amplification products using the F1 and R1 primer pair based on 12 ligation products from 6 SNP loci with two SNP alleles per SNP locus. Similar interpretations stand for F2/R1(6), F3/R1(6), F4/R1(6), F1/R2(6), F2/R2(6), F3/R2(6), and F4/R2(6).

Figure 5 is a schematic flowchart depicting an exemplary analysis of 96 CNVs in a multiplex assay by employing both fluorescent dye labeled primers and primers with stuffer sequences for amplifying ligation products. X and Y refer to primer binding sites on the probes. LSHS refers to locus-specific hybridization sequences. Stuffer L1 and stuffer L2 refer to stuffer sequences used for adjusting the size of the ligated probe. F1, F2, F3 and F4 refer to forward primers labeled with blue, green, yellow and red fluorescent dyes, respectively. R1 and R2 refer to reverse primers with different stuffer sequences. F1/R1(12) refers to 12 amplification products using the F1 and R1 primer pair based on 12 ligation products from 12 CNVs. Similar interpretations stand for F2/R1(12), F3/R1(12), F4/R1(12), F1/R2(12), F2/R2(12), F3/R2(12), and F4/R2(12).

Figure 6 is a schematic flowchart depicting an exemplary mutations screening analysis of 96 fragments overlapping upon the target region in a multiplexed assay by employing both fluorescent dye labeled primers and primers with stuffer sequences for amplifying ligation products. The labels refer to the similar components as detailed in **Figure 5**.

Figure 7 is a schematic flowchart depicting an exemplary gene expression analysis of 96 mRNAs in a multiplexed assay by employing both fluorescent dye labeled primers and primers with stuffer sequences for amplifying ligation products. The labels refer to the similar components as detailed in **Figure 5**.

Figure 8 is a schematic flowchart depicting an exemplary analysis of 96 nucleic acid targets from pathogens or transgenic plants/animals in a multiplexed assay by employing both fluorescent dye labeled primers and primers with stuffer sequences for amplifying ligation products. The labels refer to the similar components as detailed in **Figure 5**.

Figure 9 is a schematic flowchart depicting an exemplary analysis of 96 methylation target sites in a multiplexed assay by employing both fluorescent dye labeled primers and primers with stuffer sequences for amplifying ligation products. The labels refer to the similar components as detailed in **Figure 5**.

Figures 10A, 10B, 10C, 10D, and 10E are electrophoresis chromatograms depicting the electrophoresis pattern of the amplification products in a multiplexed assay of 48 SNPs. **Figure 10A** is an electrophoresis chromatogram of all 96 amplification products. **Figures 10B, 10C, 10D and 10E** are electrophoresis chromatograms for 24 amplification products produced with primers labeled with blue, green, yellow and red fluorescent dyes, respectively.

Figures 11A, 11B, 11C, 11D, 11E, 11F, 11G and 11H are electrophoresis chromatograms depicting the electrophoresis pattern of the amplification products in a multiplexed assay of 192 copy number variants. **Figure 11A and 11B** are electrophoresis chromatograms of all amplification products for the control sample panel A and panel B, respectively. **Figure 11C and 11D** are electrophoresis chromatograms of all amplification products for the patient sample panel A and panel B, respectively. **Figures 11E and 11F** are electrophoresis chromatograms of amplification products labeled with blue fluorescent dyes for the control and the patient samples, respectively. **11G and 11H** are electrophoresis chromatograms for amplification products produced with primers labeled with green fluorescent dyes for the control and the patient samples, respectively. The arrows in **Figures 11F and 11H** refer to the missing peaks corresponding to gene target sites.

Figure 12A shows a chart depicting the calculated copy numbers of all gene target sites in the dystrophin gene. The X axis refers to names of each gene target site. The Y axis refers to the calculated copy number for each gene target site. **Figure 12B** is a close-up view of a part of the chart in **Figure 12A**, showing the target sites E01A to E16B. **Figure 12C** shows the two chromatograms including the peak for DMD_E07B gene target site: one from a healthy control and the other from a male DMD patient. **Figure 12D** shows a chromatogram of DNA sequencing result of the region surrounding the DMD_07EB gene target site.

Figures 13A, 13B, 13C, 13D, 13E, 13F, 13G, 13H, 13I, and 13J are electrophoresis chromatograms depicting the electrophoresis pattern of the amplification products for measuring human chromosome 21 copy number in a control DNA sample. **Figure 13A and 13F** are electrophoresis chromatograms of all 96 amplification products for the control sample panel A and panel B, respectively. **Figures 13B, 13C, 13D, and 13E** are electrophoresis chromatograms of the 24 amplification products from the control sample panel A separately labeled with blue, green, yellow and red fluorescent dyes, respectively. **Figures 13G, 13H, 13I, and 13J** are electrophoresis chromatograms

of the 24 amplification products from the control sample panel B separately labeled with blue, green, yellow and red fluorescent dyes, respectively.

Figure 14 shows a chart depicting the calculated human chromosome 21 copy number in the five DNA samples. The X axis refers to the five samples: M0, M2, M4, M8, and M16. The Y axis refers to the calculated copy numbers. For each DNA sample, the testing was repeated three times. The p values were derived from Student's *t* test with the average copy number for M0 DNA sample as the reference.

Figure 15 shows a graph depicting the lineal correlation between the calculated copy number and the expected copy number for the five DNA samples.

DETAILED DESCRIPTION OF THE INVENTION

I. Multiplex Nucleic Acid Analysis

[0031] In one aspect, the present invention provides methods for multiplex nucleic acid analysis. In one embodiment, the method for multiplex analysis of target nucleic acids in a sample includes the steps of preparing nucleic acids that are quantitatively and qualitatively correlated to a plurality of target nucleic acids in the sample and determining quantitatively and qualitatively the nucleic acids thus prepared.

[0032] As used herein, the phrase "multiplex" or grammatical equivalents refers to the quantitative and qualitative determination of more than one target nucleic acids of interest in a sample. In one embodiment "multiplex" refers to at least about 48 different target sequences. In another embodiment "multiplex" refers to at least about 96 different target sequences. In further embodiment "multiplex" refers to at least about 192 different target sequences. In still further embodiments, "multiplex" refers to at least about 384 different target sequences. In yet still further embodiment "multiplex" refers to at least about 500 to about 100,000 different target sequences.

[0033] As used herein, the sample in which target nucleic acids exist may be a sample from a subject, including, but not limited to, bodily fluids (e.g., blood, plasma, serum, urine, sputum, spinal fluid, cerebrospinal fluid, pleural fluid, nipple aspirates, lymph fluid, fluid of the respiratory, intestinal, and genitourinary tracts, tear fluid, saliva, breast milk, fluid from the lymphatic system, semen, cerebrospinal fluid, intra-organ system fluid, ascitic fluid, tumor cyst fluid, amniotic fluid and combinations thereof); environmental samples (e.g., air, agricultural, water and soil samples); biological warfare agent samples; research samples; purified samples, such as purified genomic DNA, RNA, proteins, etc.; and raw samples (bacteria, virus, genomic DNA, etc.).

[0034] The term "subject" is intended to include all animals. In particular embodiments, the subject is a mammal, a human or nonhuman primate, a dog, a cat, a horse, a cow, other farm animals, or a rodent (e.g. mice, rats, guinea pig. etc.). A human subject may be a normal human being without observable abnormalities, e.g., a disease. A human subject may be a human being with observable abnormalities, e.g., a disease. The observable abnormalities may be observed by the human being himself, or by a medical professional. The term "subject", "patient", and "individual" are used interchangeably herein.

[0035] In one aspect, the present invention provides methods of preparing nucleic acids that are quantitatively and qualitatively correlated to a plurality of target nucleic acids in a sample. The correlation may be achieved through some mechanisms including, but not limited to, 1) specific hybridizations between complementary nucleic acids and 2) specific enzymatic recognition such as a ligation reaction to connect substantially adjacent nucleotides and a polymerase reaction to add specific nucleotide onto an existing polynucleotide based on a template sequence.

[0036] As used herein, "nucleic acid", "polynucleotide", and "oligonucleotide" are interchangeably used to indicate at least two nucleotides covalently linked together. Oligonucleotides may be generated by, e.g., chemical synthesis, restriction endonuclease digestion of plasmids or phage DNA, DNA replication, reverse transcription, or a combination thereof. One or more of the nucleotides can be modified e.g. by addition of a methyl group, a biotin or digoxigenin moiety, a fluorescent tag, or by using radioactive nucleotides.

[0037] A nucleic acid used in the present invention may contain phosphodiester bonds, although in some cases nucleic acid analogs are included that may have alternate backbones, including, e.g., phosphoramidate, phosphorothioate, phosphorodithioate, O-methylphosphoroamidite linkages, and peptide nucleic acid backbones and linkages. See, e.g., Pauwels et al., 1986, *Chemica Scripta* 26:141-9; U.S. Pat. No. 5,644,048; Briu et al., 1989, *J. Am. Chem. Soc.* 111:2321; and Carlsson et al., 1996, *Nature* 380:207. Other analog nucleic acids include those with positive backbones, non-ionic backbones, and non-ribose backbones. See, e.g., Denpcy et al., 1995, *Proc. Natl. Acad. Sci. USA* 92:6097; Jeffs et al., 1994, *J. Biomolecular NMR* 34:17; U.S. Pat. Nos. 5,386,023, 5,235,033 and 5,034,506. Modifications of the ribose-phosphate backbone may be done to facilitate the addition of labels, or to increase the stability and half-life of such molecules in physiological environments.

[0038] In some embodiments, peptide nucleic acids (PNA) may be used for nucleic acids in the present invention. The PNA backbones are substantially non-ionic under neutral conditions, in contrast to the highly charged phosphodiester backbone of naturally occurring nucleic acids. This results in two advantages. First, the PNA backbone exhibits improved

hybridization kinetics. PNAs have larger changes in the melting temperature (T_m) for mismatched versus perfectly matched basepairs. DNA and RNA typically exhibit a 2-4°C drop in T_m for an internal mismatch. With the non-ionic PNA backbone, the drop is closer to 7-9°C. This allows for better detection of mismatches. Similarly, due to their non-ionic nature, hybridization of the bases attached to these backbones is relatively insensitive to salt concentration.

[0039] Nucleic acids may be single stranded or double stranded, as specified, or contain portions of both double stranded or single stranded sequence. Nucleic acids may be DNA, genomic DNA, cDNA, RNA or a hybrid. Nucleic acids may contain any combination of deoxyribo- and ribo-nucleotides, and any combination of bases, including uracil, adenine, thymine, cytosine, guanine, inosine, xanthine, hypoxanthine, isocytosine, isoguanine, etc. In one embodiment, nucleic acids utilize isocytosine and isoguanine in nucleic acids designed to be complementary to other probes, rather than target sequences, as this reduces non-specific hybridization, as is generally described in U.S. Patent No. 5,681,702. As used herein, the term "nucleoside" includes nucleotides as well as nucleoside and nucleotide analogs, and modified nucleosides such as amino modified nucleosides. In addition, "nucleoside" includes non-naturally occurring analog structures. Thus, for example, the individual units of a peptide nucleic acid, each containing a base, are referred to herein as a nucleoside.

[0040] In some embodiments, the methods according to the present invention are directed to the multiplexed detection of target nucleic acids. The term "target nucleic acid" or grammatical equivalents herein refers to a specific nucleic acid sequence to be detected and/or quantified in the sample to be analyzed. The target nucleic acid may be a portion of a gene, a regulatory sequence, genomic DNA, cDNA, or RNA including mRNA and rRNA.

[0041] Complementary nucleic acids are capable of hybridizing to each other under normal hybridization conditions. The term "complementary" refers to sequence complementarity between regions of two nucleic acid strands or between two regions of the same nucleic acid strand. It is known that an adenine residue of a first nucleic acid region is capable of forming specific hydrogen bonds ("base pairing") with a residue of a second nucleic acid region which is antiparallel to the first region if the residue is thymine or uracil. Similarly, it is known that a cytosine residue of a first nucleic acid strand is capable of base pairing with a residue of a second nucleic acid strand which is antiparallel to the first strand if the residue is guanine. A first region of a nucleic acid is complementary to a second region of the same or a different nucleic acid if, when the two regions are arranged in an antiparallel fashion, at least one nucleotide residue of the first region is capable of base pairing with a residue of the second region. In certain embodiments, the first region comprises a first portion and the second region comprises a second portion, whereby, when the first and second portions are arranged in an antiparallel fashion, at least about 50%, at least about 75%, at least about 90%, or at least about 95% of the nucleotide residues of the first portion are capable of base pairing with nucleotide residues in the second portion. In other embodiments, all nucleotide residues of the first portion are capable of base pairing with nucleotide residues in the second portion. As used herein, the pairing of complementary nucleic acids is referred to by the terms "hybridization" or "hybridizing".

[0042] Specific enzymatic recognition may be achieved through a ligation reaction to connect substantially adjacent nucleotides or a DNA or RNA polymerase reaction to add specific nucleotide onto an existing polynucleotide based on a template sequence.

[0043] Ligases are well known and may be used for specific enzymatic recognition. See, e.g., Lehman, 1974, Science, 186: 790-797; and Engler et al, DNA Ligases, pages 3-30 in Boyer, editor, The Enzymes, Vol. 15B (Academic Press, New York, 1982). Preferred ligases include T3 DNA ligase, T4 DNA ligase, T7 DNA ligase, E. coli DNA ligase, Taq DNA ligase, Pfu ligase, and Tth ligase. Protocols for their use are well known. See, e.g., Green and Sambrook, Molecular Cloning: A Laboratory Manual (Fourth Edition): Three-volume set, Cold Spring Harbor Laboratory Press; 4th edition (June 15, 2012) and the like. Generally, ligases require that a 5' phosphate group be present for ligation to the 3' hydroxyl of an adjacent strand.

[0044] In some embodiments, the preferred ligase is one which has the least mismatch ligation. The specificity of ligase can be increased by substituting the more specific NAD⁺-dependant ligases such as E. coli ligase and (thermostable) Taq ligase for the less specific T4 DNA ligase. The use of NAD analogues in the ligation reaction further increases specificity of the ligation reaction. See, e.g., U.S. Patent No. 5,508,179.

[0045] DNA or RNA polymerases can extend a nucleic acid sequence by adding nucleotides in the presence of a template. As is well known in the art, there are a wide variety of suitable polymerases. Suitable polymerases include, but are not limited to, DNA polymerases, including the Klenow fragment of DNA polymerase I, SEQUENASE 1.0 and SEQUENASE 2.0 (U.S. Biochemical), T5 DNA polymerase, Phi29 DNA polymerase and various RNA polymerases such as from Thermus sp., Q beta replicase from bacteriophage, or SP6, T3, T4 and T7 RNA polymerases.

[0046] In some embodiments, preferred polymerases are those that are essentially devoid of a 5' to 3' exonuclease activity, so as to assure that the first probe will not be extended past the 5' end of the second probe. Exemplary enzymes lacking a 5' to 3' exonuclease activity include the Klenow fragment of the DNA Polymerase and the Stoffel fragment of DNA Taq Polymerase. (See e.g., Lawyer et al., 1989, J. Biol. Chem., 264:6427-6437; and Lawyer et al., 1993, PCR Meth. Appl., 2:275-287). Other mutant polymerases lacking 5' to 3' exonuclease activity have been generated for polymerases derived from T. vulgaris. See U.S. 6,632,645.

[0047] In other embodiments, preferred polymerases are those that lack a 3' to 5' exonuclease activity, which is commonly referred to as a proof-reading activity, and which removes bases which are mismatched at the 3' end of a primer-template duplex. Although the presence of 3' to 5' exonuclease activity provides increased fidelity in the strand synthesized, the 3' to 5' exonuclease activity found in thermostable DNA polymerases such as Tma (including mutant forms of Tma that lack 5' to 3' exonuclease activity) also degrades single-stranded DNA such as the primers used in the PCR, single-stranded templates and single-stranded PCR products. The integrity of the 3' end of an oligonucleotide primer used in a primer extension process is critical as it is from this terminus that extension of the nascent strand begins. Degradation of the 3' end leads to a shortened oligonucleotide which may result in a loss of specificity in the PCR reaction.

[0048] In still other embodiments, more preferred polymerases are thermostable polymerases. A thermostable enzyme is an enzyme that retains most of its activity after one hour at 40°C under optimal conditions. Examples of thermostable polymerases which lack both 5' to 3' exonuclease and 3' to 5' exonuclease include Stoffel fragment of Taq DNA polymerase. This polymerase lacks the 5' to 3' exonuclease activity due to genetic manipulation and no 3' to 5' activity is present as Taq polymerase is naturally lacking in 3' to 5' exonuclease activity.

[0049] The conditions for performing the addition of one or more nucleotides at the 3' end of a probe will depend on the particular enzyme used, and will generally follow the conditions recommended by the manufacturer of the enzymes used.

[0050] In some embodiments, the correlation may be achieved through a combination of different mechanisms, e.g., a combination of both specific hybridizations between complementary nucleic acids and a ligation reaction to connect substantially adjacent nucleotides; or a combination of specific hybridizations between complementary nucleic acids, a ligation reaction to connect substantially adjacent nucleotides, and a polymerase reaction to add specific nucleotide onto an existing polynucleotide based on a template sequence.

[0051] In some embodiments, the combination of both specific hybridizations between complementary nucleic acids and a ligation reaction to connect substantially adjacent nucleotides is applied to prepare nucleic acids that are quantitatively and qualitatively correlated with the target nucleic acids in a sample. As such, the method to prepare nucleic acids from a sample include the steps of 1) hybridizing probes to the target nucleic acids in the sample; and 2) ligating the hybridized probes to obtain a preparation of nucleic acids that are quantitatively and qualitatively correlated with the target nucleic acids in the sample.

[0052] In other embodiments, the combination of specific hybridizations between complementary nucleic acids, a ligation reaction to connect substantially adjacent nucleotides, and a polymerase reaction to add specific nucleotide onto an existing polynucleotide based on a template sequence is applied to prepare nucleic acids that are quantitatively and qualitatively correlated with the target nucleic acids in a sample. As such, the method to prepare nucleic acids from a sample include the steps of 1) hybridizing probes to the target nucleic acids in the sample; 2) extending one of the probes to close the gap between the probes so that the extended probes may be ligated to an adjacent probe; and 3) ligating the hybridized and extended probes to obtain a preparation of nucleic acids that are quantitatively and qualitatively correlated with the target nucleic acids in the sample.

[0053] In some embodiments, the target nucleic acid may be extracted from the sample before being hybridized to probes. Methods known for nucleic acid extraction in the art include the use of phenol/chloroform, the use of salting out procedure, the use of chaotropic salts and silica resins, the use of affinity resins, ion exchange chromatography and the use of magnetic beads. See, for example, U.S. Pat. Nos. 5,0574,26 and 4,923,978, EP Patents 0512767, WO 95/13368, WO 97/10331 and WO 96/18731. Conventional techniques of molecular biology, biochemistry, genetics, which are in the skill of the art, are explained fully in the literature. See, for instance, Green and Sambrook, *Molecular Cloning: A Laboratory Manual* (Fourth Edition): Three-volume set, Cold Spring Harbor Laboratory Press; 4th edition (June 15, 2012); Carson, Miller, and Witherow, *Molecular Biology Techniques*, Third Edition: A Classroom Laboratory Manual, Academic Press; 3 edition (November 21, 2011); Cheng and Zhang, *Molecular Genetic Pathology*, Humana Press; 1 edition (April 15, 2008).

[0054] In addition, when the target nucleic acids are preferred to be cut into a size that will facilitate handling and hybridization to the probes, particularly for genomic DNA, this may be accomplished by shearing the nucleic acid through mechanical forces (e.g. sonication) or by cleaving the nucleic acid using restriction endonucleases, or any other methods known in the art.

[0055] As used herein, "probe" refers to a known sequence of a nucleic acid that is capable of selectively binding to a target nucleic acid. More specifically, "probe" refers to an oligonucleotide designed to be sufficiently complementary to a sequence of one strand of a nucleic acid that is to be probed such that the probe and nucleic acid strand will hybridize under selected stringency conditions. Additionally, "probe" also refers to an end product which is derived by connecting one or more substantially adjacent oligonucleotides hybridized to substantially adjacent segments of a nucleic acid. For example, a "ligated probe" refers to the end product of a ligation reaction between a pair of probes.

[0056] As used herein, the term "substantially adjacent" is used in reference to nucleic acid molecules that are in close proximity to one another. The term also refers to a sufficient proximity between two nucleic acid molecules to allow the 5' end of one nucleic acid that is brought into juxtaposition with the 3' end of a second nucleic acid so that they may be

ligated by a ligase enzyme. Nucleic acid segments are defined to be substantially adjacent when the 3' end of a first probe and the 5' end of a second probe, with the first probe hybridizing to one segment and the second probe to the other segment, are sufficiently near each other to allow connection of the ends of both probes to one another. Thus, two probes are substantially adjacent, when the ends thereof are sufficiently near each other to allow connection of the ends of both probes to one another.

[0057] As such, in some embodiments of the present invention, a set of probes including 2 or more probes are designed to hybridize to a target nucleic acid. The target nucleic acid may contain several target regions; for example, a first target region of the target nucleic acid may hybridize to a first probe or a portion of the first probe, a second target region of the target nucleic acid may hybridize a second probe or a portion of the second probe. In addition, the two target regions may be adjacent or separated. When the two regions are adjacent, e.g., a first probe hybridizing to a first target region and a second probe hybridizing to a second target region, the first and the second hybridized probes may be adjacent so that a ligation reaction may connect the two probes to form a third probe. The third probe may therefore be able to hybridize to both the first region and the second region of the target nucleic acid. When the two regions are separated by one or more nucleotides, the gap between the first and the second hybridized probes may be filled with the use of a polymerase and dNTPs to extend one of the probes so that the extended probe and the other hybridized probe may be substantially adjacent to form a third probe.

[0058] The terms "first" and "second" are not meant to confer an orientation of the sequences with respect to the 5'-3' orientation of the target sequence. For example, assuming a 5'-3' orientation of the complementary target nucleic acid, the first target region may be located either 5' to the second region, or 3' to the second region.

[0059] As such in one embodiment, the probes of the present invention are designed to be complementary to a target nucleic acid, such that the probes hybridize to the target nucleic acids. This complementarity need not be perfect; there may be any number of base pair mismatches that will interfere with hybridization between the target nucleic acid and the single stranded probe sequence. However, if the number of mismatches is so many so that no hybridization can occur under certain hybridization conditions, the sequence is not a complementary target sequence.

[0060] In addition, these probes may take on a variety of configurations and may have a variety of structural components. In some embodiments, a probe may be an allele specific probe or a locus specific probe. An allele specific probe includes an allele-specific hybridization sequence (ASHS) portion that hybridizes to a target nucleic acid and discriminates between alleles, or hybridizes to a target nucleic acid and is modified in an allele specific manner. A locus specific probe includes a locus-specific hybridization sequence (LSHS) portion that hybridizes to a target nucleic acid in a locus specific manner, but does not necessarily discriminate between alleles. A locus specific probe also may be modified, i.e. extended as described below, such that it includes information about a particular allele, but the locus specific primer itself does not discriminate between alleles. The length of the ASHS or LSHS may be designed to confer sufficient specificity for the probe to hybridize to the target nucleic acids. With the general guidance that the longer the ASHS or LSHS sequence, the more specific they binds to the target nucleic acids, a skilled person in the art has the knowledge to varying the length and decide the length of the ASHS or LSHS in order to achieve his/her testing goals as described in detail herein, e.g., in the Examples.

[0061] In other embodiments, a probe may include one or more segments in addition to ASHS or LAHS. In some instance, the additional segment of the probe may be a primer binding site, onto which a primer may bind in a polymerase chain reaction. In other instance, the additional segment of the probe may be a stuffer sequence, which may make the ligation product vary in length and thereby be distinguished on the basis of fragment sizes. The length of the primer binding site and the stuffer sequence may be designed by a person skilled in the art to suit his/her testing goals. For example, a primer binding site of the probes may be about 15-20 nucleotides in length, with 18 being especially preferred. For another example, the stuffer sequence of the probes may be about 2-100 nucleotides depending on the needs to distinguish different target nucleic acids. As such, the stuffer sequence is a nucleic acid that is generally not native to the target sequence, but is added or inserted in the probe sequence. Preferred stuffer sequences are those that are not found in a genome, e.g., a human genome, and they do not have undesirable structures, such as hairpin loops.

[0062] In some embodiments, double stranded target nucleic acids are denatured to render them single stranded so as to permit hybridization of the target nucleic acids and the probes. The denaturation may be achieved, among other suitable means, by treating the target nucleic acids with heat, alkali, or both heat and alkali. See, e.g., Green and Sambrook, *Molecular Cloning: A Laboratory Manual* (Fourth Edition): Three-volume set, Cold Spring Harbor Laboratory Press; 4th edition (June 15, 2012). In some embodiments, the denaturing may be carried out at temperature about 90 °C to about 99 °C for about 5 seconds to 30 minutes. In a preferred embodiment, the denaturation may be carried out at about 98°C for 5 minutes.

[0063] The use of different stringency conditions such as variations in hybridization temperature and buffer composition may be used to determine the presence or absence of mismatches between a single stranded target nucleic acid and a probe. With regard to temperature, differences in the number of hydrogen bonds as a function of basepairing between perfect matches and mismatches can be exploited as a result of their different T_m s. Under a defined ionic strength, pH and nucleic acid concentration, the T_m is the temperature at which 50% of the probes complementary to the target

hybridize to the target sequence at equilibrium. Accordingly, a hybridized nucleic acid comprising perfect complementarity will melt at a higher temperature than one comprising at least one mismatch, with all other parameters being equal. The other parameters include the length of the hybridized nucleic acid, the nature of the backbone (i.e. naturally occurring or nucleic acid analog), the assay solution composition, and the composition of the nucleic acid, e.g., the G-C content.

[0064] High stringency conditions are those that result in perfect matches remaining in hybridization complexes, while imperfect matches melt off. On the other hand, low stringency conditions are those that allow the formation of hybridization complexes with both perfect and imperfect matches. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures.

[0065] A guide to the hybridization of nucleic acids is found in Tijssen, Techniques in Biochemistry and Molecular Biology-Hybridization with Nucleic Acid Probes, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10°C lower than the thermal melting point (T_m) for the specific sequence at defined ionic strength and pH. Stringent conditions may be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 3°C for short probes (e.g. 10 to 50 nucleotides) and at least about 6°C for long probes (e.g. greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. In another embodiment, less stringent hybridization conditions are used; for example, moderate or low stringency conditions may be used, as are known in the art. See, e.g., Tijssen, *supra*.

[0066] Similarly, variations in buffer composition may be used to elucidate the presence or absence of a mismatch at the detection position. Suitable conditions include, but are not limited to, formamide concentration. Thus, for example, "low" or "permissive" stringency conditions include formamide concentrations of 0 to 10%, while "high" or "stringent" conditions utilize formamide concentrations of 40%. Low stringency conditions include NaCl concentrations of 1 M, and high stringency conditions include concentrations of 0.3 M. Furthermore, low stringency conditions include MgCl₂ concentrations of 10 mM, moderate stringency as 1-10 mM, and high stringency conditions include concentrations of 1 mM.

[0067] Ligase catalyzes the covalent bonding between two nucleotides adjacent to each other. The ligation reaction is facilitated by a complementary strand holding the two nucleotides comprising the 3' and 5' ends of two polynucleotides with no gaps between the two ends in close proximity. In addition, the ligation reaction requires that there is a phosphate group exposed on the 5' end or hydroxyl group exposed on the 3' end.

[0068] Ligation can be carried out using any enzyme capable of ligating nucleotides. In some embodiments, Taq DNA ligase is used to ligate the two adjacent oligonucleotide probes hybridized to the target nucleic acid. Taq DNA Ligase catalyzes the formation of a phosphodiester bond between juxtaposed 5' phosphate and 3' hydroxyl termini of two adjacent oligonucleotide probes which are hybridized to a complementary target DNA. The ligation will occur only if the oligonucleotides are perfectly paired to the complementary target DNA and have no gaps between them; therefore, a single-base substitution may be detected if no ligation product is generated.

[0069] The condition for the ligation reaction depends on what ligase is used. For a thermal stable ligase, the ligase retains activities at elevated temperatures. For example, Taq DNA ligase remains active at 45-65 °C. For a non-thermal stable ligase, the ligase is active at lower temperatures. For example, T4 DNA ligase is active at around 16°C. As such, in one embodiment, the temperature for ligation reaction may be from about 4 °C to about 70 °C depending on which ligase is used.

[0070] The amount of the ligase and the duration of the ligation reaction may depend on the amount of probes and target nucleic acids. A person skilled in the art may adjust the amount of the ligase added in a reaction and the duration of the reaction so that a desirable ligation product may be achieved. For example, in some embodiments of the present invention, 40 units of Taq DNA ligase is used for a 20 µl ligation reaction. One unit is defined as the amount of enzyme required to give 50% ligation of the 12-base pair cohesive ends of 1 µg of BstEII-digested λ DNA in a total reaction volume of 50 µl in 15 minutes at 45°C.

[0071] In some embodiments, the duration of the ligation reaction is about 1 minute to 48 hours. In other embodiments, the duration of the ligation reaction is about 16 hours. In other embodiments, the steps of denaturation, hybridization, and ligation are repeated several times so that more probes may hybridize to the target nucleic acid or hybridize to the newly ligated probe. For example, the mixture of the probes and the target nucleic acids may be held at about 90 °C to about 99 °C for about 5 seconds to about 30 seconds for denaturation, and then at about 4°C to about 58 °C for about 1 minute to 48 hours for hybridization and ligation, and the cycles may be repeated to 100 times. In some preferred embodiments, for example, the mixture of the probes and the target nucleic acids may be held at about 95 °C for about 30 seconds for denaturation, and then at 58 °C for about 4 hours for hybridization and ligation, and the cycles may be repeated 4 times.

[0072] In other embodiments, there is a gap between the hybridized probes on the target nucleic acid. In some instances, the gap may be filled by another probe that is complementary to the gap in the target nucleic acid. This gap-filling probe is designed so that when it is hybridized to the nucleic acid, its two ends are substantially adjacent to the other two probes. In other instances, the gap may be filled by extending one of the probes hybridized to the target nucleic acid. This probe extension may be carried out using a DNA polymerase and dNTPs. A description of DNA polymerase

is described *supra*. For both gap filling methods, a ligation reaction is performed to ligate the substantially adjacent gap-filling probe with the other two probes or ligate the substantially adjacent extended probe with the other probe to obtain ligation products. The ligation reaction may be carried out with suitable parameters including, but not limited to, the type of the ligase, the amount of the ligase, and the duration of the ligation reaction as describe above. *Supra*.

[0073] In some embodiments, the ligation products are then analyzed directly to determine the presence, absence, or quantity of target nucleic acids in the sample. In other embodiments, the ligation products are then amplified to obtain amplification products which are analyzed to determine the presence, absence, or quantity of target nucleic acids in the sample.

[0074] As used herein, "amplification" refers to the increase in the number of copies of a particular nucleic acid. Copies of a particular nucleic acid made in an amplification reaction are called "amplicons" or "amplification products".

[0075] Nucleic acid amplification methods include, without limitation, polymerase chain reaction (PCR) (U.S. Patent Nos. 4,683,195, 4,683,202, 4,800,159, and 5,219,727) and its variants such as in situ polymerase chain reaction (U.S. Patent No. 5,538,871), quantitative polymerase chain reaction (U.S. Patent No. 5,219,727), and nested polymerase chain reaction (U.S. Patent No. 5,556,773).

[0076] In some embodiments, the amplification process is achieved through PCR. The PCR amplification process results in the exponential increase of discrete DNA fragments whose length is defined by the 5' ends of the oligonucleotide primers.

[0077] In some embodiments, universal primer binding sites are included in the probes and universal primers are used to amplify ligation products for all target nucleic acids. Alternatively, "sets" of universal primer binding sites are included in "sets" of corresponding probes, and "sets" of universal primers are used to amplify "sets" of the ligation products either simultaneously or sequentially to obtain "sets" of amplification products for further analysis.

[0078] Accordingly, in some embodiments of the present invention, "sets" of probes are provided for multiplex nucleic acid analysis with each set of probes including a first probe and a second probe. Multiplexity here refers to at least two target nucleic acids, with more than 10 being preferred, depending on the assay, sample and purpose of the test. In some embodiments the multiplexity refers to more than 48, 96, 192, or 384 target nucleic acids.

[0079] As used herein, the term "primer" refers to an oligonucleotide, sometimes produced synthetically, which is capable of acting as a point of initiation of nucleic acid sequence synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a template nucleic acid strand is induced, i.e. in the presence of different nucleotide triphosphates and a polymerase in an appropriate buffer and at a suitable temperature. A primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being substantially complementary to the template.

[0080] In some embodiments, an oligonucleotide GTTTCTT was included in the 5' portion of a primer sequence. The addition of the oligonucleotide GTTTCTT may help facilitate the non-templated addition of adenosine on the 3' end of PCR products when polymerases such as Taq polymerase are used in the PCR. See, e.g., Brownstein et al., Modulation of Non-Templated Nucleotide Addition by TaqDNA Polymerase: Primer Modifications that Facilitate Genotyping, Bio-Techniques 20:1004-1010 (June 1996). The consistent addition of adenosine may help to have most or all PCR products consistently have an adenosine end. As such, the genotyping result of these PCR products can be consistent. Without the consistent addition of adenosine, a PCR product may not vary in size by one base pair and the genotyping result of the PCR product may not be consistent. In some embodiments, the reverse primer used for amplifying the ligation products includes oligonucleotide GTTTCTT or its functional equivalents, e.g., GTTTCTTG.

[0081] In some embodiments, one or more of the nucleotides of the primer may be modified by adding a detectable moiety, e.g., a methyl group, a biotin or digoxigenin moiety, or a fluorescent tag. For some instances, the moiety is a fluorescent dye and the dye may be, but not limited to, FAM (5-or 6-carboxyfluorescein), VIC, NED, PET, Fluorescein, FITC, IRD-700/800, CY3, CY5, CY3.5, CY5.5, HEX, TET, TAMRA, JOE, ROX, BODIPY TMR, Oregon Green, Rhodamine Green, Rhodamine Red, Texas Red, and Yakima Yellow. See, e.g., U.S. Patent Publication No. 20110151459 for fluorescent dyes that may be used to label primers in the present invention and the U.S. Patent Publication No. 20110151459.

[0082] In one embodiment, the multiplexity of nucleic acid analysis is increased by adding a detectable moiety to the primers used for amplifying ligation products. In one example, the moieties used for labeling the primers are FAM-blue, VIC-green, NED-yellow and PET-red (Life Technologies, Inc.). As shown in **Figure 1**, to analyze target nucleic acids T01, T02, T03, and T04 in a sample according to one embodiment of the present invention, two probes (here referred to as the right probe and the left probe as they appear in the figure) for each target site are designed so that each probe contains a locus specific hybridization sequence (LSHS) and a primer binding site. The right probe contains a primer binding site Y. The primer binding site Y may be shared by the right probes for all target nucleic acids. In contrast, the left probe for each target nucleic acid contains a unique primer binding sequence (X1, X2, X3 and X4). The primer binding sequences are incorporated in the ligation products. To amplify the ligation product, a pair of primers is designed for each ligation product corresponding to each target site. Because the left probes have four unique primer binding

sites, four unique forward primers (F1, F2, F3 and F4) are designed corresponding to the four unique primer binding sites, respectively. In the example, the forward primer F1 is labeled with FAM-blue fluorescent dye, F2 with VIC-green, F3 with NED-yellow, and F4 with PET-red. The reverse primer R binds to the primer binding site Y. As such, the amplification product for target T01 is labeled with FAM-blue fluorescent dye. And the amplification products for targets T02, T03, and T04 are labeled with VIC-green, NED-yellow, and PET-red, respectively. The fluorescent labeled amplification products may then be analyzed by capillary electrophoresis on the basis of the different fluorescent dyes that each product is labeled with. The amplification products differentially labeled with fluorescent dyes may be distinguished even if the amplification products for different target nucleic acids are of the same length.

[0083] In another embodiment, the multiplexity of nucleic acid analysis is increased by varying the length of primers used for amplifying ligation products. To vary the length of primers, in one example, a stuffer sequence may be inserted into the primers. The stuffer sequence may be incorporated into the amplification product during the PCR reaction; i.e., the primer may be extended to form the amplification product to incorporate the stuffer sequence. As such, the addition of a stuffer sequence may help distinguish the amplification products on the basis of fragment sizes. In one embodiment as illustrated in **Figure 2**, a stuffer sequence is inserted in primer R2 so that the amplification product from target T02 has a bigger fragment size than the product from target T01. As shown in **Figure 2**, to detect target nucleic acids T01 and T02 in a sample according to one embodiment of the present invention, two probes (here referred to as the right probe and the left probe as they appear in the figure) are designed for each target nucleic acid so that each probe contain a locus specific hybridization sequence and a primer binding sequence. The left probe contains a primer binding sequence X, which is shared by left probes for both target sites. In contrast, the right probe contains a unique primer binding sequence (Y1 or Y2). The primer binding sequences may be incorporated into the ligation products. To amplify the ligation products, a pair of primers is designed for each ligation product corresponding to each target site. The forward primer binds to the primer binding site X shared by the two ligation products. The primers binding to the unique primer binding site Y1 and Y2 are designed to have a stuffer sequence inserted in the 5' portion. In one example, the reverse primer R2 contains a stuffer sequence so that R2 is longer than R1 and the amplification product from F/R2 is longer than from F/R1 if the ligation products for the two target sites are of the same length. In some embodiments, the stuffer sequence may have about 10 to about 500 nucleotides. In other embodiments, the stuffer sequence may have about 10 to about 60 nucleotides. In some preferred embodiments, the length of each and every primer is no more than 125 nucleotides. In other preferred embodiments, the length of each and every primer is no more than 75 nucleotides. The amplification products may then be analyzed by capillary electrophoresis on the basis of the different fragment size. The amplification products thus obtained may be distinguished even if the amplification products are labeled with the same fluorescent dye and the ligation products are of the same length for different target nucleic acids.

[0084] In still other embodiment, the multiplexity of nucleic acid analysis is increased by a combination of the use of primers labeled with fluorescent dyes and primers inserted with stuffer sequences. For example, as illustrated in **Figure 3A**, the forward primers are labeled with four fluorescent dyes: F1-FAM-blue, F2-VIC-green, F3, NED-yellow, and F4-PET-red. In addition, the reverse primer R2 contains a stuffer sequence but R1 does not contain a stuffer sequence. As such, the combination may give rise to an eight fold increase in terms of the multiplexity for determining the ligation products; eight ligation product of the same length may be distinguished by determining the corresponding amplification products on the basis of fragment sizes and fluorescent labels.

[0085] In still other embodiments, the multiplexity of the nucleic acid analysis according to the present invention may be further increased by inserting stuffer sequences in the probes to be hybridized to target nucleic acids and ligated to form ligation products. The insertion of stuffer sequences in probes may produce ligation products with unique sequence length corresponding to a particular target nucleic acid. For example, to distinguish 16 target nucleic acids T01-T16 in a sample according to one embodiment of the present invention, two locus-specific probes (here referred to as the right probe and the left probe as they appear in the figure) are designed for each target nucleic acid. The left probe contains a locus specific hybridization sequence and a universal primer binding sequence X. The right probe for each target nucleic acid contains locus specific hybridization sequence, a stuffer sequence and a universal primer binding sequence Y. The stuffer sequence for each target nucleic acid may vary in length, e.g., for T01, the right probe has no stuffer sequence; for T02, the right probe has a stuffer sequence of 2 nucleotides; for T03, 4 nucleotides; for T04, 6 nucleotides; for T05, 8 nucleotides; for T06, 10 nucleotides...and for T16, 30 nucleotides. Consequently, the ligation product for each target nucleic acid has a unique fragment size because the stuffer sequences in the probes are incorporated into the ligation product. The ligation products are optionally further amplified with a pair of universal primers that binds to the universal primer binding sites X and Y. As such, the fragment sizes of the amplification products match the fragment sizes of the ligation products and therefore may be determined on the basis of fragment sizes. As such, if without the stuffer sequences in the probes, the ligation products are of the same length, the addition of the stuffer sequences in the probes makes it possible to distinguish the ligation product and/or the amplification product for each target nucleic acid on the basis of fragment size. In this example, the multiplexity for target nucleic acid analysis increases 16 folds. Thus, as exemplified in this embodiment, the fold increase of multiplexity may depend on the number of stuffer sequences with different length that are inserted in the locus-specific probes. If 12 stuffer sequences of varying length are inserted

in the locus-specific probes for each target nucleic acid, the multiplexity for target nucleic acid analysis may increase 12 fold.

[0086] Therefore, according to the present invention, the methods of increasing multiplexity by inserting stuffer sequences in probes may be combined with the method of increasing multiplexity by labeling the primers with detectable moieties and/or inserting stuffer sequences in primers. For example, by both labeling primers with fluorescent dyes and inserting stuffer sequences in probes, 64 amplification products corresponding to 64 target nucleic acids may be distinguished by capillary electrophoresis on the basis of fluorescent dyes and fragment size (see **Figure 1**, bottom chart). For another example, by inserting stuffer sequences in both primers and probes, 24 amplification products corresponding to 24 target nucleic acids may be distinguished by capillary electrophoresis on the basis of fragment size (see **Figure 2**, bottom chart). For still another example, by labeling primers with fluorescent dyes and inserting stuffer sequences in primers and probes, 96 amplification products corresponding to 96 target nucleic acids may be distinguished by capillary electrophoresis on the basis of fluorescent dyes and fragment size (see **Figure 3**, bottom chart).

[0087] The analysis of amplification products may be by any method that can separate DNA fragments on the basis of size, mass, fluorescent moieties, or other measurable properties. These methods include, but not limited to, agarose gel electrophoresis followed by capillary electrophoresis (CE), DNA sequencing, ethidium bromide or DNA staining, microarray, and flow cytometry.

[0088] For example, capillary electrophoresis may identify DNA fragments on the basis of both fluorescent moieties and fragment sizes. In some embodiments, capillary electrophoresis is performed by injecting the DNA fragments into a capillary, filled with polymer. The DNA is pulled through the tube by the application of an electric field, which separates the fragments such that the smaller fragments travel faster through the capillary. The fragments are then detected on the basis of fluorescent dyes that are attached to the primers used in PCR. This allows multiple fragments to be amplified and run simultaneously in a multiplexed manner. Sizes are assigned using labeled DNA size standards that are added to each sample. In capillary electrophoresis the intensity of signal of an amplification product is the number of relative fluorescence units (rfus) of its corresponding peak. The intensity value correlates with the amount of labeled amplification product.

[0089] In some embodiments, capillary electrophoresis is the preferred method for analyzing the amplification products of the present invention. Capillary electrophoresis devices are known in the art. Capillary electrophoresis devices useful according to the invention include, but are not limited to, ABI 3130XL Genetic Analyzer by Applied Biosystems (Foster City, California); MegaBACE 1000 Capillary Array Electrophoresis System by Amersham Pharmacia Biotech (Piscataway, N.J.); CEQ™ 8000 Genetic Analytic System by Beckman Coulter (Fullerton, Calif.); Agilent 2100 Bioanalyzer by Caliper Technologies (Mountain View, Calif.); and iCE280 System by Convergent Bioscience Ltd. (Toronto, Canada).

[0090] As is the case throughout this invention disclosure, including the background, the summary, the detailed description and the claims, the designation of the "left" probe and the "right" probe are arbitrary and are interchangeable. For example, the left probe have the features described for the right probe and the right probe may have the features for the left probe. In addition, the designation of the "first" primer and the "second" primer, or the "forward" primer and the "reverse" primer are also arbitrary and interchangeable. Further, when an oligo primer binding to a primer binding site on a template DNA in a PCR amplification reaction, the oligo primer is meant to bind either to the positive strand or to the negative strand of the template DNA. A primer is reversely complementary to a binding site on a single-stranded DNA when the primer is complementary to the opposite strand of the single-stranded DNA. As is well known in the art for a PCR reaction to proceed successfully, the forward primer and the reverse primer do not bind to the same strand of a DNA template. Instead, the forward primer and the reverse primer bind to different strands of a DNA template if an exponential amplification is desired.

II. Applications of Multiplex Nucleic Acid Analysts

[0091] Methods of multiplex nucleic acid analysis according to the present invention may be applied to detect various genetic aberrations, including, but not limited to, single nucleotide polymorphisms (SNPs), gene copy number variants (CNVs), chromosomal abnormalities (e.g., insertions, deletions and duplications), gene mutations (e.g., single nucleotide changes, insertions, and deletions), nucleic acid modifications (e.g., methylation, acetylation and phosphorylations), and abnormal gene expression. Some aberrations may be qualitative changes, e.g., the presence or absence of a SNP. Other aberrations may be quantitative, e.g., the change of gene copy number. The quantitative change may be big or small. For example, in a genomic DNA sample from a Down's syndrome patient, the copy number of chromosome 21 increases 50%. In contrast, in a blood sample of a pregnant woman conceiving a Down's syndrome fetus, the copy number of chromosome 21 may increase less than 10%.

[0092] The terms "analyzing", "determining", "measuring", "assessing", "assaying", "evaluating", and any grammatical equivalents are used interchangeably to refer to any form of quantitative or qualitative measurement, and include determining if a characteristic, trait, or feature is present or not. An analysis of nucleic acids may be relative or absolute. For example, an increase of copy number of a nucleic acid in a sample may be measured in relative to the copy number

of the nucleic acid in a reference sample.

Multiplex SNP detection.

[0093] Recent human genomics research indicates that the genomic makeup between any two humans has over 99.9% similarity. The relatively small number of variations in DNA between individuals gives rise to differences in phenotypic traits, and may be related to many human diseases, susceptibility to various diseases, or response to treatment of disease. Variations in DNA between individuals occur in both coding and non-coding regions, and include changes of a single nucleotide, as well as insertions and deletions of nucleotides. Changes of a single nucleotide in the genome are referred to as single nucleotide polymorphisms, or "SNPs." The occurrences of SNPs in the genome are becoming correlated to the presence of and/or susceptibility to various diseases and conditions. As these correlations and other advances in human genetics are being made, medicine and personal health in general are moving toward a customized approach in which a patient will make appropriate medical and other choices in consideration of his or her genomic information, among other factors. Thus, there is a need to provide individuals and their care-givers with information specific to the individual's personal genome toward providing personalized medical and other decisions.

[0094] A multiplex SNP detection method according to the present invention is illustrated in **Figure 4**. In the example, 48 SNP sites are determined in a single assay simultaneously. For illustrative purposes, one SNP has a C or T nucleotide at the polymorphic position. A set of probes (here referred to as the right probe and the left probe as they appear in the figure) are designed. The left probe contains an allele-specific hybridization sequence (ASHS) with the SNP recognition nucleotide "C" or "T" on one end, a primer binding site X, a stuffer A sequence between the ASHS and the primer binding site, and a stuffer L1 sequence between the stuffer A sequence and the primer binding site. The stuffer A sequence helps distinguish the alleles for each SNP site. The right probe contains a locus-specific hybridization sequence (LSHS), a primer binding site Y, and a stuffer L2 sequence between the LSHS and the primer binding site. By varying the length of stuffer L1 and L2 sequences, a six-fold multiplexity may be achieved for the ligation products; ligation products for six SNP sites can be distinguished on the basis of fragment size. In some instances, a mismatch at a position 2, 3, or 4 nucleotides away from the SNP recognition site is introduced into the probe. These mismatches may help increase the specificity of the hybridization of the ASHS to the target site. See, e.g., Luo et al., Improving the fidelity of *Thermus thermophilus* DNA ligase, *Nucleic Acids Res.* 1996 Aug 1;24(15):3071-8.

[0095] In addition, by labeling the forward primers with FAM-blue, VIC-green, NED-yellow and PET-red and inserting a stuffer sequence in the reverse primer R2, the multiplexity for the SNP analysis is increased to 96 ($=6 \times 2 \times 4 \times 2$). As such, the multiplex SNP detection method may analyze 48 SNP sites simultaneously with each SNP site having two alleles. A person skilled in the art may increase the multiplexity for SNP detection by varying the parameters, e.g., inserting stuffer sequences in more probes, labeling the primers with more fluorescent tags, or inserting stuffer sequences in more primers. As such, the multiplexity may be increased to 192, 384, 768, or more if desirable.

Multiplex CNV detection.

[0096] As used herein, CNVs refer to variations in the number of copies of a nucleic acid sequence that contains 2 or more nucleotides in a test sample in comparison with the copy number of the nucleic acid sequence present in a reference sample. CNVs may include deletions, microdeletions, insertions, microinsertions, duplications, multiplications, inversions, translocations and complex multisite variants. CNVs may also encompass chromosomal aneuploidies and partial aneuploidies that may cause many genetic diseases, e.g., Down's syndrome, Turner's syndrome, diGeorge syndrome, Angelman syndrome, Cri-du-chat, Kallmann syndrome, Miller-Dieker syndrome, Prader-Willi syndrome (PWS), Smith-Magenis syndrome, Steroid sulfatase deficiency (X-linked ichthyosis), Williams syndrome, and Wolf-Hirschhorn syndrome.

[0097] An exemplary multiplex CNV detection method according to the present invention is illustrated in **Figure 5**. In the example, 96 CNVs are measured in a single assay simultaneously. For each CNV, a set of probes (here referred to as the right probe and the left probe as they appear in the figure) are designed. The left probe contains a locus-specific hybridization sequence (LSHS), a primer binding site X, and a stuffer L1 sequence between the LSHS and the primer binding site. The right probe contains a locus-specific hybridization sequence (LSHS), a primer binding site Y, and a stuffer L2 sequence between the LSHS and the primer binding site. By varying the length of stuffer L1 and stuffer L2 sequences, a 12-fold multiplexity may be achieved for the ligation products; ligation products for 12 CNVs can be distinguished on the basis of fragment size. In addition, by labeling the forward primers with FAM-blue, VIC-green, NED-yellow and PET-red and inserting a stuffer sequence in one of the reverse primers R2, the multiplexity for the CNVs analysis may be increased to 96 ($=12 \times 4 \times 2$). As such, the multiplex CNV detection method may analyze 96 CNVs simultaneously. A person skilled in the art may increase the multiplexity for CNV detection by varying the parameters, e.g., inserting stuffer sequences in more probes, labeling the primers with more fluorescent tags, or inserting stuffer sequences in more primers. As such, the multiplexity may be increased to 192, 384, 768, or more if desirable.

[0098] In some embodiments, the fluorescent intensity of each peak was compared to a standard value to determine the copy number of each CNV site. For example, if the intensity decreases in half compared to the standard value, the copy number may be considered as decreased in half. On the other hand, if the intensity increases 50% compared to the standard value, the copy number may be considered as increased 50%. A standard value may be a fluorescent intensity value specific to a testing system. A testing system means the whole experimental system including the reagents, primers, probes, procedures and devices used for the testing the CNVs. One test sample is considered to share the same testing system with another test sample if the whole experimental system is the same except that the initial DNA sample to be tested is different. As such a standard value may be obtained for a specific testing system based on prior testing results of DNA samples for which copy numbers of the CNV sites are known.

[0099] In other embodiments, a control sample is used to generate control fluorescent intensity values for the same CNV sites. The copy number for each CNV site is known for the control sample. When the peak intensity for a CNV site in a test sample is compared to the peak intensity for the same CNV site in the control sample, the copy number of the CNV site may be determined. For example, as shown in **Figure 5** bottom chart, arrow 1 points to a CNV site for which the test sample has about 50% copy number of the number in the control sample because the peak intensity decreases about 50% when compared to the peak intensity for the same CNV site in the control sample. For another example, arrow 2 points to a CNV site for which the test sample has about 150% copy number of the number in the control sample because the peak fluorescent intensity in the test sample increases about 50% when compared to the peak for the same CNV site in the control sample.

[0100] In other preferred embodiments, the peak values for each CNV site are normalized against peak values for one or more reference target sites. A CNV copy number change may be detected by comparing the normalized peak value for a CNV site in a test sample to the normalized standard value for the same CNV site in the testing system or the normalized peak value for the same CNV site in a control sample. The normalization may correct the variations, e.g., the amount of DNA templates used for probe hybridization, the amount of ligation probes used for probe hybridization, the amount of PCR primers for amplifying ligation products, the ligation efficiency of each set of probes, and the amount of ligation products used for PCR amplification, in obtaining the peak values between different samples and/or between different CNV sites.

[0101] To obtain peak values of reference target sites, probes for the reference target sites may be designed in a similar manner and used simultaneously with the probes for the CNV sites in the hybridization and ligation reactions. In addition, probes for the reference target sites and probes for the CNV sites may share the same primer binding sites so that the same set of primers may be used to amplify the ligation products for both the reference sites and the CNV sites.

[0102] As such in some instances, peak values for a plurality of reference target sites are obtained in parallel with the peak values for a plurality of CNV sites so that the peak value for each of the plurality of the CNV sites may be normalized against the peak value for each of the plurality of the reference target sites. For example, peak values for 6 reference target sites may be obtained together with the peak values for 6 CNV sites. In this example, 6 sets of probes for the 6 reference target sites and 6 sets of probes for the 6 CNV sites are added to a sample and ligation products are obtained after hybridization and ligation reactions. A single set of primers may be used to amplify the ligation products and peak values are measured by analyzing the amplification products by capillary electrophoresis.

[0103] In some embodiments, the normalization of the peak value for a CNV site in a sample is to obtain a ratio (here referred to as R) of the peak value for the CNV site against the peak value for a reference target site. As such, the copy number of a CNV site in a test sample may be measured by comparing the ratio for the CNV site in the test sample (here referred to as R_{test}) with the standard ratio for the same CNV site in the testing system (here referred to as R_{standard}) or with the ratio for the same CNV site in a control sample (here referred to as R_{control}).

[0104] In some instances, the copy number of a CNV site in a test sample is measured by comparing the ratio for the CNV site in the test sample (R_{test}) with the standard ratio for the same CNV site in the testing system (R_{standard}). The copy number of the CNV site in the testing system is known (here referred to as C_{standard}). In this case, the copy number of the CNV site in the test sample (here referred to as C_{test}) may be calculated as follow: $C_{\text{test}} = C_{\text{standard}} \times R_{\text{test}} / R_{\text{standard}}$.

[0105] In other instances, the copy number of a CNV site in a test sample may be measured by comparing the ratio for the CNV site in the test sample (R_{test}) with the ratio for the same CNV site in a control sample (R_{control}). The copy number of the CNV site in the testing system is known (here referred to as C_{control}). In this case, the copy number of the CNV site in the test sample (C_{test}) may be calculated as follow: $C_{\text{test}} = C_{\text{control}} \times R_{\text{test}} / R_{\text{control}}$.

[0106] As such, when 6 reference target sites are introduced to normalize the peak value for a CNV site, six copy number measurements (C_{test}) for the CNV site may be derived on the basis of the six peak values for the six reference target sites. Based on the six C_{test} measurements, the copy number of the CNV site is obtained according to certain statistical analysis. In one embodiment, the median value of the six C_{test} measurements is deemed the copy number of the CNV site. In another embodiment, the average value of the six C_{test} measurements is deemed the copy number of the CNV site.

[0107] In a multiplex CNV analysis method, multiple sets of primers may be used to amplify ligation products in a single tube PCR reaction. In this case, each set of primers is used to amplify a group of ligation products from both CNV sites

and reference target sites so that peak values of CNV sites maybe normalized against peak values of the reference target sites in the same group. Depending on probe designs, the number of CNV sites or reference target sites in each group may vary from 1 to 24. In some embodiments, there are 6 CNV sites and 6 reference target sites in each group. In other embodiments, there are 12 CNV sites and 12 reference target sites in each group. In still other embodiments, there are 9 CNV sites and 3 reference target sites in each group. In further other embodiments, there are 16 CNV sites and 8 reference target sites in each group.

[0108] The selection of a reference target site is sometimes based on criteria including, but not limited to, the copy number of each reference target site being stable in different samples and the sequences of the reference target sites being unique and not prone to interfering the reactions of the CNV sites of interest. In addition, when a plurality of reference target sites are used in a group, each reference target site is preferably from a different chromosome. In some embodiments, the quality of detecting copy number changes may be improved by increasing the number of reference target sites.

[0109] In some embodiments, when normalization is performed and normalized peak values for each CNV site are obtained for the test sample and the control sample, a comparison of the normalized peak values in the test sample with the values in the control sample may be carried out to determine any change of copy number of each CNV site. For example, if a normalized peak value for a CNV site in the test sample is 1.0 and the normalized peak value for the same CNV site in the control sample (the copy number for the CNV site is known to be 2) is 2.0, the copy number of the CNV site in the test sample is determined to be 1, about half of the copy number in the control sample. For another example, if a normalized peak value for a CNV site in the test sample is 1.0 and the normalized standard peak value for the testing system (the copy number for the CNV site is known to be 2) is 1.0, the copy number of the CNV site in the test sample is determined to be 2, about the same with the copy number in the control sample.

Mutation screening

[0110] DNA mutations refer to nucleotide changes in the genome in comparison to a wild type genome. "Wild-type" refers to a gene or gene product which has the characteristics of that gene or gene product when isolated from a naturally occurring source. A wild-type gene is that which is most frequently observed in a population and is thus arbitrarily designed the "normal" or "wild-type" form of the gene. In contrast, "mutant" refers to a gene or gene-product having at one or more sites a different nucleic acid sequence when compared to the wild-type gene or gene product.

[0111] In some embodiments, mutations in a gene may be screened by a method according to the present invention. An exemplary multiplex mutation screening method according to the present invention is illustrated in **Figure 6**. In the example, 96 target sites are screened in a single assay simultaneously. For each target site, a set of probes (here referred to as the right probe and the left probe as they appear in the figure) are designed. The left probe contains a locus-specific hybridization sequence (LSHS), a primer binding site X and a stuffer L1 sequence between the LSHS and the primer binding site X. The right probe contains a locus-specific hybridization sequence (LSHS), a primer binding site Y, and a stuffer L2 sequence between the LSHS and the primer binding site Y. By varying the length of stuffer sequences, a 12-fold multiplexity may be achieved for the ligation products; ligation products for 12 target sites can be distinguished on the basis of fragment size. In addition, by labeling the forward primers with FAM-blue, VIC-green, NED-yellow and PET-red and inserting a stuffer sequence in one of the reverse primers R2, the multiplexity for the mutation screening may be increased to 96 (=12 x 4 x 2). As such, the multiplex mutation screening method may analyze 96 target sites simultaneously. A person skilled in the art would consider increase the multiplexity for mutation screening by varying the parameters, e.g., inserting stuffer sequences in more probes, labeling the primers with more fluorescent tags, or inserting stuffer sequences in more primers. As such, the multiplexity may be increased to 192, 384, 768, or more if desirable.

[0112] As shown in **Figure 6**, sets of probes are designed to overlap with each other. The probes may target the positive stand, the negative stand, or both the positive and the negative stands. In all cases, similar to the rationale in **Figure 5**, the peak intensity for each target site is compared to a standard value or the peak intensity of the same target site in a control sample. A standard value may be derived in a similar way as described in **Figure 5**. As shown in **Figure 6** bottom charts, the arrow points to a peak with about half the intensity of the same target site in the control sample. The decreased peak intensity suggests that the target site may contain a mutation. This is so because the two probes corresponding to the target site covers a mutation and therefore cannot hybridize to the target site to form a proper ligation product and finally result in no amplification product. This exemplary mutation screening method would provide a preliminary result as to the location of a mutation in a target nucleic acid. This may facilitate further sequencing analysis to identify the exact mutation.

Multiplex RNA analysis

[0113] In some embodiments, the target nucleic acids in a sample are RNA and the analysis of the target nucleic acids

is to determine the presence, absence, or quantity of the RNA in the sample. In some instances according to the present invention, the RNA is directly used for probe hybridization, probe ligation, ligation product amplification, and amplification product analysis. In other instances, the RNA is reverse-transcribed into complementary DNA (cDNA) before probe hybridization and further steps. As is known in the art, reverse transcription of RNA into cDNA may be accomplished using reverse transcriptase. The analysis of RNA may help determine gene expression levels if the RNA is a transcription product of a gene.

[0114] As such, in some embodiments, target RNAs may be analyzed using a multiplex method according to the present invention. An exemplary multiplex target RNA analysis method according to the present invention is illustrated in **Figure 7**. In the example, 96 target RNAs or its reverse transcribed cDNAs are analyzed in a single assay simultaneously. For each target RNA or cDNA, a set of probes (here referred to as the right probe and the left probe as they appear in the figure) are designed. The left probe contains a locus-specific hybridization sequence (LSHS), a primer binding site X, and a stuffer L1 sequence between the LSHS and the primer binding site X. The right probe contains a locus-specific hybridization sequence (LSHS), a primer binding site Y, and a stuffer L2 sequence between the LSHS and the primer binding site Y. By varying the length of stuffer sequences, a 12-fold multiplexity may be achieved for the ligation products; ligation products for 12 target sites can be distinguished on the basis of fragment size. In addition, by labeling the forward primers with FAM-blue, VIC-green, NED-yellow and PET-red and inserting a stuffer sequence in one of the reverse primers R2, the multiplexity for the mutation screening is increased to 96 (=12 x 4 x 2). As such, the multiplex gene expression analysis may measure 96 target RNAs simultaneously. A person skilled in the art would consider increase the multiplexity for target RNA analysis by varying the parameters, e.g., inserting stuffer sequences in more probes, labeling the primers with more fluorescent tags, or inserting stuffer sequences in more primers. As such, the multiplexity may be increased to 192, 384, 768, or more if desirable.

[0115] In some embodiments, as shown in **Figure 7** and similar to the rationale in **Figure 5**, the peak values for each RNA target is normalized against reference target sites before determining the presence, absence or amount of RNA copy number changes between different samples. Reference target sites may be any RNA, for example, RNA of house-keeping genes including, but not limited to, histone, β -actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or hypoxanthine-guanine phosphoribosyltransferase (HPRT) genes. In a multiplex RNA analysis, multiple groups of target RNA and reference target sites may be analyzed simultaneously. The same set of primers is used to amplify ligation products for both the target RNA and the reference target sites within each group. The copy number of a target RNA is determined by comparing the normalized peak values for a target RNA in the test sample to the normalized peak values for the target RNA in the control sample. If the value increases 2 times, the copy number of the target RNA increase 2 times. Other aspects of the peak value normalization and copy number measurement methods are similar to those described for the normalization of peak values for CNV sites against reference target sites and therefore are not repeated here.

Multiplex detection of pathogens and transgenic organism

[0116] Multiplex nucleic acid analysis methods according to the present invention may be applied to detect nucleic acids from pathogens and transgenic organisms and thereby identify the pathogens and transgenic organisms. Pathogens, including, but not limited to, a bacterium, a virus, a protozoan, a parasite, a mold, or a fungus, may cause diseases or other conditions in an animal. Traditionally, several methods including bacteriological analysis, virus isolation and culture, histopathology and an enzyme-linked immunosorbent assay (ELISA) (Adams and Thompson, 2008, Rev. Sci. Technol. 27, 197-209) have been developed for the phenotypic characterization and identification of pathogens. Alternatively, molecular diagnosis based on polymerase chain reaction (PCR), RT-PCR, (Dhar et al., 2002, J. Virol. Methods 104, 69-82; Nishizawa et al., 1995, J. Gen. Virol. 76, 1563-1569) or quantitative real-time PCR (DallaValle et al., 2005, Vet. Microbiol. 110, 167-179) using specific primer sets for nucleic acid amplification has been demonstrated for diagnosis of diseases. The development of a new multiplex, rapid, accurate, and sensitive diagnostic methods for the identification of pathogens may help in treating, controlling, or even eradicating many pathogen-related diseases. In addition, transgenic organisms, including transgenic plants, e.g., corn, rice, soybean and cotton and transgenic animals, e.g., cow, pig, sheep and dog may sometimes need to be identified.

[0117] One aspect of the present invention provides a multiplex detection of pathogens and transgenic organisms. In some embodiments, target nucleic acids specific to pathogens or transgenic organisms are obtained for multiplex analysis. In some instances, nucleic acids specific to pathogens or transgenic organisms may be DNA unique to the pathogens or the transgenic organisms or unique to the family of the pathogens or the transgenic organisms. The pathogens or transgenic organisms may be from many sources. For example, in screening blood and other bodily fluids and tissues for pathogenic and non-pathogenic bacteria, viruses, parasites, fungi and the like, or transgenic genes, the sources may be blood and other bodily fluid or tissue samples, which may be obtained from living animals or deceased animals. In other instances, nucleic acids specific to a pathogen or transgenic organism may be RNA unique to the pathogens or transgenic organisms or unique to the family of the pathogens or transgenic organisms. For example, RNA viruses such

as HIV, HBV, and HCV viruses contain unique RNA molecules and the detection of these unique RNA molecules helps determine the presence, absence or quantity of these RNA viruses.

[0118] In some embodiments, the target nucleic acids are amplified before the multiplex analysis. Such amplification is needed when the amount of target nucleic acids obtained from pathogens or transgenic organisms is limited. In other embodiments, when the target nucleic acids are RNA, a reverse transcription step may be optionally performed to convert the RNA into cDNA before the multiplex analysis.

[0119] An exemplary multiplex target nucleic acid analysis method for detecting pathogens and transgenic organisms according to the present invention is illustrated in **Figure 8**. In the example, 96 target nucleic acids are analyzed in a single assay simultaneously. For each target nucleic acid, a set of probes (here referred to as the right probe and the left probe as they appear in the figure) are designed. The left probe contains a locus-specific hybridization sequence (LSHS), a primer binding site X, and a stuffer L1 sequence between the LSHS and the primer binding site X. The right probe contains a locus-specific hybridization sequence (LSHS), a primer binding site Y, and a stuffer L2 sequence between the LSHS and the primer binding site Y. By varying the length of stuffer sequences, a 12-fold multiplexity may be achieved for the ligation products; ligation products for 12 target nucleic acids can be distinguished on the basis of fragment size. In addition, by labeling the forward primers with FAM-blue, VIC-green, NED-yellow and PET-red and inserting a stuffer sequence in one of the reverse primers R2, the multiplexity for the multiplex pathogen and transgenic organism detection is increased to 96 (=12 x 4 x 2). As such, the multiplex pathogen and transgenic organism detection analysis may assay 96 target nucleic acids simultaneously. A person skilled in the art would consider increase the multiplexity for target nucleic acid analysis for detecting pathogens and transgenic organisms by varying the parameters, e.g., inserting stuffer sequences in more probes, labeling the primers with more fluorescent tags, or inserting stuffer sequences in more primers. As such, the multiplexity may be increased to 192, 384, 768, or more if desirable.

[0120] As a person skilled in the art understands based on the disclosure of the present invention, the presence of peaks corresponding to target nucleic acids indicates the presence of the corresponding pathogens or transgenic organisms. As shown in **Figure 8** bottom chart, the arrows point to two peaks corresponding to two target nucleic acids, indicating the presence of two corresponding pathogens. The absence of peaks corresponding to the remaining 86 target nucleic acids indicates the absence of the 86 corresponding pathogens. The encircled peaks are positive controls, whose presence indicates the testing system is functional.

[0121] In other embodiments, the method according to the present invention may be applied to measure quantitatively the amount of pathogens and transgenic organisms. The rationale behind this application procedure for this purpose are similar to those described for quantitatively measure RNA expression levels in the multiplex RNA analysis section *supra* except that exogenous nucleic acids may be used as reference target sites. In some embodiments, a certain amount of exogenous DNA or RNA fragments are added into samples and used as reference target sites. For example, in measuring the load of HIV virus in a plasma samples, the same amount of exogenous RNA fragments may be mixed with the same amount of plasma from each sample before RNA extraction. The quantitative evaluation of HIV load may be carried out by quantitatively measuring the HIV RNA level by normalizing the peak values for HIV RNA against the peak values for the exogenous reference RNA fragments.

Multiplex DNA Methylation Analysis

[0122] Epigenetic modifications of genomic DNA, e.g., changes in DNA methylation patterns are related to many diseases or other health conditions. Abnormal methylation of normally unmethylated CpG-rich areas, also known as CpG-islands, have been associated with transcriptional inactivation of many disease genes, e.g., tumor suppressor genes, DNA repair genes, metastasis inhibitor genes. See, e.g., Jain PK, Epigenetics: the role of methylation in the mechanism of action of tumor suppressor genes, Ann N Y Acad Sci. 2003 Mar;983:71-83. There is a need for a multiplex, quick, and accurate method for detecting DNA methylation patterns, which may aid the diagnosis, prognosis, prediction, and evaluation of treatment plan for underlying diseases or other health conditions.

[0123] An exemplary multiplex DNA methylation detection method according to the present invention is illustrated in **Figure 9**. In the example, 96 target methylation sites are analyzed in a single assay simultaneously. In some instances, DNA bearing the target methylation sites in a test sample may be treated with methylation sensitive restriction endonucleases, e.g., HpaII or HhaI, so that unmethylated DNA are cleaved at a position near or close to the methylation site. The treatment of methylation sensitive restriction endonucleases may be carried out before, at the same time, or after the probe hybridization step. In some embodiments, the treatment is carried out before the probe hybridization step. In this case, if the DNA in the test sample is cleaved, the probes designed to hybridize near or close to the methylation site on the target DNA may not be ligated and no amplification products may be produced. In contrast, if the DNA in the test sample is methylated at the methylation site and therefore cannot be cleaved by the methylation sensitive restriction endonuclease, the probes hybridized near or close to the methylation site on the target DNA may be ligated and the corresponding amplification products may be produced. In other embodiments, the treatment is carried out at the same time or after the probe hybridization step. In this case, probes may hybridize to the target DNA near or close to the

methylation site, the methylation sensitive endonuclease may cleave the hybridized probe/target DNA duplex if the target DNA is not methylated and therefore no amplification product is produced. In contrast, if the target DNA is methylated, the methylation sensitive endonuclease may not cleave the hybridized probe/target DNA duplex and therefore amplification product is produced.

[0124] In both situations, as shown in **Figure 9**, in an exemplary multiplex methylation detection method according to the present invention, for each target methylation site, a set of probes (here referred to as the right probe and the left probe as they appear in the figure) are designed. The left probe contains a locus-specific hybridization sequence (LSHS) and a primer binding site X, and a stuffer L1 sequence between the LSHS and the primer binding site X. The right probe contains a locus-specific hybridization sequence (LSHS), a primer binding site Y, and a stuffer L2 sequence between the LSHS and the primer binding site Y. By varying the length of stuffer L1 and stuffer L2 sequences, a 12-fold multiplexity may be achieved for the ligation products; ligation products for 12 target methylation sites can be distinguished on the basis of fragment size. In addition, by labeling the forward primers with FAM-blue, VIC-green, NED-yellow and PET-red and inserting a stuffer sequence in one of the reverse primers R2, the multiplexity for the methylation detection may be increased to 96 ($=12 \times 4 \times 2$). As such, the multiplex methylation detection may measure 96 target methylation sites simultaneously. A person skilled in the art may increase the multiplexity for methylation detection by varying the parameters, e.g., inserting stuffer sequences in more probes, labeling the primers with more fluorescent tags, or inserting stuffer sequences in more primers. As such, the multiplexity may be increased to 192, 384, 768, or more if desirable.

[0125] In other instances, the DNA in the test sample is treated with bisulfite so that unmethylated cytosines (here referred to as "C") are converted into uracils. The bisulfite treatment therefore makes nucleotide changes at unmethylation sites, converting an unmethylated "C" into "U" in the target DNA. In contrast, methylated "C" remains as "C" after bisulfite treatments. As such, specific probes are designed to bind either to the methylated allele or the unmethylated allele of the test DNA: the methylated allele with "Cs" at methylated C sites and "Us" at the unmethylated C sites, and the unmethylated allele with "Us" at all C sites. Depending on which allele needs to be detected, the design of these specific probes is briefly described below.

[0126] As shown in **Figure 9**, 96 methylation sites may be detected in a single assay simultaneously when a bisulfite treated is first performed on the target DNA. For illustrative purposes, a set of probes (here referred to as the right probe and the left probe as they appear in the figure) are designed. The left probe contains a methylation-specific hybridization sequence (MSHS) with the methylation site recognition nucleotides "G", a primer binding site X, a stuffer L1 sequence between the MSHS and the primer binding site X. The right probe contains a methylation-specific hybridization sequence (MSHS) with the methylation site recognition nucleotides "G", a primer binding site Y, and a stuffer L2 sequence between the MSHS and the primer binding site. By varying the length of stuffer L1 and stuffer L2 sequences, a 12-fold multiplexity may be achieved for the ligation products; ligation products for 12 methylation sites can be distinguished on the basis of fragment size. In addition, by labeling the forward primers with FAM-blue, VIC-green, NED-yellow and PET-red and inserting a stuffer sequence in one of the reverse primers, the multiplexity for the methylation detection may be increased to 96 ($=12 \times 4 \times 2$). As such, the multiplex methylation detection may measure 96 target methylation sites simultaneously. A person skilled in the art may increase the multiplexity for methylation detection by varying the parameters, e.g., inserting stuffer sequences in more probes, labeling the primers with more fluorescent tags, or inserting stuffer sequences in more primers. As such, the multiplexity may be increased to 192, 384, 768, or more if desirable. In some embodiments, when only unmethylated alleles need to be detected, the unmethylation specific probes are used instead of the methylation specific hybridization probes. In other embodiments, when both methylated and unmethylated alleles need to be detected, both methylated and unmethylated specific probes are used. The rationale for designing the methylated and unmethylated specific probes is similar to those described supra, and therefore is not repeated here.

[0127] In both the method using methylation sensitive restriction enzymes and the method using bisulfite treatment, the analysis of the amplification products may determine the presence or absence of methylated nucleotides in a target DNA. In the method using methylation sensitive restriction enzymes, amplification peaks appear only if the DNA is methylated. In the method using bisulfite treatment and MSHS probes which have the methylation site recognition nucleotides "G", amplification peaks appear only if the DNA is methylated. Therefore, in both methods, the presence of peaks indicates the presence of methylated nucleotides in the target DNA, and the absence of peaks indicates the absence of methylated nucleotides in the target DNA. As shown in **Figure 9** bottom chart, the arrows point to the peaks in the amplification product, indicating that the corresponding methylation sites contain methylated nucleotides in the test sample. The absence of peaks corresponding to other methylation sites indicates that those other methylation sites in the test sample contain no methylated nucleotides. The encircled peaks are positive control, whose presence indicates the testing system is functional.

[0128] In some embodiments, the multiplex DNA methylation analysis may determine the relative amount of methylated DNA in a sample. To determine the relative amount of methylated DNA in a sample, the peak value for each target methylation site in a test sample is first normalized to the peak value of the reference target site, and then compared to the normalized peak value in a control sample. The selection of a reference target site is sometimes based on criteria including, but not limited to, the copy number of each reference target site being stable after methylation sensitive

restriction enzyme digestion or bisulfite treatment in different samples and the sequences of the reference target sites being unique and not prone to interfering the reactions of the methylation sites of interest. In addition, when a plurality of reference target sites are used in a group, each reference target site is preferably from a different chromosome. In a multiplex DNA methylation analysis, multiple groups of target methylation sites and reference target sites may be analyzed simultaneously. The same set of primers is used to amplify ligation products for both the target methylation sites and the reference target sites within each group. The relative amount of methylated DNA in the target methylation site is determined by comparing the normalized peak value for a target methylation site in the test sample to the normalized peak value for the target methylation site in the control sample. If the value increases 2 times, the relative amount of methylated DNA in the target methylation site increases 2 times. Other aspects of the normalization method are similar to those described for the normalization of peak values for CNV sites against reference target sites and therefore are not repeated here.

III. Detection Of Small Copy Number Changes

[0129] In another aspect, the present invention is a method for detecting a small quantitative variation of a nucleic acid between two samples. A small quantitative variation of a nucleic acid may be a small copy number change of a nucleic acid, e.g., a gene, a part of a chromosome, or a whole chromosome. As used herein, a small quantitative variation of a nucleic acid refers to any copy number changes that are less than 50%. Indeed, in some embodiments of the present invention, the method may detect small copy number changes of about 0.1% to about 30%. In other embodiments, the method may detect small copy number changes of about 8%, 6%, 4%, 2%, 1%, or 0.1 %.

[0130] For example, the copy number of human chromosome 21, human chromosome 18, human chromosome 13, human chromosome region 22q11.2, or the pseudoautosomal regions of human chromosomes X or Y in the maternal blood may change in a small scale if the fetus harbors a different copy number of those chromosome regions. In one instance, if a fetus has Down's syndrome, the copy number of chromosome 21 may increase in a small scale in maternal blood. The increase is usually less than 10%. As such, a method according to the present invention may be used to detect small copy number changes of human chromosome 21, human chromosome 18, human chromosome 13, human chromosome region 22q11.2, or the pseudoautosomal regions of human chromosomes X or Y in maternal blood. The pseudoautosomal regions (PAR1 and PAR2) of the human X and Y chromosomes are not inherited in a strictly sex-linked fashion and may be used to detect the copy number of the X and Y chromosome pairs. See, e.g., Mangs and Morris, The Human Pseudoautosomal Region (PAR): Origin, Function and Future, *Curr Genomics*. 2007 April; 8(2): 129-136.

[0131] In one embodiment, the method for detecting small copy number changes of a nucleic acid in a test sample comprises the steps of measuring the copy number of a plurality of target sites within the nucleic acid in the test sample, and determining the copy number of the nucleic acid by statistically analyzing the measured copy number for each of the plurality of target sites.

[0132] According to this present invention, to detect small copy number changes of a nucleic acid, a plurality of target sites in the nucleic acid are chosen for analysis so that a statistically significant result may be obtained to quantitatively determine a copy number change for the nucleic acid. As used herein, a plurality of target sites refers to more than about 5 target sites, preferably more than about 10 target sites, more preferably more than 100 target sites. The number of target sites may increase to about 100-500 if a more sensitive detection is desirable. A person skilled in the art may decide the number of target sites based on the disclosure of the present invention or empirically according to prior testing results.

[0133] The measurement of copy numbers for each of the plurality of target sites may be accomplished by many techniques, including a multiplex nucleic acid analysis method similar to the CNV detection method as detailed *supra*, a multiplex nucleic acid analysis employing DNA sequencing techniques, and real-time PCR.

[0134] For one example, a multiplex nucleic acid analysis method similar to the CNV detection method as detailed *supra* is used. Similar to the scheme shown in **Figure 5**, the copy number of 96 target sites may be measured in a single assay simultaneously. The descriptions of the designs of probes, primers and testing procedures are not repeated here except that the statistical analysis of peak intensities is described below.

[0135] In some embodiments, the fluorescent peak intensity of each target site in a test sample is measured and compared to a standard peak value of the same target site in the testing system to determine the copy number of the target site. A standard value may be a fluorescent intensity value corresponding to the target site in the specific testing system. A testing system means the whole experimental system including the reagents, primers, probes, procedures and devices used for the testing the copy number changes. One test sample is considered to share the same testing system with another test sample if the whole experimental system is the same except the initial DNA sample to be tested. As such a standard value may be obtained for a specific testing system based on prior testing results of DNA samples. If the DNA samples are from normal or wild-type subject, the standard value is a normal or wild-type standard value. If the DNA samples are from an abnormal subject, the standard value is an abnormal standard value.

[0136] In other embodiments, a control sample is used to generate control fluorescent peak intensity values for the target sites. The copy number for each target site is known for the control sample. When the fluorescent peak value for a target site in a test sample is obtained and compared to the peak value for the same target site in the control sample, the copy number of the target site may be determined.

[0137] In some preferred embodiments, similar to the normalization and copy number measurement methods described for CNV copy number change detection *supra*, the peak values for each gene target site (i.e., target DNA of interest, which is not necessarily within a gene but can be within non-coding genomic DNA) are normalized against peak values for one or more reference target sites. The copy number changes are measured by comparing the normalized peak value for each gene target site in the test sample to the normalized standard value for the same gene target site in the testing system or the normalized peak value for the same gene target site in the control sample. The normalization may correct the variations, e.g., the amount of DNA templates used for probe hybridization, the amount of ligation probes and PCR primers, the ligation efficiency of each set of probes, and the amount of DNA used for PCR amplification, in obtaining the peak values between different samples and between different gene target sites. To obtain peak values of reference target sites, probes for the reference target sites may be designed in a similar manner and used simultaneously with the probes for the gene target sites in the hybridization and ligation reactions. In addition, probes for the reference target sites and probes for the gene target sites may share the same primer binding sites so that the same set of primers may be used to amplify the ligation products for both the reference sites and the gene target sites.

[0138] As such in some instances, peak values for a plurality of reference target sites are obtained in parallel with the peak values for a plurality of gene target sites so that the peak value for each of the plurality of the gene target sites may be normalized against the peak value for each of the plurality of the reference target sites. The number of reference target sites may be about 1 to about 100, and the number of gene target sites may be about 1 to about 100. For one example, peak values for 6 reference target sites may be obtained together with the peak values for 6 gene target sites. In this example, 6 sets of probes for the 6 reference target sites and 6 sets of probes for the 6 gene target sites are added to a sample and ligation products are obtained after hybridization and ligation reactions. For another example, peak values for 100 reference target sites may be obtained together with the peak values for 100 gene target sites. In this example, 100 sets of probes for the 100 reference target sites and 100 sets of probes for the 100 gene target sites are added to a sample and ligation products are obtained after hybridization and ligation reactions. In both examples, a single set of primers may be used to amplify the ligation products and peak values are measured by analyzing the amplification products by capillary electrophoresis.

[0139] In some embodiments, the number of reference target sites used for a gene target site may affect the sensitivity of detecting copy number changes of the gene target site. The more reference target sites are used, the smaller of the copy number change of the gene target site may be detected. Accordingly, when it is desirable to detect more copy number changes, for example a 0.1 % change of cancer cell-associated copy number variant, it is desirable to use relatively more reference target sites for each gene target site. A person skilled in the art may increase the number of reference target sites when it is apparent that more sensitive detection of copy number changes is desired.

[0140] In some embodiments, the normalization of the peak value for a gene target site in a sample is to obtain a ratio (here referred to as R) of the peak value for the gene target site against the peak value for a reference target site. As such, the copy number of a gene target site in a test sample may be measured by comparing the ratio for the gene target site in the test sample (here referred to as R_{test}) with the standard ratio for the same gene target site in the testing system (here referred to as R_{standard}) or with the ratio for the same gene target site in a control sample (here referred to as R_{control}).

[0141] In some instances, the copy number of a gene target site in a test sample is measured by comparing the ratio for the gene target site in the test sample (R_{test}) with the standard ratio for the same gene target site in the testing system (R_{standard}). The copy number of the gene target site in the testing system is known (here referred to as C_{standard}). In this case, the copy number of the gene target site in the test sample (here referred to as C_{test}) may be calculated as follow:

$$C_{\text{test}} = C_{\text{standard}} \times R_{\text{test}} / R_{\text{standard}}$$

[0142] In other instances, the copy number of a gene target site in a test sample may be measured by comparing the ratio for the gene target site in the test sample (R_{test}) with the ratio for the same gene target site in a control sample (R_{control}). The copy number of the gene target site in the testing system is known (here referred to as C_{control}). In this case, the copy number of the gene target site in the test sample (C_{test}) may be calculated as follow: $C_{\text{test}} = C_{\text{control}} \times$

$$R_{\text{test}} / R_{\text{control}}$$

[0143] As such, when 6 reference target sites are introduced to normalize the peak value for a gene target site, six copy number measurements (C_{test}) for the gene target site may be derived on the basis of the six peak values for the six reference target sites. Based on the six C_{test} measurements, the copy number of the gene target site is obtained according to certain statistical analysis. In one embodiment, the median value of the six C_{test} measurements is deemed the copy number of the gene target site. In another embodiment, the average value of the six C_{test} measurements is deemed the copy number of the gene target site.

[0144] Based on the copy number for each of the plurality of target sites, the copy number for the nucleic acid may be determined by methods including, but not limited to, taking the average of the copy numbers of all target sites or the

median value of the copy numbers for all target sites, or taking the average of the copy numbers of all target sites or the median value of the copy numbers of all target sites after abandoning some egregious values if desirable.

[0145] In some embodiments, the testing for each gene target site in the nucleic acid may be repeated so that multiple copy number calculation results may be obtained. For example, the testing for each gene target site within a nucleic acid may be repeated three times and three copy number calculation results for the nucleic acid may be obtained. The average or median value of the three copy number calculation results may be deemed as the copy number of the nucleic acid. As such, the number of repeats of the testing may also affect the sensitivity of the method. If a more sensitive detection of copy number changes is desired, the testing may be repeated more times. A person skilled in the art may increase the number of repeated testing if he or she desires to detect smaller copy number changes, e.g., 0.1%.

[0146] As such, the sensitivity of detecting small copy number changes of a nucleic acid in a sample may be influenced by many factors including, but not limited to, the number of gene target sites within the nucleic acid, the number of reference target sites used for each gene target site, and the number of repeated testing for each gene target site. The increase of the number of gene target sites within the nucleic acid, the number of reference target sites used for each gene target site, and/or the number of repeated testing for each gene target site may enhance the sensitivity of the detection. A person skilled in the art may adjust the numbers according to the present invention if a more sensitive detection is needed in measuring very small copy number changes of a nucleic acid of interest in a sample.

[0147] Various statistical methods may be applied to calculate the copy number of each target site based on experimental results and determine the copy number of the nucleic acid based on the calculated copy number of each target site. A specific example of statistical analysis of small copy number changes is detailed below in **Example 4**: "Detection of chromosome 21 copy number changes."

[0148] For another example, real-time PCR may be used to detect the copy number of each of the plurality of the selected target sites as is known in the art. Real-time PCR may determine the copy number of a target site in a monoplex or multiplex manner. Based on the copy numbers for each of the plurality of target sites, the copy number for the nucleic acid may be determined by methods including, but not limited to, taking the average of the copy numbers of all target sites or the median value of the copy numbers for all target sites, or taking the average of the copy numbers of all target sites or the median value of the copy numbers of all target sites after abandoning some egregious values if desirable.

IV. Kits For Multiplex Nucleic Acid Analysis and Small Copy Number Change Detection

[0149] In yet another aspect of the present invention, a kit is provided for multiplex nucleic acid analysis and small copy number change detection. In one embodiment, the kit for assaying nucleic acids in a sample include one or more sets of probes corresponding to a target nucleic acid so that the probes in each set, when hybridized to the target nucleic acid may be ligated to form a third probe. In another embodiment, the kit further includes one or more sets of primers for amplifying the third probe.

[0150] Each set of probes may include a first probe having a first portion at least partially complementary to a first region of the target nucleic acid and a second portion as a first primer binding site, and a second probe having a first portion at least partially complementary to a second region of the target nucleic acid and a second portion as a second primer binding site. In some instances, the 5' end of the first probe is essentially adjacent to the 3' end of the second probe and the first and the second probes may be ligated to form a third probe. In other instances, the 5' end of the first probe is not adjacent to the 3' end of the second probe and the first and the second probes may not be ligated to form a third probe without filling the gaps. Gap filling may be achieved by another probe that can hybridize to the gap on the target nucleic acid or by extending one of the two probes in a polymerase reaction. For example, the 3' end of the second probe may be extended to fill the gap until the extended 3' end is substantially adjacent to the 5' end of the first probe.

[0151] In some embodiments, the kit may also contain reagents including, but not limited to, a ligase, e.g., a Taq DNA ligase or a T4 DNA ligase, a buffer for a ligation reaction, a DNA polymerase, e.g., a Taq DNA polymerase, a buffer for polymerase chain reaction, or a combination thereof.

[0152] In some embodiments, the set of primers in a kit may include a first primer at least partially complementary to the first primer binding site and a second primer at least partially complementary to the second primer binding site. In some instances, at least one primer of the set of primers is labeled with a detectable moiety. The moiety may be an oligonucleotide tag or a fluorescent dye such as a fluorescein fluorophore. A fluorophore may be FAM (5-or 6-carboxyfluorescein), VIC, NED, PET, Fluorescein, FITC, IRD-700/800, CY3, CY5, CY3.5, CY5.5, HEX, TET, TAMRA, JOE, ROX, BODIPY TMR, Oregon Green, Rhodamine Green, Rhodamine Red, Texas Red, and Yakima Yellow.

[0153] In some embodiments, an oligonucleotide GTTCTT was included in the 5' portion of at least one of the primers. In some preferred embodiments, the reverse primer used for amplifying the ligation products includes oligonucleotide GTTCTT or its functional equivalents, e.g., GTTCTTG.

[0154] In some embodiments, at least one primer of the set of primers includes a stuffer sequence with a length of about 10-500 nucleotides. The stuffer sequence in some primers may have about 10 to about 500 nucleotides. The stuffer sequence in other primers may be about 10 to about 60 nucleotides. In some preferred embodiments, no primer

has more than about 125 nucleotides. In other preferred embodiments, no primer has more than about 75 nucleotides.

[0155] In other embodiments, at least one probe of the set of probes includes a stuffer sequence with a length of about 1-200 nucleotides. In other instances, the stuffer sequence has about 1 to about 55 nucleotides. In some preferred embodiments, the third probe has no more than about 250 nucleotides. In other preferred embodiments, the third probe has no more than about 140 nucleotides. In still some preferred embodiments, no probe has more than about 125 nucleotides. In still some other preferred embodiments, no probe has more than about 70 nucleotides. In some further preferred embodiments, no probe has more than about 60 nucleotides.

[0156] In some embodiments, the target nucleic acid is a dystrophin gene and the kit is for detecting Duchenne muscular dystrophy. The sets of probes in the kit comprise one or more probe pairs selected from SEQ ID NOs: 158-541.

[0157] In other embodiments, the target nucleic acid is on human chromosome 21 and the kit is for detecting fetal Down's syndrome in maternal blood. The sets of probes in the kit comprise one or more probe pairs selected from SEQ ID NOs: 559-942.

V. Examples

[0158] It should be understood that this invention is not limited to the particular methodologies, protocols and reagents, described herein. The terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention.

[0159] In addition, the reactions outlined below may be accomplished in a variety of ways, as will be appreciated by those in the art. Components of the reaction may be added simultaneously, or sequentially, in different orders, with preferred embodiments outlined below. In addition, the reaction may include a variety of other reagents which may be included in the assays. These include reagents like salts, buffers, neutral proteins, e.g. albumin, detergents, etc., which may be used to facilitate optimal hybridization and detection, and/or reduce non-specific or background interactions. Also reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, etc., may be used, depending on the sample preparation methods and purity of the target.

[0160] While the present invention has been disclosed with reference to certain embodiments, numerous modifications, alterations, and changes to the described embodiments are possible without departing from the full scope of the invention, as described in the appended specification and claims. Other features, objects, and advantages of the disclosed subject matter will be apparent from the detailed description, figures, examples and claims. Methods and materials substantially similar or equivalent to those described herein can be used in the practice or testing of the presently disclosed subject matter.

Example 1: multiplex SNP detection

[0161] This example demonstrates a multiplex SNP detection method according to the present invention. In this example, 48 SNPs were detected simultaneously in a blood sample by employing fluorescent dye labeled forward primers and varying the length of the reverse primer by adding a stuffer sequence according to the scheme in **Figure 4**.

[0162] The 48 SNPs were rs1056893, rs1058588, rs10790286, rs10791649, rs11107, rs11155787, rs11161732, rs1249950, rs12719860, rs1359185, rs1572983, rs2161916, rs2231926, rs2241280, rs2241571, rs2241802, rs2279072, rs2294092, rs2297129, rs2304035, rs2304102, rs2305150, rs2306331, rs2401751, rs2779500, rs2986014, rs3182535, rs3731631, rs3736582, rs3749877, rs3809806, rs3816800, rs4141253, rs4362, rs4371677, rs469783, rs4829830, rs4920098, rs624821, rs625372, rs639225, rs6784322, rs6892205, rs894344, rs934472, rs938883, rs9389034, and rs9791113. All SNP names and other information may be found in the National Center for Biotechnology Information dbSNP database. For each SNP, three probes were designed: the 3' probe (the right probe), and two 5' probes (the left probe) corresponding to two alleles. The 3' and 5' probes were designed in a manner so that when both are hybridized to the target sequence under a suitable condition, there is no gap between the two probes. The probes were synthesized by Life Technologies Corporation. The names, sequence ID numbers, primer binding sequences, and stuffer sequences for each of the 144 (3 x 48) probes are shown in **Table 1**.

[0163] For the 3' probe, the 5' end nucleotide was phosphorylated to provide a phosphate which would be connected to the hydroxyl group in the 3' end nucleotide of the 5' probe. Each 3' probe included a locus specific hybridization sequence (LSHS) in the 5' portion followed by a stuffer L2 sequence, and a primer binding sequence Y in the 3' portion. In some 3' probes, SNP_Y1 sequence, SEQ ID NO: 155 was used. In other 3' probes, SNP-Y2 sequence, SEQ ID NO: 156 was used.

[0164] Two different 5' probes were designed for each SNP with each 5' probe corresponding to a different allele. The 3' portion of each 5' probe was an allele-specific hybridization sequence (ASHS) and the 3' end nucleotide corresponded to the specific nucleotide in each individual allele. The 5' portion of each 5' probe had a primer binding sequence X. In this example, four primer binding sequence X: SNP_X1, SEQ ID NO: 151; SNP_X2, SEQ ID NO: 152; SNP_X3, SEQ ID NO: 153; SNP_X4, SEQ ID NO: 154 were used in the 5' probes. In some 5' probes, a stuffer A sequence was inserted

between the 3' portion and the 5' portion of the 5' probes. In addition, in some 5' probes, a stuffer L1 sequence was inserted between the 5' portion and the stuffer A sequence. The position of the stuffer A and stuffer L1 sequences are interchangeable.

[0165] In addition, four forward primers and two reverse primers were designed for amplifying the ligation products. The four forward primers (SNP_F1, SEQ ID NO: 145; SNP_F2, SEQ ID NO: 146; SNP_F3, SEQ ID NO: 147; and SNP_F4, SEQ ID NO: 148) had unique sequences that were consistent with the four primer binding sequence X (SNP_X1, SEQ ID NO: 151; SNP_X2, SEQ ID NO: 152; SNP_X3, SEQ ID NO: 153; SNP_X4, SEQ ID NO: 154), respectively. The four forward primers (SNP_F1, SNP_F2, SNP_F3, and SNP_F4) were labeled on the 5' ends with four different fluorescent dyes: FAM-blue, VIC-green, NED-yellow, and PET-red, respectively. The two reverse primers (SNP_R1, SEQ ID NO: 149; and SNP_R2, SEQ ID NO: 150) had unique sequences that were reversely complementary to the primer binding sequence Y (SNP_Y1 sequence, SEQ ID NO: 155 and SNP_Y2 sequence, SEQ ID NO: 156), respectively. The SNP_R primer also had a stuffer sequence SNP_R-Buffer, SEQ ID NO: 157 in the 5' portion. All primers and probes were synthesized by Life Technologies Corporation.

[0166] To perform the 48 multiplex SNP detection assay, a ligation product was first generated. Briefly, genomic DNA was extracted from a 2ml whole blood sample using the classic phenol:chloroform method. The blood sample was collected from a healthy volunteer at Shanghai Ruijin Hospital, Shanghai, China. From the extracted genomic DNA, 100-200 microgram (μ g) DNA was dissolved in 10 microliter (μ l) 1xTE buffer (10mM Tris.Cl, pH8.0, 1mM EDTA from Sigma-Aldrich). The dissolved genomic DNA was denatured at 98°C for 5 minutes and then immediately cooled down on ice. At the same time, a 2x ligation premix solution was prepared according to the following formula: a 10 μ l 2x ligation premix was made of 2 μ l 10xTaq ligase buffer, 1 μ l 40U/ μ l Taq Ligase from NewEngland Biolabs, Inc., 1 μ l ProbeMix (each probe with a final concentration of 0.005 micromolar in 1xTE), and 6 μ l ddH₂O (Distilled Milli-Q water from Milli-Q Advantage A10, Millipore). 10 μ l 2X ligation premix was mixed with the denatured 10 μ l genomic DNA and the mixture was allowed to undergo 4 cycles of denaturation, hybridization and ligation under the following conditions: 95 °C for 30 seconds, and then 58 °C for 4 hours. The ligation product thus obtained could be stored on ice for same day use or frozen in -20 °C for future use.

[0167] With the ligation product, an amplification step was then performed to obtain an amplification product. Briefly, a PCR reaction was performed using the amplification product as the template. The PCR reaction mixture was prepared as follows: a 20 μ l reaction system was made by mixing 2 μ l 10x PCR buffer (Qiagen, Germany), 2 μ l 2.5mM dNTP mix (2.5mM each of dATP, dTTP, dCTP and dGTP from Takara Bio Inc.), 2 μ l primer mix (SNP_F1, SNP_F2, SNP_F3, SNP_F4, SNP_R1 and SNP_R2 at final concentrations of 1 μ M, 1 μ M, 1 μ M, 1 μ M, 2 μ M and 2 μ M, respectively), 1 μ l Ligation product, 0.2 μ l 5U/ μ l HotStarTaq Plus Taq DNA polymerase (Qiagen, Germany), and 12.8 μ l ddH₂O. The PCR mixture was allowed to undergo a polymerase chain reaction under the following conditions: 95 °C for 2 minutes, followed by 35 cycles of 94 °C for 20 second, 57°C for 40 second, and 72 °C for 1.5 minutes, and after the 35th cycle, the reaction mixture was kept at 60 °C for 1 hour. To analyze the amplification product, 1 μ l of the amplification product was first diluted with ddH₂O 10 times into 10 μ l. Then 1 μ l was taken out of the 10 μ l diluted amplification product and mixed with 0.1 μ l GeneScan™ 500 LIZ® size standard (Life Technologies, Inc.) and 8.9 μ l Hi-Di formamide (Life Technologies, Inc.). The mixture was denatured at 95 °C for 5 minutes and analyzed with capillary electrophoresis by ABI3130XL according to manufacturer's manual. The capillary electrophoresis data was processed using Genemapper 4.0.

Table 1. Names, sequence ID numbers, primer binding sites and stuffer sequences in the probes used for multiplex SNP detection. The names for 3' probe are in the format: SNP name_3, e.g., rs1056893_3 refers to the 3' probe for the SNP rs1056893. The names for the 5' probe are in the format: SNP name_polymorphic nucleotide, e.g., rs1056893_C refers to the 5' probe for the SNP rs1056893 allele C; and similarly, rs1056893_T refers to the 5' probe for the SNP rs1056893 allele T.

SEQ ID NO.	Probe Name	Sequence X	Stuffer L1	Stuffer A	Stuffer L2	Sequence Y
1	rs1056893_3				ATTA	Y2
2	rs1056893_C	X3				
3	rs1056893_T	X3		TT		
4	rs1058588_3					Y2
5	rs1058588_C	X2				
6	rs1058588_T	X2		TT		
7	rs10790286_3				ATT	Y2
8	rs10790286_C	X4				
9	rs10790286_T	X4		TT		
10	rs10791649_3				ATTACGCGATTAC	Y2

EP 2 893 034 B9

(continued)

	SEQ ID NO.	Probe Name	Sequence X	Stuffer L1	Stuffer A	Stuffer L2	Sequence Y
5	11	rs10791649_A	X4		TT		
	12	rs10791649_G	X4				
	13	rs11107_3				ATTACGCGATTACG	Y1
	14	rs11107_A	X2	A	TT		
10	15	rs11107_G	X2	A			
	16	rs11155787_3				ATTACGCGATTAC	Y1
	17	rs11155787_C	X3	A			
	18	rs11155787_T	X3	A	TT		
	19	rs11161732_3				ATTACGCGA	Y1
15	20	rs11161732_A	X1	ATTA	TT		
	21	rs11161732_G	X1	ATTA			
	22	rs1249950_3				ATTAC	Y1
	23	rs1249950_C	X1				
20	24	rs1249950_T	X1		TT		
	25	rs12719860_3				ATTACGCGATTAC	Y2
	26	rs12719860_A	X1	ATTAC	TT		
	27	rs12719860_C	X1	ATTAC			
	28	rs1359185_3				ATTACGCGATTA	Y2
25	29	rs1359185_A	X4	ATT	TT		
	30	rs1359185_G	X4	ATT			
	31	rs1572983_3				ATTACGCGA	Y2
	32	rs1572983_C	X2				
30	33	rs1572983_T	X2		TT		
	34	rs2161916_3				A	Y2
	35	rs2161916_A	X4		TT		
	36	rs2161916_G	X4				
	37	rs2231926_3				ATTACGC	Y1
35	38	rs2231926_A	X4		TT		
	39	rs2231926_G	X4				
	40	rs2241280_3				ATTACGCGAT	Y2
	41	rs2241280_A	X1	ATTA	TT		
40	42	rs2241280_G	X1	ATTA			
	43	rs2241571_3				AT	Y1
	44	rs2241571_C	X2				
	45	rs2241571_T	X2		TT		
	46	rs2241802_3				ATTACGCGAT	Y2
45	47	rs2241802_A	X4		TT		
	48	rs2241802_G	X4				
	49	rs2279072_3				ATTACGCGATT	Y2
	50	rs2279072_C	X2				
50	51	rs2279072_T	X2		TT		
	52	rs2294092_3				ATTACGCGATT	Y1
	53	rs2294092_C	X1	ATTAC	TT		
	54	rs2294092_G	X1	ATTAC			
	55	rs2297129_3					Y2
55	56	rs2297129_A	X3		TT		
	57	rs2297129_G	X3				
	58	rs2304035_3				A	Y2

EP 2 893 034 B9

(continued)

	SEQ ID NO.	Probe Name	Sequence X	Stuffer L1	Stuffer A	Stuffer L2	Sequence Y
5	59	rs2304035_A	X1		TT		
	60	rs2304035_G	X1				
	61	rs2304102_3				ATTACGCGATTA	Y2
	62	rs2304102_A	X1		TT		
10	63	rs2304102_G	X1				
	64	rs2305150_3				ATTACGCGATTA	Y1
	65	rs2305150_C	X2	ATT			
	66	rs2305150_T	X2	ATT	TT		
	67	rs2306331_3				ATTACGCG	Y1
15	68	rs2306331_C	X2				
	69	rs2306331_T	X2		TT		
	70	rs2401751_3				ATTA	Y1
	71	rs2401751_A	X2		TT		
20	72	rs2401751_G	X2				
	73	rs2779500_3				ATTACGCGA	Y1
	74	rs2779500_C	X1		TT		
	75	rs2779500_G	X1				
	76	rs2986014_3				ATT	Y1
25	77	rs2986014_C	X3				
	78	rs2986014_T	X3		TT		
	79	rs3182535_3					Y1
	80	rs3182535_A	X3		TT		
30	81	rs3182535_G	X3				
	82	rs3731631_3				ATTACGCGATTAC	Y2
	83	rs3731631_A	X2	AT	TT		
	84	rs3731631_G	X2	AT			
	85	rs3736582_3				ATTA	Y2
35	86	rs3736582_C	X2		TT		
	87	rs3736582_G	X2				
	88	rs3749877_3				ATTACGC	Y2
	89	rs3749877_A	X3		TT		
	90	rs3749877_G	X3				
40	91	rs3809806_3				ATTACGCG	Y1
	92	rs3809806_C	X4				
	93	rs3809806_T	X4		TT		
	94	rs3816800_3				AT	Y1
45	95	rs3816800_C	X1		TT		
	96	rs3816800_G	X1				
	97	rs4141253_3				ATTACGCGATT	Y1
	98	rs4141253_C	X3				
	99	rs4141253_T	X3		TT		
50	100	rs4362_3				ATTACGC	Y2
	101	rs4362_C	X4				
	102	rs4362_T	X4		TT		
	103	rs4371677_3				ATTACGCGATT	Y2
55	104	rs4371677_A	X3		TT		
	105	rs4371677_G	X3				
	106	rs469783_3				ATTACGCGATT	Y2

(continued)

	SEQ ID NO.	Probe Name	Sequence X	Stuffer L1	Stuffer A	Stuffer L2	Sequence Y
5	107	rs469783_C	X3	ATTACGCGAT			
	108	rs469783_T	X3	ATTACGCGAT	TT		
	109	rs4829830_3				ATTACGCGAT	Y1
	110	rs4829830_A	X2		TT		
10	111	rs4829830_C	X2				
	112	rs4920098_3				ATTACG	Y2
	113	rs4920098_C	X1				
	114	rs4920098 T	X1		TT		
	115	rs624821_3				ATTACGCGATTA	Y1
15	116	rs624821_A	X4				
	117	rs624821_T	X4		TT		
	118	rs625372_3					Y1
	119	rs625372_C	X4				
20	120	rs625372_T	X4		TT		
	121	rs639225_3				ATTACGCGATTA	Y2
	122	rs639225_A	X2	ATTA	TT		
	123	rs639225_G	X2	ATTA			
	124	rs6784322_3				ATTACGCGATTACG	Y1
25	125	rs6784322_A	X4	A			
	126	rs6784322_T	X4	A	TT		
	127	rs6892205_3				ATTACGCGA	Y1
	128	rs6892205_A	X3	AT	TT		
	129	rs6892205_G	X3	AT			
30	130	rs894344_3				ATTACGCGAT	Y1
	131	rs894344_A	X1		TT		
	132	rs894344_G	X1				
	133	rs934472_3				AT	Y1
35	134	rs934472_A	X4		TT		
	135	rs934472_C	X4				
	136	rs938883_3				ATTACGCGATTACGC	Y2
	137	rs938883_C	X3	A			
	138	rs938883_T	X3	A	TT		
40	139	rs9389034_3				ATTACGCG	Y1
	140	rs9389034_C	X3				
	141	rs9389034_T	X3		TT		
	142	rs9791113_3				ATTACGCG	Y2
45	143	rs9791113_C	X1		TT		
	144	rs9791113_G	X1				

[0168] As shown in **Figures 10A-E**, the amplification products obtained in the assay could be separated and the peaks corresponding to each SNP allele could be individually identified by capillary electrophoresis. All the amplification products were from the same PCR reaction. **Figure 10A** showed the chromatograms of all amplification products which were labeled with four different fluorescent dyes. Each peak represented one amplification product corresponding to an individual SNP allele. **Figures 10B, 10C, 10D, and 10E**, which were individually derived from **Figure 10A**, showed the chromatograms for amplification products labeled with blue, green, yellow and red, respectively. As seen in **Figures 10B, 10C, 10D, and 10E**, the peaks from the amplification products labeled with the same fluorescent dye could be individually identified on the basis of different fragment sizes. The two alleles for each SNP were separated on the basis of a fragment size difference of about 2 nucleotides.

[0169] For example, SNP rs3816800 had two alleles with G and C, respectively. The ligated probe size for A1 allele

was 95 base pair (bp) and the size for A2 97 bp; and the CE reference size for A1 allele was 92.46 and the size for A2 was 94.56. As shown in **Figure 10B**, there were two peaks one with the fragment size of about 93 bp and the other with the fragment size of about 95 bp. Therefore, the genotype for SNP rs3816800 was determined to be heterozygous G/C.

[0170] A CE reference size for a SNP allele was obtained as follows: first, the corresponding set of probes were used to perform hybridization, ligations and amplification in a DNA sample harboring the SNP allele, and then the amplification products were analyzed by capillary electrophoresis to obtain the CE reference size for the SNP allele with the presence of a size standard. For example, the set of probes rs11161732_3, SEQ ID NO: 19 and rs11161732_A SEQ ID NO: 20 were used to obtain the CE reference size for the rs11161732_A allele and the CE reference size was determined to be 114.64.

[0171] For another example, SNP rs1249950 had two alleles with C and T, respectively. The ligated probe size for A1 allele was 100 bp and the size for A2 102 bp; and the CE reference size for A1 allele was 97.82 and the size for A2 was 100.11. As shown in **Figure 10B**, there was only one peak with the fragment size of about 100 bp. Therefore, the genotype for SNP rs1249950 was determined to be homozygous T/T.

[0172] As such, the genotypes of all the 48 SNP were determined according to the capillary electrophoresis results (see **Table 2**). These results were all consistent with the results obtained by direct DNA sequencing.

Table 2. The capillary electrophoresis results in terms of the ligation product size and genotypes of 48 SNPs.

A1 refers to Allele #1; A2 refers to Allele #2; A1LP refers to Allele #1 Ligation Product Size; A2LP refers to Allele #2 Ligation Product Size; REF refers to reference; and CE refers to Capillary Electrophoresis.

SNP rs#	A1	A2	PCR Primers	A1LP SIZE	A2LP SIZE	A1LP CE REF SIZE	A2LP CE REF SIZE	GENOTYPE
rs11161732	G	A	SNP_ F1/SNP_ R1	115	117	112.56	114.64	G/A
rs1249950	C	T	SNP_ F1/SNP_ R1	100	102	97.82	100.11	T/T
rs2294092	G	C	SNP_ F1/SNP_ R1	120	122	118.23	120.55	C/C
rs2779500	G	C	SNP_ F1/SNP_ R1	105	107	103.2	105.41	G/C
rs3816800	G	C	SNP_ F1/SNP_ R1	95	97	92.46	94.56	G/C
rs894344	G	A	SNP_ F1/SNP_ R1	110	112	107.75	109.69	G/G
rs12719860	C	A	SNP_ F1/SNP_ R2	154	156	153.51	155.7	C/C
rs2241280	G	A	SNP_ F1/SNP_ R2	149	151	147.53	149.88	A/A
rs2304035	G	A	SNP_ F1/SNP_ R2	128	130	126.12	128.23	G/G
rs2304102	G	A	SNP_ F1/SNP_ R2	144	146	143.77	146.15	G/A
rs4920098	C	T	SNP_ F1/SNP_ R2	134	136	130.98	133.25	T/T

EP 2 893 034 B9

(continued)

	SNP rs#	A1	A2	PCR Primers	A1LP SIZE	A2LP SIZE	A1LP CE REF SIZE	A2LP CE REF SIZE	GENOTYPE
5	rs9791113	G	C	SNP_ F1/SNP_ R2	139	141	135.06	137.18	G/C
10	rs11107	G	A	SNP_ F2/SNP_ R1	115	117	113.01	114.86	G/A
	rs2241571	C	T	SNP_ F2/SNP_ R1	95	97	93.51	95.49	C/T
15	rs2305150	C	T	SNP_ F2/SNP_ R1	120	122	118.24	120.33	C/T
	rs2306331	C	T	SNP_ F2/SNP_ R1	105	107	103.44	105.59	C/T
20	rs2401751	G	A	SNP_ F2/SNP_ R1	100	102	98.97	101.18	G/A
25	rs4829830	C	A	SNP_ F2/SNP_ R1	110	112	109.48	111.44	C/C
	rs1058588	C	T	SNP_ F2/SNP_ R2	128	130	126.65	128.78	C/C
30	rs1572983	C	T	SNP_ F2/SNP_ R2	139	141	136.39	138.43	C/T
	rs2279072	C	T	SNP_ F2/SNP_ R2	144	146	142.77	145.27	C/C
35	rs3731631	G	A	SNP_ F2/SNP_ R2	149	151	149.5	151.89	G/A
40	rs3736582	G	C	SNP_ F2/SNP_ R2	134	136	132.01	134.03	G/C
	rs639225	G	A	SNP_ F2/SNP_ R2	154	156	154.65	156.72	G/A
45	rs11155787	C	T	SNP_ F3/SNP_ R1	115	117	112.56	114.86	C/T
50	rs2986014	C	T	SNP_ F3/SNP_ R1	100	102	97.59	100.11	C/T
	rs3182535	G	A	SNP_ F3/SNP_ R1	95	97	92.3	94.93	G/A
55	rs4141253	C	T	SNP_ F3/SNP_ R1	110	112	107.53	109.38	C/C

EP 2 893 034 B9

(continued)

	SNP rs#	A1	A2	PCR Primers	A1LP SIZE	A2LP SIZE	A1LP CE REF SIZE	A2LP CE REF SIZE	GENOTYPE
5	rs6892205	G	A	SNP_ F3/SNP_ R1	120	122	117.01	119.3	G/G
10	rs9389034	C	T	SNP_ F3/SNP_ R1	105	107	102.45	104.59	C/C
	rs1056893	C	T	SNP_ F3/SNP_ R2	134	136	131.68	133.7	T/T
15	rs2297129	G	A	SNP_ F3/SNP_ R2	129	131	126.95	128.85	G/A
	rs3749877	G	A	SNP_ F3/SNP_ R2	139	141	137.29	139.63	A/A
20	rs4371677	G	A	SNP_ F3/SNP_ R2	144	146	142.65	145.15	G/A
25	rs469783	C	T	SNP_ F3/SNP_ R2	154	156	153.63	155.58	C/C
	rs938883	C	T	SNP_ F3/SNP_ R2	149	151	148.29	150.59	C/T
30	rs2231926	G	A	SNP_ F4/SNP_ R1	102	104	104.62	106.56	G/A
	rs3809806	C	T	SNP_ F4/SNP_ R1	107	109	109.25	111.22	C/C
35	rs624821	A	T	SNP_ F4/SNP_ R1	112	114	114.09	116.13	A/A
40	rs625372	C	T	SNP_ F4/SNP_ R1	92	94	93.83	94.87	T/T
	rs6784322	A	T	SNP_ F4/SNP_ R1	117	119	118.09	120.18	A/T
45	rs934472	C	A	SNP_ F4/SNP_ R1	97	99	98.51	100.64	C/C
50	rs10790286	C	T	SNP_ F4/SNP_ R2	131	133	133	135.14	C/T
	rs10791649	G	A	SNP_ F4/SNP_ R2	146	148	148.51	151.06	G/A
55	rs1359185	G	A	SNP_ F4/SNP_ R2	151	153	153.16	155.24	A/A

(continued)

SNP rs#	A1	A2	PCR Primers	A1LP SIZE	A2LP SIZE	A1LP CE REF SIZE	A2LP CE REF SIZE	GENOTYPE
rs2161916	G	A	SNP_ F4/SNP_ R2	125	127	126.73	128.78	A/A
rs2241802	G	A	SNP_ F4/SNP_ R2	141	143	141.98	144.4	G/G
rs4362	C	T	SNP_ F4/SNP_ R2	136	138	137.52	139.63	C/T

Example 2: Multiplex detection of Copy Number Variants

[0173] This example demonstrates a multiplex CNV detection method according to the present invention. In this example, there were 192 target sequences including 129 target sites in the Duchenne muscular dystrophy (DMD) gene, and 63 target reference sites on chromosomes X, Y, 2-12, 14, 16-20. DMD is a recessive X-linked form of muscular dystrophy, which results in muscle degeneration. The disorder is caused by mutations in the dystrophin gene, located on the human X chromosome, which codes for the protein dystrophin, an important structural component within muscle tissue that provides structural stability to the dystroglycan complex (DGC) of the cell membrane. Approximately two-thirds of the mutations in DMD are copy number variants of one or more exons in the dystrophin gene. The exemplary multiplex CNV detection method employed fluorescent dye labeled forward primers and reverse primers with stuffer sequences according to the scheme in **Figure 5**.

[0174] Specifically, probes were designed to cover 192 target sites with at least one target in each of the 79 exons of the DMD gene. Exons 22-42, 58-60, and 62-79 contained one target site. Exons 1, 3-15, 18-20, 47, 49, 54-57, and 61 contained two target sites. Exons 2, 16-17, 21, 43-46, 48, and 50-53 contain three target sites. For each target site, a probe pair was designed including a 3' probe (the left probe) and a 5' probe (the right probe). The 3' and 5' probes were designed in a manner so that when both are hybridized to the target sequence under a suitable condition, there is no gap between the two probes. The probes were synthesized by Life Technologies Corporation. The names, sequence ID numbers, primer binding sites and stuffer sequences of the 384 probes (192 probe pairs) for the 192 target sites are shown in **Table 3**.

[0175] For each 3' probe, the 5' end nucleotide was phosphorylated to provide a phosphate which would be connected to the hydroxyl group in the 3' end nucleotide of the 5' probe. Each 3' probe included a locus specific hybridization sequence (LSHS) in the 5' portion followed by a stuffer L2 sequence, and a primer binding sequence Y in the 3' portion. In this example, four primer binding sequence Y (MDM_Y1, SEQ ID NO: 554; MDM_Y2, SEQ ID NO: 555; MDM_Y3, SEQ ID NO: 556; MDM_Y4, SEQ ID NO: 557) were used in the 3' probes.

[0176] Each 5' probe included a locus specific hybridization sequence (LSHS) in the 3' portion followed by a stuffer L1 sequence and a primer binding sequence X in the 5' portion. In this example, four primer binding sequence X (MDM_X1, SEQ ID NO: 550; MDM_x2, SEQ ID NO: 551; MDM_X3, SEQ ID NO: 552; MDM_X4, SEQ ID NO: 553) were used in the 5' probes.

[0177] In addition, four forward primers and four reverse primers were designed for amplifying the ligation products. The four forward primers (DMD_F1, SEQ ID NO: 542; DMD_F2, SEQ ID NO: 543; DMD_F3, SEQ ID NO: 544; and DMD_F4 SEQ ID NO: 545) had unique sequences that were consistent with the four primer binding sequence X (MDM_X1, SEQ ID NO: 550; MDM_x2, SEQ ID NO: 551; MDM_X3, SEQ ID NO: 552; MDM_X4, SEQ ID NO: 553), respectively. The four forward primers: DMD_F1, DMD_F2, DMD_F3, and DMD_F4 were labeled on the 5' ends with four different fluorescent dyes: FAM-blue, VIC-green, NED-yellow, and PET-red, respectively. The four reverse primers (DMD_R1, SEQ ID No: 546; DMD_R2, SEQ ID No: 547; DMD_R3, SEQ ID No: 548; and DMD_R4, SEQ ID No: 549) had unique sequences there were reversely complementary to the four primer binding sequence Y (MDM_Y1, SEQ ID NO: 554; MDM_Y2, SEQ ID NO: 555; MDM_Y3, SEQ ID NO: 556; MDM_Y4, SEQ ID NO: 557), respectively. The DMD_R2 and DMD_R4 reverse primers also had a stuffer sequence DMD_R_Stuffer, SEQ ID No: 558 in the 5' portion. All primers and probes were synthesized by Life Technologies Corporation.

Table 3. Names, sequence ID numbers, primer binding sites and stuffer sequences in the probes used in the multiplex CNV analysis for DMD gene. X1, X2, X3 and X4 refer to MDM_X1, SEQ ID NO: 550; MDM_x2, SEQ ID NO: 551; MDM_X3, SEQ ID NO: 552; MDM_X4, SEQ ID NO: 553, respectively. Y1, Y2, Y3 and Y4 refer to MDM_Y1, SEQ ID NO: 554; MDM_Y2, SEQ ID NO: 555; MDM_Y3, SEQ ID NO: 556; MDM_Y4, SEQ ID NO: 557, respectively.

SEQ ID NO.	Probe NAME	Sequence X	Stuffer L1	Stuffer L2	Sequence Y
158	ChrX_A_3			ATTACGCGA	Y4
159	ChrX_A_5	X2	ATTACG		
160	ChrX_B_3			ATTACG	Y3
161	ChrX_B_5	X1			
162	ChrX_C_3			ATTAC	Y3
163	ChrX_C_5	X3	ATTAC		
164	ChrY_A_3				Y3
165	ChrY_A_5	X3			
166	ChrY_B_3			ATTACGCGA	Y3
167	ChrY_B_5	X3	ATTACGCGATT		
168	ChrY_C_3				Y4
169	ChrY_C_5	X3	A		
170	DMD_E01A_3			ATTACGCGATT	Y4
171	DMD_E01A_5	X4	AT		
172	DMD_E01B_3			ATTAC	Y4
173	DMD_E01B_5	X4	ATTA		
174	DMD_E02A_3			ATTACGCGATT	Y4
175	DMD_E02A_5	X4	ATTACGC		
176	DMD_E02B_3			ATTACGC	Y4
177	DMD_E02B_5	X4	ATTACGC		
178	DMD_E02C_3			AT	Y4
179	DMD_E02C_5	X4			
180	DMD_E03A_3			ATTACGC	Y1
181	DMD_E03A_5	X4			
182	DMD_E03B_3			AT	Y2
183	DMD_E03B_5	X4			
184	DMD_E04A_3			ATT	Y1
185	DMD_E04A_5	X1			
186	DMD_E04B_3			ATTACGC	Y2

EP 2 893 034 B9

(continued)

	SEQ ID NO.	Probe NAME	Sequence X	Stuffer L1	Stuffer L2	Sequence Y
5	187	DMD_ E04B_5	X4			
	188	DMD_ E05A_3			ATTACGCG	Y1
10	189	DMD_ E05A_5	X3			
	190	DMD_ E05B_3			ATTACG	Y2
	191	DMD_ E05B_5	X1	ATTACG		
15	192	DMD_ E06A_3			A	Y2
	193	DMD_ E06A_5	X3			
20	194	DMD_ E06B_3			ATTACGCG	Y2
	195	DMD_ E06B_5	X1			
25	196	DMD_ E07A_3			ATT	Y2
	197	DMD_ E07A_5	X1			
	198	DMD_ E07B_3			ATTACGCGA	Y1
30	199	DMD_ E07B_5	X3	ATT		
	200	DMD_ E08A_3			ATT	Y1
35	201	DMD_ E08A_5	X2			
	202	DMD_ E08B_3			ATTAC	Y2
	203	DMD_ E08B_5	X2	ATT		
40	204	DMD_ E09A_3			ATTACGCGA	Y2
	205	DMD_ E09A_5	X1			
45	206	DMD_ E09B_3			ATT	Y2
	207	DMD_ E09B_5	X3			
50	208	DMD_ E10A_3			ATTACGCG	Y1
	209	DMD_ E10A_5	X3			
	210	DMD_ E10B_3			ATTACG	Y1
55	211	DMD_ E10B_5	X2	ATT		

EP 2 893 034 B9

(continued)

	SEQ ID NO.	Probe NAME	Sequenc e X	Stuffer L1	Stuffer L2	Sequence Y
5	212	DMD_ E11A_3			ATTA	Y2
	213	DMD_ E11A_5	X1			
10	214	DMD_ E11B_3			ATTAC	Y2
	215	DMD_ E11B_5	X2	ATTA		
	216	DMD_ E12A_3			ATTACGC	Y1
15	217	DMD_ E12A_5	X4	A		
	218	DMD_ E12B_3			ATTACGCGAT	Y2
20	219	DMD_ E12B_5	X4			
	220	DMD_ E13A_3			ATTACGCG	Y2
25	221	DMD_ E13A_5	X3			
	222	DMD_ E13B_3			ATTACGCGA	Y1
	223	DMD_ E13B_5	X2			
30	224	DMD_ E14A_3			AT	Y2
	225	DMD_ E14A_5	X4			
35	226	DMD_ E14B_3			ATTAC	Y1
	227	DMD_ E14B_5	X1	ATTACGCG		
	228	DMD_ E15A_3			ATTACGC	Y1
40	229	DMD_ E15A_5	X4	ATTA		
	230	DMD_ E15B_3			ATTACGCG	Y1
45	231	DMD_ E15B_5	X2	A		
	232	DMD_ E16A_3			ATT	Y4
50	233	DMD_ E16A_5	X2	ATTAC		
	234	DMD_ E16B_3			ATTA	Y4
	235	DMD_ E16B_5	X2			
55	236	DMD_ E16C_3			ATTAC	Y4

EP 2 893 034 B9

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	SEQ ID NO.	Probe NAME	Sequenc e X	Stuffer L1	Stuffer L2	Sequence Y
5	237	DMD_ E16C_5	X1	ATTACGCG		
	238	DMD_ E17A_3			A	Y3
10	239	DMD_ E17A_5	X2	A		
	240	DMD_ E17B_3				Y4
15	241	DMD_ E17B_5	X1			
	242	DMD_ E17C_3			ATTAC	Y3
	243	DMD_ E17C_5	X2			
20	244	DMD_ E18A_3			ATTACGCGATTA	Y1
	245	DMD_ E18A_5	X2	A		
25	246	DMD_ E18B_3			ATTACGC	Y2
	247	DMD_ E18B_5	X4			
	248	DMD_ E19A_3			ATTA	Y2
30	249	DMD_ E19A_5	X1			
	250	DMD_ E19B_3			ATTACGCGATT	Y2
35	251	DMD_ E19B_5	X4			
	252	DMD_ E20A_3			ATT	Y2
40	253	DMD_ E20A_5	X2			
	254	DMD_ E20B_3			ATTACG	Y1
	255	DMD_ E20B_5	X4			
45	256	DMD_ E21A_3			A	Y4
	257	DMD_ E21A_5	X1			
50	258	DMD_ E21B_3				Y3
	259	DMD_ E21B_5	X4			
	260	DMD_ E21C_3			ATTA	Y3
55	261	DMD_ E21C_5	X2	ATT		

EP 2 893 034 B9

(continued)

	SEQ ID NO.	Probe NAME	Sequence X	Stuffer L1	Stuffer L2	Sequence Y
5	262	DMD_ E22A_3				Y3
	263	DMD_ E22A_5	X1			
10	264	DMD_ E23A_3			ATT	Y3
	265	DMD_ E23A_5	X4			
	266	DMD_ E24A_3			A	Y3
15	267	DMD_ E24A_5	X4			
	268	DMD_ E25A_3				Y4
20	269	DMD_ E25A_5	X2			
	270	DMD_ E26A_3			ATT	Y3
25	271	DMD_ E26A_5	X3			
	272	DMD_ E27A_3				Y3
	273	DMD_ E27A_5	X3			
30	274	DMD_ E28A_3			ATT	Y3
	275	DMD_ E28A_5	X4	A		
35	276	DMD_ E29A_3			AT	Y3
	277	DMD_ E29A_5	X1	ATTACGC		
	278	DMD_ E30A_3			A	Y4
40	279	DMD_ E30A_5	X2			
	280	DMD_ E31A_3			ATTAC	Y3
45	281	DMD_ E31A_5	X4	ATTACGCGATTACGC		
	282	DMD_ E32A_3			ATTACGCGAT	Y4
50	283	DMD_ E32A_5	X3	ATT		
	284	DMD_ E33A_3			ATTACG	Y4
	285	DMD_ E33A_5	X3			
55	286	DMD_ E34A_3			AT	Y4

EP 2 893 034 B9

(continued)

	SEQ ID NO.	Probe NAME	Sequenc e X	Stuffer L1	Stuffer L2	Sequence Y
5	287	DMD_ E34A_5	X1	ATTACGCGATT		
	288	DMD_ E35A_3			ATTA	Y4
10	289	DMD_ E35A_5	X3			
	290	DMD_ E36A_3			ATTA	Y3
	291	DMD_ E36A_5	X4	ATTACG		
15	292	DMD_ E37A_3			ATTACGC	Y4
	293	DMD_ E37A_5	X2	ATTACGC		
20	294	DMD_ E38A_3			ATTA	Y4
	295	DMD_ E38A_5	X3	AT		
25	296	DMD_ E39A_3			ATTACG	Y4
	297	DMD_ E39A_5	X3	ATTACGC		
	298	DMD_ E40A_3			ATT	Y3
30	299	DMD_ E40A_5	X1	ATTACGCG		
	300	DMD_ E41A_3			ATTA	Y3
35	301	DMD_ E41A_5	X1			
	302	DMD_ E42A_3			ATTACG	Y3
40	303	DMD_ E42A_5	X1	A		
	304	DMD_ E43A_3			ATT	Y3
	305	DMD_ E43A_5	X4			
45	306	DMD_ E43B_3				Y3
	307	DMD_ E43B_5	X3			
50	308	DMD_ E43C_3			ATTA	Y4
	309	DMD_ E43C_5	X2	ATTACGCGA		
	310	DMD_ E44A_3			ATTA	Y1
55	311	DMD_ E44A_5	X2			

EP 2 893 034 B9

(continued)

	SEQ ID NO.	Probe NAME	Sequenc e X	Stuffer L1	Stuffer L2	Sequence Y
5	312	DMD_ E44B_3			ATTACGCG	Y2
	313	DMD_ E44B_5	X3	ATT		
10	314	DMD_ E44C_3			AT	Y2
	315	DMD_ E44C_5	X3	ATTA		
	316	DMD_ E45A_3			ATT	Y1
15	317	DMD_ E45A_5	X4	A		
	318	DMD_ E45B_3			ATTACGCGA	Y1
20	319	DMD_ E45B_5	X1	A		
	320	DMD_ E45C_3			ATTACGCG	Y1
25	321	DMD_ E45C_5	X1	ATTACG		
	322	DMD_ E46A_3			ATTACGCGA	Y4
	323	DMD_ E46A_5	X3	ATTACG		
30	324	DMD_ E46B_3			ATTACG	Y4
	325	DMD_ E46B_5	X1			
35	326	DMD_ E46C_3			ATT	Y4
	327	DMD_ E46C_5	X2	ATT		
40	328	DMD_ E47A_3			A	Y1
	329	DMD_ E47A_5	X4			
	330	DMD_ E47B_3			ATTACGCGATTA	Y2
45	331	DMD_ E47B_5	X1	A		
	332	DMD_ E48A_3			ATTACG	Y3
50	333	DMD_ E48A_5	X3	AT		
	334	DMD_ E48B_3				Y3
	335	DMD_ E48B_5	X2			
55	336	DMD_ E48C_3			ATTACGCGAT	Y3

EP 2 893 034 B9

(continued)

	SEQ ID NO.	Probe NAME	Sequenc e X	Stuffer L1	Stuffer L2	Sequence Y
5	337	DMD_ E48C_5	X3	ATTACGC		
	338	DMD_ E49A_3			ATTACGCGAT	Y1
10	339	DMD_ E49A_5	X1			
	340	DMD_ E49B_3			ATTACGCG	Y1
	341	DMD_ E49B_5	X2			
15	342	DMD_ E50A_3			ATTA	Y4
	343	DMD_ E50A_5	X4			
20	344	DMD_ E50B_3				Y4
	345	DMD_ E50B_5	X4			
25	346	DMD_ E50C_3			ATTACG	Y4
	347	DMD_ E50C_5	X4	ATTAC		
	348	DMD_ E51A_3			ATTACGCG	Y1
30	349	DMD_ E51A_5	X3	ATTACGCGA		
	350	DMD_ E51B_3			ATT	Y1
35	351	DMD_ E51B_5	X3			
	352	DMD_ E51C_3			ATTACGCGAT	Y2
	353	DMD_ E51C_5	X1			
40	354	DMD_ E52A_3			ATTA	Y2
	355	DMD_ E52A_5	X2			
45	356	DMD_ E52B_3			ATTAC	Y1
	357	DMD_ E52B_5	X1			
50	358	DMD_ E52C_3			ATTACGCGATTAC	Y2
	359	DMD_ E52C_5	X2			
	360	DMD_ E53A_3			ATTAC	Y3
55	361	DMD_ E53A_5	X1	ATTACG		

EP 2 893 034 B9

(continued)

	SEQ ID NO.	Probe NAME	Sequence X	Stuffer L1	Stuffer L2	Sequence Y
5	362	DMD_ E53B_3			A	Y3
	363	DMD_ E53B_5	X1			
10	364	DMD_ E53C_3				Y3
	365	DMD_ E53C_5	X2			
15	366	DMD_ E54A_3			ATTACGCG	Y2
	367	DMD_ E54A_5	X2			
	368	DMD_ E54B_3			A	Y2
20	369	DMD_ E54B_5	X4			
	370	DMD_ E55A_3			ATTACGCG	Y1
25	371	DMD_ E55A_5	X3			
	372	DMD_ E55B_3			ATTA	Y1
	373	DMD_ E55B_5	X3			
30	374	DMD_ E56A_3			ATTACGCGA	Y2
	375	DMD_ E56A_5	X2			
35	376	DMD_ E56B_3				Y2
	377	DMD_ E56B_5	X4	ATTA		
40	378	DMD_ E57A_3			ATT	Y2
	379	DMD_ E57A_5	X3			
	380	DMD_ E57B_3			ATTACGCG	Y2
45	381	DMD_ E57B_5	X3			
	382	DMD_ E58A_3			ATTACGC	Y3
50	383	DMD_ E58A_5	X2	ATT		
	384	DMD_ E59A_3			ATTA	Y3
	385	DMD_ E59A_5	X2			
55	386	DMD_ E60A_3			ATT	Y1

EP 2 893 034 B9

(continued)

	SEQ ID NO.	Probe NAME	Sequence X	Stuffer L1	Stuffer L2	Sequence Y
5	387	DMD_ E60A_5	X4			
	388	DMD_ E61A_3			ATT	Y1
10	389	DMD_ E61A_5	X3			
	390	DMD_ E61B_3			AT	Y1
15	391	DMD_ E61B_5	X4	ATTACGCGA		
	392	DMD_ E62A_3			ATTA	Y1
	393	DMD_ E62A_5	X2			
20	394	DMD_ E63A_3			ATTACGC	Y4
	395	DMD_ E63A_5	X2			
25	396	DMD_ E64A_3			ATTACGCG	Y2
	397	DMD_ E64A_5	X3			
	398	DMD_ E65A_3			ATTA	Y2
30	399	DMD_ E65A_5	X2			
	400	DMD_ E66A_3			ATTACGCGA	Y1
35	401	DMD_ E66A_5	X1			
	402	DMD_ E67A_3			ATTAC	Y1
40	403	DMD_ E67A_5	X1			
	404	DMD_ E68A_3			ATTACGCG	Y4
	405	DMD_ E68A_5	X3	ATT		
45	406	DMD_ E69A_3			AT	Y3
	407	DMD_ E69A_5	X1			
50	408	DMD_ E70A_3			ATTAC	Y3
	409	DMD_ E70A_5	X3	AT		
	410	DMD_ E71A_3			ATTAC	Y4
55	411	DMD_ E71A_5	X1			

EP 2 893 034 B9

(continued)

	SEQ ID NO.	Probe NAME	Sequence X		Stuffer L1	Stuffer L2	Sequence Y
5	412	DMD_ E72A_3				ATTAC	Y3
	413	DMD_ E72A_5	X2	AT			
10	414	DMD_ E73A_3				ATTA	Y3
	415	DMD_ E73A_5	X2	ATT			
	416	DMD_ E74A_3				ATTAC	Y3
15	417	DMD_ E74A_5	X4	ATT			
	418	DMD_ E75A_3					Y3
20	419	DMD_ E75A_5	X4				
	420	DMD_ E76A_3				AT	Y4
25	421	DMD_ E76A_5	X1				
	422	DMD_ E77A_3				ATTACGC	Y4
	423	DMD_ E77A_5	X1	ATTACGCGA			
30	424	DMD_ E78A_3					Y4
	425	DMD_ E78A_5	X3				
35	426	DMD_ E79A_3				ATTACGCGA	Y4
	427	DMD_ E79A_5	X1	ATTAC			
	428	REF10p_ A_3				ATTACGCG	Y2
40	429	REF10p_ A_5	X1				
	430	REF10p_ B_3				ATTAC	Y4
45	431	REF10p_ B_5	X4				
	432	REF10p_ C_3				ATTACGCGATT	Y3
50	433	REF10p_ C_5	X4				
	434	REF10q_ A_3					Y3
	435	REF10q_ A_5	X2				
55	436	REF10q_ B_3				ATTACGCGATTA	Y1

EP 2 893 034 B9

(continued)

	SEQ ID NO.	Probe NAME	Sequenc e X	Stuffer L1	Stuffer L2	Sequence Y
5	437	REF10q_ B_5	X3	ATTACGCGATTACGCG		
	438	REF11p_ A_3			ATTACGC	Y1
10	439	REF11p_ A_5	X4			
	440	REF11p_ B_3			ATTACGCGATTAC	Y3
	441	REF11p_ B_5	X2	AT		
15	442	REF11q_ A_3			ATTACGCGATTAC	Y2
	443	REF11q_ A_5	X4	ATTACGCGATTA		
20	444	REF11q_ B_3			ATTACG	Y3
	445	REF11q_ B_5	X4			
25	446	REF12p_ A_3			ATTACGCGATTACG	Y2
	447	REF12p_ A_5	X4	ATT		
	448	REF12q_ A_3			ATTACGC	Y1
30	449	REF12q_ A_5	X3			
	450	REF12q_ B_3			ATTACGCGA	Y4
35	451	REF12q_ B_5	X1			
	452	REF14q_ A_3				Y2
	453	REF14q_ A_5	X2			
40	454	REF16p_ A_3			ATTACGCGATTAC	Y2
	455	REF16p_ A_5	X2	ATTACG		
45	456	REF16p_ B_3			ATTACGCGATTACG	Y4
	457	REF16p_ B_5	X2	ATTACG		
50	458	REF16q_ A_3			ATTACGCGAT	Y3
	459	REF16q_ A_5	X1	ATTAC		
	460	REF16q_ B_3				Y2
55	461	REF16q_ B_5	X3			

EP 2 893 034 B9

(continued)

	SEQ ID NO.	Probe NAME	Sequence X	Stuffer L1	Stuffer L2	Sequence Y
5	462	REF17q_A_3			ATTACGCGATTA	Y2
	463	REF17q_A_5	X1	ATTACGCGATTACG		
10	464	REF18p_A_3			ATTACGCGATTA	Y1
	465	REF18p_A_5	X1	AT		
	466	REF18p_B_3			ATTACGC	Y3
15	467	REF18p_B_5	X2			
	468	REF19q_A_3			ATTACGCGATTAC	Y2
20	469	REF19q_A_5	X3	ATTACGC		
	470	REF19q_B_3				Y4
25	471	REF19q_B_5	X1			
	472	REF19q_C_3				Y3
	473	REF19q_C_5	X4			
30	474	REF20p_A_3			ATTACGCGATT	Y1
	475	REF20p_A_5	X2	ATTA		
35	476	REF20p_B_3			ATTACGCGA	Y4
	477	REF20p_B_5	X2			
	478	REF20q_A_3			ATTACGCGATTA	Y2
40	479	REF20q_A_5	X1	ATTACGC		
	480	REF20q_B_3			ATTACGCGATTACGCGA	Y4
45	481	REF20q_B_5	X4			
	482	REF20q_C_3			ATT	Y3
50	483	REF20q_C_5	X3			
	484	REF2p_A_3			ATTACGCGAT	Y1
	485	REF2p_A_5	X3	ATTACGCGA		
55	486	REF2p_B_3				Y4

EP 2 893 034 B9

(continued)

	SEQ ID NO.	Probe NAME	Sequence X	Stuffer L1	Stuffer L2	Sequence Y
5	487	REF2p_B_5	X2			
	488	REF2q_A_3			ATTACGCGATTACG	Y4
10	489	REF2q_A_5	X3	ATTACG		
	490	REF2q_B_3			ATTACGCGATTA	Y2
	491	REF2q_B_5	X2	ATTACGCGAT		
15	492	REF3p_A_3			ATTACG	Y3
	493	REF3p_A_5	X1			
20	494	REF3p_B_3			ATTACGCGATTAC	Y4
	495	REF3p_B_5	X4	ATTACGCGATTAC		
25	496	REF3p_C_3				Y2
	497	REF3p_C_5	X4			
	498	REF3p_D_3			ATTACGCGA	Y1
30	499	REF3p_D_5	X1			
	500	REF3q_A_3			ATTACGCGATTACG	Y1
35	501	REF3q_A_5	X4	ATTACGCGATTAC		
	502	REF3q_B_3			ATTACGCGA	Y4
40	503	REF3q_B_5	X3	A		
	504	REF3q_C_3			A	Y2
	505	REF3q_C_5	X3			
45	506	REF4q_A_3				Y4
	507	REF4q_A_5	X3			
50	508	REF4q_B_3			ATTACGC	Y1
	509	REF4q_B_5	X2			
	510	REF5p_A_3			A	Y1
55	511	REF5p_A_5	X2			

EP 2 893 034 B9

(continued)

	SEQ ID NO.	Probe NAME	Sequence X	Stuffer L1	Stuffer L2	Sequence Y
5	512	REF5q_A_3			ATTACGCGATTACGCG	Y1
	513	REF5q_A_5	X1	ATTACGCGATTACG		
10	514	REF6p_A_3				Y1
	515	REF6p_A_5	X3			
	516	REF6p_B_3				Y3
15	517	REF6p_B_5	X1			
	518	REF6q_A_3			ATTACGCGATTACG	Y1
20	519	REF6q_A_5	X2	ATTACGCGATT		
	520	REF6q_B_3			ATTACGCGATTACGCG	Y4
25	521	REF6q_B_5	X1	ATTAC		
	522	REF7p_A_3			ATTACGC	Y2
	523	REF7p_A_5	X4			
30	524	REF7p_B_3			ATTACGCGATT	Y3
	525	REF7p_B_5	X3	ATTAC		
35	526	REF8p_A_3			ATTACGCGA	Y1
	527	REF8p_A_5	X4	ATTACG		
	528	REF8p_B_3			ATTAC	Y2
40	529	REF8p_B_5	X1			
	530	REF8q_A_3			A	Y3
45	531	REF8q_A_5	X3			
	532	REF8q_B_3			A	Y1
50	533	REF8q_B_5	X1	ATTACGC		
	534	REF9p_A_3			ATTACGCGATTAC	Y2
	535	REF9p_A_5	X3	ATTACGCGATT		
55	536	REF9p_B_3				Y1

(continued)

SEQ ID NO.	Probe NAME	Sequence X	Stuffer L1	Stuffer L2	Sequence Y
537	REF9p_B_5	X4			
538	REF9q_A_3				Y4
539	REF9q_A_5	X4			
540	REF9q_B_3			ATTACGCGA	Y2
541	REF9q_B_5	X2			

[0178] To perform the 192 multiplex CNV detection assay, a ligation product was first generated. Briefly, genomic DNA was extracted from 2ml whole blood samples using the classic phenol:chloroform method. The blood samples were collected from a male DMD patient and a healthy male volunteer at Shanghai Ruijin Hospital, Shanghai, China. The genomic DNA from the male DMD patient was the test sample. The genomic DNA from the healthy volunteer was the control sample. From the extracted genomic DNA, 100-200 microgram (μg) DNA was dissolved in 10 microliter (μl) 1xTE buffer (10mM Tris.Cl, pH8.0, 1mM EDTA from Sigma-Aldrich). The dissolved genomic DNA was denatured at 98 °C for 5 minutes and then immediately cooled down on ice. At the same time, a 2x ligation premix solution was prepared according to the following formula: a 10 μl 2x ligation premix was made of 2 μl 10xTaq ligase buffer, 1 μl 40U/ μl Taq Ligase from NewEngland Biolabs, Inc., 1 μl ProbeMix (the 384 probes with a final concentration of 0.005 micromolar for each probe in 1xTE), and 6 μl ddH₂O (Distilled Milli-Q water from Milli-Q Advantage A10, Millipore). 10 μl 2xligation premix was mixed with the denatured 10 μl genomic DNA and the mixture was allowed to undergo 4 cycles of denaturation, hybridization, and ligation under the following conditions: 95 °C for 30 seconds, and then 58 °C for 4 hours. The ligation product thus obtained could be stored on ice for same day use or freezed in -20 °C for future use.

[0179] With the ligation product, an amplification step was then performed to obtain an amplification product. Briefly, two PCR reactions were performed using the same amplification product as template DNA. One PCR reaction had DMD_F1, DMD_F2, DMD_F3, DMD_F4, DMD_R1 and DMD_R2 as primers. The other PCR reaction had DMD_F1, DMD_F2, DMD_F3, DMD_F4, DMD_R3 and DMD_R4 as primers. The PCR reaction mixture was prepared as follows: a 20 μl reaction system was made by mixing 2 μl 10x PCR buffer (Qiagen, Germany), 2 μl 2.5mM dNTP mix (2.5mM each of dATP, dTTP, dCTP and dGTP from Takara Bio Inc.), 2 μl primer mix (DMD_F1, DMD_F2, DMD_F3, DMD_F4, DMD_R1 and DMD_R2 at final concentrations of 1 μM , 1 μM , 1 μM , 1 μM , 2 μM and 2 μM , respectively; or DMD_F1, DMD_F2, DMD_F3, DMD_F4, DMD_R3 and DMD_R4 at final concentrations of 1 μM , 1 μM , 1 μM , 1 μM , 2 μM and 2 μM , respectively), 1 μl Ligation product, 0.2 μl 5U/ μl HotStarTaq Plus Taq DNA polymerase (Qiagen, Germany), and 12.8 μl ddH₂O. The PCR mixture was allowed to undergo a polymerase chain reaction under the following conditions: 95 °C for 2 minutes; followed by 35 cycles of 94 °C for 20 second, 57 °C for 40 second, and 72 °C for 1.5 minutes; and after the 35th cycle, the reaction mixture was kept in 60 °C for 1 hour. To analyze the amplification product, 1 μl of the amplification product was first diluted with ddH₂O 10 times into 10 μl . Then 1 μl was taken out of the 10 μl diluted amplification product and mixed with 0.1 μl GeneScan™ 500 LIZ® size standard (Life Technologies, Inc.) and 8.9 μl Hi-Di formamide (Life Technologies, Inc.). The mixture was detoured at 95 °C for 5 minutes and run through capillary electrophoresis by ABI3130XL according to manufacturer's manual. The chromatograms from the two PCR reactions were designated as Panel A and Panel B, respectively. The capillary electrophoresis data was processed using Genemapper 4.0 to obtain peak intensity values for each amplification product.

[0180] As shown in **Figures 11A-H**, the amplification products obtained in the assay could be separated by capillary electrophoresis and the peaks could be individually identified on the chromatograms. **Figures 11A** and **11B** showed the chromatograms of all amplification products for the control sample panel A and panel B, respectively. Each panel represented the analysis of amplification products from one PCR reaction. **Figures 11C** and **11D** showed the chromatograms of all amplification products for the patient sample panel A and panel B, respectively. Each peak represented one amplification product corresponding to an individual target site.

[0181] **Figures 11E** and **11F** showed the chromatograms for amplification products labeled with blue fluorescent dyes for the control sample and the patient sample, respectively. **Figures 11G** and **11H** showed the chromatograms for amplification products labeled with green fluorescent dyes for the control sample and the patient sample, respectively. As seen in **Figures 11E-11H**, the peaks from the amplification products labeled with the same fluorescent dye could be identified on the basis of different fragment sizes. By comparing the chromatograms from the control and the patient

samples, the presence or absence of peaks of similar fragment sizes may be determined. For example, when comparing the chromatograms in **Figures 11E** and **11F**, the peaks for the target sites E22A, E41A, E40A, E29A, E21A and E34A were missing in the chromatogram from the patient sample, indicating the deletion of the sequences in the DMD gene corresponding to these target sites. For another example, when comparing the chromatograms in **Figures 11G** and **11H**, the peaks for the target sites E18A, and E20A are missing in the chromatogram from the patient sample, indicating the deletion of the sequences in the DMD gene corresponding to these target sites.

[0182] Alternatively, in another method to analyze the electrophoresis data, a ratio for the test sample (R_{test}) was obtained for each DMD, chromosome X, and chromosome Y target sites (herein also referred to as gene target sites) by dividing the peak intensity value of the gene target site with the peak intensity value of each of the reference target sites in the same group. The same PCR primer pair was used for each gene target site and the reference target site in the same group. For example, As shown in **Table 4**, in one group which shared the same primer pair DMD_F1/DMD_R1, there were amplification products for 12 probe loci (herein also referred to as probe target sites) including 8 gene target loci: DMD_E04A, DMD_E14B, DMD_E45B, DMD_E45C, DMD_E49A, DMD_E52B, DMD_E66A, and DMD_E67A; and 4 reference target loci: REF18p_A, REF3p_D, REF5q_A, and REF8q_B. To obtain the ratio for the locus DMD_E04A in the test sample (R_{test}), the peak intensity for the locus DMD_E04A was divided by the peak intensity for each of the reference target sites REF18p_A, REF3p_D, REF5q_A, and REF8q_B so that four ratios (R_{test}) were derived.

[0183] Similarly, a ratio (R_{control}) in the control sample was obtained for each gene target site. Assuming the copy number of the target nucleic acid in the control sample (C_{control}) is 1, the copy number of each gene target site (C_{test}) was then calculated according to the formula: $C_{\text{test}} = C_{\text{control}} \times R_{\text{test}} / R_{\text{control}}$.

[0184] The copy number for a gene target site in the test sample equals to the ratio between the R_{test} and the R_{control} , assuming the copy number of the target nucleic acid in the control sample is 1 (e.g., there is one X chromosome in a male patient). Because four reference target sites were introduced for each gene target site, four R_{test} and therefore four C_{test} were obtained for each gene target site. The median value the four C_{test} was deemed as the copy number for that gene target site in the test sample. The copy numbers thus calculated for each gene target site are shown in **Table 4**. Copy numbers for gene target sites corresponding to exons 18-41 were zero, indicating that these exons were deleted in the DMD patient sample.

Table 4. Copy number calculation results based on peak intensity for each gene target site. LP SIZE refers to Ligation Product Size; REF refers to Reference; CE refers to Capillary Electrophoresis; Non-PAR refers to non-pseudoautosomal region.

PANEL	PROBE LOCUS	TARGET REGION	PCR PRIMERS	LP SIZE	CE REF SIZE	COPY NUMBER MEASUREMENT
A	DMD_E04A	DMD EXON04	DMD_F1/DMD_R1	101	97.95	1.02
A	DMD_E14B	DMD EXON14	DMD_F1/DMD_R1	125	122.35	1.02
A	DMD_E45B	DMD EXON45	DMD_F1/DMD_R1	116	113.84	1.08
A	DMD_E45C	DMD EXON45	DMD_F1/DMD_R1	122	120.03	1.07
A	DMD_E49A	DMD EXON49	DMD_F1/DMD_R1	113	110.3	1.04
A	DMD_E52B	DMD EXON52	DMD_F1/DMD_R1	104	102.87	1.03
A	DMD_E66A	DMD EXON66	DMD_F1/DMD_R1	110	108.1	1
A	DMD_E67A	DMD EXON67	DMD_F1/DMD_R1	98	94.86	1.07
A	REF18p_A	REFERENCE	DMD_F1/DMD_R1	119	117.75	/
A	REF3p_D	REFERENCE	DMD_F1/DMD_R1	107	104.93	/
A	REF5q_A	REFERENCE	DMD_F1/DMD_R1	128	124.92	/
A	REF8q_B	REFERENCE	DMD_F1/DMD_R1	102	100.21	/

EP 2 893 034 B9

(continued)

	PANEL	PROBE LOCUS	TARGET REGION	PCR PRIMERS	LP SIZE	CE REF SIZE	COPY NUMBER MEASUREMENT
5	A	DMD_E05B	DMD EXON05	DMD_ F1/DMD_R2	164	163.68	0.98
	A	DMD_E06B	DMD EXON06	DMD_ F1/DMD_R2	148	147.96	0.99
10	A	DMD_E07A	DMD EXON07	DMD_ F1/DMD_R2	139	137.04	1.04
	A	DMD_E09A	DMD EXON09	DMD_ F1/DMD_R2	151	150.45	0.97
	A	DMD_E11A	DMD EXON11	DMD_ F1/DMD_R2	136	134.22	1.06
15	A	DMD_E19A	DMD EXON19	DMD_ F1/DMD_R2	142	140.22	0
	A	DMD_E47B	DMD EXON47	DMD_ F1/DMD_R2	159	160.53	1.08
20	A	DMD E51C	DMD EXON51	DMD_ F1/DMD_R2	154	154.36	0.96
	A	REF10p_A	REFERENCE	DMD_ F1/DMD_R2	133	130.34	/
25	A	REF17q_A	REFERENCE	DMD_ F1/DMD_R2	166	166.35	/
	A	REF20q_A	REFERENCE	DMD_ F1/DMD_R2	157	157.72	/
	A	REF8p_B	REFERENCE	DMD_ F1/DMD_R2	145	144.59	/
30	B	DMD_E22A	DMD EXON22	DMD_ F1/DMD_R3	104	102.07	0
	B	DMD_E29A	DMD EXON29	DMD_ F1/DMD_R3	125	122.73	0
35	B	DMD_E40A	DMD EXON40	DMD_ F1/DMD_R3	122	119.3	0
	B	DMD_E41A	DMD EXON41	DMD_ F1/DMD_R3	110	108.32	0
	B	DMD_E42A	DMD EXON42	DMD_ F1/DMD_R3	116	113.83	1.12
40	B	DMD_E53A	DMD EXON53	DMD_ F1/DMD_R3	128	124.34	1.1
	B	DMD_E53B	DMD EXON53	DMD_ F1/DMD_R3	101	99.46	1.01
45	B	DMD_E69A	DMD EXON69	DMD_ F1/DMD_R3	98	96.33	1.05
	B	ChrX_B	ChrX Non- PAR	DMD_ F1/DMD_R3	113	110.95	0.96
50	B	REF16q_A	REFERENCE	DMD_ F1/DMD_R3	119	116.64	/
	B	REF3p_A	REFERENCE	DMD_ F1/DMD_R3	107	105.37	/
	B	REF6p_B	REFERENCE	DMD_ F1/DMD_R3	95	92.77	/
55	B	DMD_E16C	DMD EXON16	DMD_ F1/DMD_R4	162	163.37	1.01

EP 2 893 034 B9

(continued)

	PANEL	PROBE LOCUS	TARGET REGION	PCR PRIMERS	LP SIZE	CE REF SIZE	COPY NUMBER MEASUREMENT
5	B	DMD_E17B	DMD EXON17	DMD_ F1/DMD_R4	139	138.03	0.99
	B	DMD_E21A	DMD EXON21	DMD_ F1/DMD_R4	141	140.14	0
10	B	DMD_E34A	DMD EXON34	DMD_ F1/DMD_R4	160	160.74	0
	B	DMD_E46B	DMD EXON46	DMD_ F1/DMD_R4	150	150.99	0.98
15	B	DMD_E71A	DMD EXON71	DMD_ F1/DMD_R4	148	148.7	0.99
	B	DMD_E76A	DMD EXON76	DMD_ F1/DMD_R4	136	134.94	0.98
	B	DMD_E77A	DMD EXON77	DMD_ F1/DMD_R4	166	165.44	0.94
20	B	DMD_E79A	DMD EXON79	DMD_ F1/DMD_R4	159	157.44	0.99
	B	REF12q_B	REFERENCE	DMD_ F1/DMD_R4	145	142.57	/
25	B	REF19q_B	REFERENCE	DMD_ F1/DMD_R4	133	131.12	/
	B	REF6q_B	REFERENCE	DMD_ F1/DMD_R4	157	154.42	/
	A	DMD_E08A	DMD EXON08	DMD_ F2/DMD_R1	101	99.78	0.97
30	A	DMD_E10B	DMD EXON10	DMD_ F2/DMD_R1	116	114.26	0.97
	A	DMD_E13B	DMD EXON13	DMD_ F2/DMD_R1	113	111.65	0.96
35	A	DMD_E15B	DMD EXON15	DMD_ F2/DMD_R1	125	123.2	0.91
	A	DMD_E18A	DMD EXON18	DMD_ F2/DMD_R1	122	120.8	0
40	A	DMD_E44A	DMD EXON44	DMD_ F2/DMD_R1	104	103.18	1.02
	A	DMD_E49B	DMD EXON49	DMD_ F2/DMD_R1	110	108.73	1.04
	A	DMD_E62A	DMD EXON62	DMD_ F2/DMD_R1	98	96.8	0.96
45	A	REF20p_A	REFERENCE	DMD_ F2/DMD_R1	119	118.73	/
	A	REF4q_B	REFERENCE	DMD_ F2/DMD_R1	107	105.96	/
50	A	REF5p_A	REFERENCE	DMD_ F2/DMD_R1	95	92.81	/
	A	REF6q_A	REFERENCE	DMD_ F2/DMD_R1	128	126.84	/
	A	DMD_E08B	DMD EXON08	DMD_ F2/DMD_R2	163	164.24	0.95
55	A	DMD_E11B	DMD EXON11	DMD_ F2/DMD_R2	154	155.4	0.97

EP 2 893 034 B9

(continued)

	PANEL	PROBE LOCUS	TARGET REGION	PCR PRIMERS	LP SIZE	CE REF SIZE	COPY NUMBER MEASUREMENT
5	A	DMD_E20A	DMD EXON20	DMD_ F2/DMD_R2	139	138.23	0
	A	DMD_E52A	DMD EXON52	DMD_ F2/DMD_R2	142	141.19	0.94
10	A	DMD_E52C	DMD EXON52	DMD_ F2/DMD_R2	160	161.38	0.93
	A	DMD_E54A	DMD EXON54	DMD_ F2/DMD_R2	148	147.35	0.97
	A	DMD_E56A	DMD EXON56	DMD_ F2/DMD_R2	151	151.48	0.99
15	A	DMD_E65A	DMD EXON65	DMD_ F2/DMD_R2	136	134.87	0.94
	A	REF14q_A	REFERENCE	DMD_ F2/DMD_R2	133	131.43	/
20	A	REF16p_A	REFERENCE	DMD_ F2/DMD_R2	157	158.24	/
	A	REF2q_B	REFERENCE	DMD_ F2/DMD_R2	166	167.39	/
25	A	REF9q_B	REFERENCE	DMD_ F2/DMD_R2	145	145.43	/
	B	DMD_E17A	DMD EXON17	DMD_ F2/DMD_R3	99	96.61	1.03
	B	DMD_E17C	DMD EXON17	DMD_ F2/DMD_R3	113	111.55	1.03
30	B	DMD_E21C	DMD EXON21	DMD_ F2/DMD_R3	125	123.15	0
	B	DMD_E48B	DMD EXON48	DMD_ F2/DMD_R3	104	102.95	0.97
35	B	DMD_E53C	DMD EXON53	DMD_ F2/DMD_R3	101	99.67	1.03
	B	DMD_E58A	DMD EXON58	DMD_ F2/DMD_R3	122	120.59	1
	B	DMD_E59A	DMD EXON59	DMD_ F2/DMD_R3	110	108.73	0.96
40	B	DMD_E72A	DMD EXON72	DMD_ F2/DMD_R3	116	115.09	1.02
	B	DMD_E73A	DMD EXON73	DMD_ F2/DMD_R3	128	126.65	0.98
45	B	REF10q_A	REFERENCE	DMD_ F2/DMD_R3	93	92.06	/
	B	REF11p_B	REFERENCE	DMD_ F2/DMD_R3	119	118.29	/
50	B	REF18p_B	REFERENCE	DMD_ F2/DMD_R3	107	106.2	/
	B	DMD_E16A	DMD EXON16	DMD_ F2/DMD_R4	154	155.18	0.96
	B	DMD_E16B	DMD EXON16	DMD_ F2/DMD_R4	148	149.05	1.18
55	B	DMD_E25A	DMD EXON25	DMD_ F2/DMD_R4	139	138.79	0

EP 2 893 034 B9

(continued)

	PANEL	PROBE LOCUS	TARGET REGION	PCR PRIMERS	LP SIZE	CE REF SIZE	COPY NUMBER MEASUREMENT
5	B	DMD_E30A	DMD EXON30	DMD_ F2/DMD_R4	136	134.72	0
	B	DMD_E37A	DMD EXON37	DMD_ F2/DMD_R4	163	163.48	0
10	B	DMD_E43C	DMD EXON43	DMD_ F2/DMD_R4	160	160.74	0.87
	B	DMD_E46C	DMD EXON46	DMD_ F2/DMD_R4	166	166.08	1.18
	B	DMD_E63A	DMD EXON63	DMD_ F2/DMD_R4	151	151.38	1.06
15	B	ChrX_A	ChrX Non- PAR	DMD_ F2/DMD_R4	148	146.02	0.9
	B	REF16p_B	REFERENCE	DMD_ F2/DMD_R4	157	157.23	/
20	B	REF20p_B	REFERENCE	DMD_ F2/DMD_R4	145	142.32	/
	B	REF2p_B	REFERENCE	DMD_ F2/DMD_R4	133	131.1	/
25	A	DMD_E05A	DMD EXON05	DMD_ F3/DMD_R1	116	113.74	0.99
	A	DMD_E07B	DMD EXON07	DMD_ F3/DMD_R1	122	119.71	0.99
	A	DMD_E10A	DMD EXON10	DMD_ F3/DMD_R1	113	110.62	1.03
30	A	DMD_E51A	DMD EXON51	DMD_ F3/DMD_R1	125	122.06	0.96
	A	DMD_E51B	DMD EXON51	DMD_ F3/DMD_R1	101	97.89	0.95
35	A	DMD_E55A	DMD EXON55	DMD_ F3/DMD_R1	110	107.27	0.99
	A	DMD_E55B	DMD EXON55	DMD_ F3/DMD_R1	104	101.74	0.93
	A	DMD_E61A	DMD EXON61	DMD_ F3/DMD_R1	98	94.88	1.1
40	A	REF10q_B	REFERENCE	DMD_ F3/DMD_R1	128	125.01	/
	A	REF12q_A	REFERENCE	DMD_ F3/DMD_R1	107	104.7	/
45	A	REF2p_A	REFERENCE	DMD_ F3/DMD_R1	119	115.98	/
	A	REF6p_A	REFERENCE	DMD_ F3/DMD_R1	95	92.7	/
50	A	DMD_E06A	DMD EXON06	DMD_ F3/DMD_R2	136	134.22	1
	A	DMD_E09B	DMD EXON09	DMD_ F3/DMD_R2	142	140.95	0.94
	A	DMD_E13A	DMD EXON13	DMD_ F3/DMD_R2	154	153.36	1.02
55	A	DMD_E44B	DMD EXON44	DMD_ F3/DMD_R2	160	160.42	0.88

EP 2 893 034 B9

(continued)

	PANEL	PROBE LOCUS	TARGET REGION	PCR PRIMERS	LP SIZE	CE REF SIZE	COPY NUMBER MEASUREMENT
5	A	DMD_E44C	DMD EXON44	DMD_ F3/DMD_R2	163	163.71	0.95
	A	DMD_E57A	DMD EXON57	DMD_ F3/DMD_R2	139	137.9	0.96
10	A	DMD_E57B	DMD EXON57	DMD_ F3/DMD_R2	148	147.35	0.93
	A	DMD_E64A	DMD EXON64	DMD_ F3/DMD_R2	151	150.11	0.97
15	A	REF16q_B	REFERENCE	DMD_ F3/DMD_R2	133	130.13	/
	A	REF19q_A	REFERENCE	DMD_ F3/DMD_R2	157	157.24	/
	A	REF3q_C	REFERENCE	DMD_ F3/DMD_R2	145	143.98	/
20	A	REF9p_A	REFERENCE	DMD_ F3/DMD_R2	166	166.34	/
	B	DMD_E26A	DMD EXON26	DMD_ F3/DMD_R3	110	107.5	0
25	B	DMD_E27A	DMD EXON27	DMD_ F3/DMD_R3	101	99.78	0
	B	DMD_E43B	DMD EXON43	DMD_ F3/DMD_R3	98	95.66	0.96
	B	DMD_E48A	DMD EXON48	DMD_ F3/DMD_R3	118	115.51	1
30	B	DMD_E48C	DMD EXON48	DMD_ F3/DMD_R3	128	125.33	1.2
	B	DMD_E70A	DMD EXON70	DMD_ F3/DMD_R3	115	112.29	1.01
35	B	ChrX_C	ChrX Non- PAR	DMD_ F3/DMD_R3	122	119.94	1.07
	B	ChrY_A	ChrY Non- PAR	DMD_ F3/DMD_R3	104	101.87	1.01
40	B	ChrY_B	ChrY Non- PAR	DMD_ F3/DMD_R3	130	126.33	1.06
	B	REF20q_C	REFERENCE	DMD_ F3/DMD_R3	107	104.85	/
	B	REF7p_B	REFERENCE	DMD_ F3/DMD_R3	119	118.09	/
45	B	REF8q_A	REFERENCE	DMD_ F3/DMD_R3	95	93.55	/
	B	DMD_E32A	DMD EXON32	DMD_ F3/DMD_R4	163	162.74	0
50	B	DMD_E33A	DMD EXON33	DMD_ F3/DMD_R4	142	140.58	0
	B	DMD_E35A	DMD EXON35	DMD_ F3/DMD_R4	148	147.63	0
	B	DMD_E38A	DMD EXON38	DMD_ F3/DMD_R4	151	150.99	0
55	B	DMD_E39A	DMD EXON39	DMD_ F3/DMD_R4	166	164.62	0

EP 2 893 034 B9

(continued)

	PANEL	PROBE LOCUS	TARGET REGION	PCR PRIMERS	LP SIZE	CE REF SIZE	COPY NUMBER MEASUREMENT
5	B	DMD_E46A	DMD EXON46	DMD_ F3/DMD_R4	163	160.1	0.96
	B	DMD_E68A	DMD EXON68	DMD_ F3/DMD_R4	157	154.02	0.95
10	B	DMD_E78A	DMD EXON78	DMD_ F3/DMD_R4	136	133.08	0.98
	B	ChrY_C	ChrY Non- PAR	DMD_ F3/DMD_R4	139	136.74	1.05
	B	REF2q_A	REFERENCE	DMD_ F3/DMD_R4	157	157.2	/
15	B	REF3q_B	REFERENCE	DMD_ F3/DMD_R4	146	143.15	/
	B	REF4q_A	REFERENCE	DMD_ F3/DMD_R4	133	131.02	/
20	A	DMD_E03A	DMD EXON03	DMD_ F4/DMD_R1	110	111.76	1.03
	A	DMD_E12A	DMD EXON12	DMD_ F4/DMD_R1	114	113.87	0.95
	A	DMD_E15A	DMD EXON15	DMD_ F4/DMD_R1	119	119.22	0.98
25	A	DMD_E20B	DMD EXON20	DMD_ F4/DMD_R1	107	109.13	0
	A	DMD_E45A	DMD EXON45	DMD_ F4/DMD_R1	96	98.31	0.95
30	A	DMD_E47A	DMD EXON47	DMD_ F4/DMD_R1	98	100	0.98
	A	DMD_E60A	DMD EXON60	DMD_ F4/DMD_R1	101	102.36	0.98
35	A	DMD_E61B	DMD EXON61	DMD_ F4/DMD_R1	122	122.48	0.96
	A	REF11p_A	REFERENCE	DMD_ F4/DMD_R1	104	105.44	/
	A	REF3q_A	REFERENCE	DMD_ F4/DMD_R1	125	126.72	/
40	A	REF8p_A	REFERENCE	DMD_ F4/DMD_R1	116	117.23	/
	A	REF9p_B	REFERENCE	DMD_ F4/DMD_R1	92	94.43	/
45	A	DMD_E03B	DMD EXON03	DMD_ F4/DMD_R2	130	132.06	0.97
	A	DMD_E04B	DMD EXON04	DMD_ F4/DMD_R2	148	151.02	1.07
50	A	DMD_E12B	DMD EXON12	DMD_ F4/DMD_R2	160	164.03	1
	A	DMD_E14A	DMD EXON14	DMD_ F4/DMD_R2	139	141.8	1.02
	A	DMD_E18B	DMD EXON18	DMD_ F4/DMD_R2	151	154.36	0
55	A	DMD_E19B	DMD EXON19	DMD_ F4/DMD_R2	157	160.11	0

EP 2 893 034 B9

(continued)

	PANEL	PROBE LOCUS	TARGET REGION	PCR PRIMERS	LP SIZE	CE REF SIZE	COPY NUMBER MEASUREMENT
5	A	DMD_E54B	DMD EXON54	DMD_ F4/DMD_R2	136	137.26	1.01
	A	DMD_E56B	DMD EXON56	DMD_ F4/DMD_R2	145	148.68	1.14
10	A	REF11q_A	REFERENCE	DMD_ F4/DMD_R2	163	167.72	/
	A	REF12p_A	REFERENCE	DMD_ F4/DMD_R2	154	158.37	/
	A	REF3p_C	REFERENCE	DMD_ F4/DMD_R2	133	134.98	/
15	A	REF7p_A	REFERENCE	DMD_ F4/DMD_R2	142	144.34	/
	B	DMD_E21B	DMD EXON21	DMD_ F4/DMD_R3	98	100.62	0
20	B	DMD_E23A	DMD EXON23	DMD_ F4/DMD_R3	95	97.78	0
	B	DMD_E24A	DMD EXON24	DMD_ F4/DMD_R3	107	107.99	0
	B	DMD_E28A	DMD EXON28	DMD_ F4/DMD_R3	113	114.88	0
25	B	DMD_E31A	DMD EXON31	DMD_ F4/DMD_R3	128	126.18	0
	B	DMD_E36A	DMD EXON36	DMD_ F4/DMD_R3	121	120.6	0
30	B	DMD_E43A	DMD EXON43	DMD_ F4/DMD_R3	110	110.95	1.05
	B	DMD_E74A	DMD EXON74	DMD_ F4/DMD_R3	122	124.12	1.01
35	B	DMD_E75A	DMD EXON75	DMD_ F4/DMD_R3	101	102.59	1
	B	REF10p_C	REFERENCE	DMD_ F4/DMD_R3	116	118.4	/
	B	REF11q_B	REFERENCE	DMD_ F4/DMD_R3	104	106.31	/
40	B	REF19q_C	REFERENCE	DMD_ F4/DMD_R3	92	94.78	/
	B	DMD_E01A	DMD EXON01	DMD_ F4/DMD_R4	160	163.69	0.9
45	B	DMD_E01B	DMD EXON01	DMD_ F4/DMD_R4	155	158.11	1.08
	B	DMD_E02A	DMD EXON02	DMD_ F4/DMD_R4	167	168.9	0.9
	B	DMD_E02B	DMD EXON02	DMD_ F4/DMD_R4	169	171.8	0.99
50	B	DMD_E02C	DMD EXON02	DMD_ F4/DMD_R4	145	147.83	1.02
	B	DMD_E50A	DMD EXON50	DMD_ F4/DMD_R4	148	152.18	0.97
55	B	DMD_E50B	DMD EXON50	DMD_ F4/DMD_R4	133	135.04	1.01

(continued)

PANEL	PROBE LOCUS	TARGET REGION	PCR PRIMERS	LP SIZE	CE REF SIZE	COPY NUMBER MEASUREMENT
B	DMD_E50C	DMD EXON50	DMD_ F4/DMD_R4	157	161.27	0.98
B	REF10p_B	REFERENCE	DMD_ F4/DMD_R4	142	144.28	/
B	REF20q_B	REFERENCE	DMD_ F4/DMD_R4	154	155.12	/
B	REF3p_B	REFERENCE	DMD_ F4/DMD_R4	163	166.76	/
B	REF9q_A	REFERENCE	DMD_ F4/DMD_R4	130	131.56	/

Example 3. Multiplex Point Mutation Screening

[0185] This example provides an exemplary method of multiplex point mutation screening according to the present invention. In this example, as in **Example 2**, there were 192 target sequences including 129 sites in the Duchenne muscular dystrophy (DMD) gene, and 63 reference sites on chromosomes X, Y, 2-12, 14, 16-20. Some mutations causing DMD are point mutations in the dystrophin gene. The multiplex point mutation screening in the DMD gene employed fluorescent dye labeled forward primers and reverse primers with stuffer sequences according to the scheme in **Figure 6**.

[0186] In this exemplary mutation screening method, the same probes and primers as those designed and used in **Example 2** were used for the hybridization, ligation, and amplification reactions except that the test genomic DNA was obtained from another male DMD patient. The amplification products were analyzed through capillary electrophoresis, the peak intensities were obtained, and the copy numbers for each target site were calculated as detailed in **Example 2**. For sake of brevity, the descriptions of the probes, primers, ligation reaction, amplification reaction, data procurement, and copy number calculation, are not repeated in this example.

[0187] The copy number thus calculated for all gene target sites are shown in **Figure 12A** and partially shown in **Figure 12B**. The copy number for E07B (exon 7B gene target site in the dystrophin gene) was zero, suggesting that the probes for this site could not match perfectly with the target site. On the other hand, the copy number for E07A was about 1, meaning that part of exon7 had a normal copy number and there was no whole exon 7 deletion in the test DNA sample. The lack of amplification product for the E07B gene target site could also be seen in the chromatograms (**Figure 12C**), which showed that the peak corresponding to E07B gene target site was missing in the DMD test panel (lower panel) when compared to the peak in the control panel (upper panel). The E07B gene target site was then sequenced to find that there was an adenosine ("A") insertion in the E07B probe region. See the adenosine position as indicated by the arrow in **Figure 12D**. Because of the adenosine insertion in exon 7 of the dystrophin gene, the DMD_07B_5 probe SEQ ID NO: 294 was unable to anneal to the target site, leading to the disappearance of the peak corresponding to the Exon07B target site.

[0188] Therefore, the multiplex mutation screening method can identify gene regions where mutations may exist. The screening may be carried out simultaneously with the detection for CNVs, as demonstrated in **Examples 2 and 3** where the same set of probes and primers were used and the same procedure was followed to obtain electrophoresis data.

Example 4. Detection Of Human Chromosome 21 Copy Number Changes

[0189] This example demonstrates a method of detecting human chromosome 21 copy number changes according to the present invention. In this example, a mere 2% increase of the copy number of human chromosome 21 in a sample could be detected. The method employed fluorescent dye labeled forward primers and reverse primers with stuffer sequences according to the scheme in **Figure 5**.

[0190] In the exemplary method, probes were designed to cover 192 target sites including 96 sites on human chromosome 21, and 96 reference sites on human chromosomes 2-12, 14, and 16-20. For each target site, a probe pair was designed including a 3' probe (the right probe) and a 5' probe (the left probe). The 3' and 5' probes were designed in a manner so that when both are hybridized to the target sequence under a suitable condition, there is no gap between the two probes. The probes were synthesized by Life Technologies Corporation. The names, sequence ID numbers, primer binding sequences, and stuffer sequences of the 384 probes (192 probe pairs) are shown in **Table 5**. The probe pairs for the target sites on Chromosome 21 were SEQ ID NOs: 559-750. The probe pairs for the reference sites were SEQ ID NOs: 751-942.

[0191] Similar to the design in **Example 2**, for each 3' probe, the 5' end nucleotide was phosphorylated to provide a phosphate which would be connected to the hydroxyl group in the 3' end nucleotide of the 5' probe. Each 3' probe included a locus specific hybridization sequence (LSHS) in the 5' portion followed by a stuffer L2 sequence, and a primer binding sequence Y in the 3' portion. In this example, four primer binding sequence Y: Chr21_Y1, SEQ ID NO: 955; Chr21_Y2, SEQ ID NO: 956; Chr21_Y3, SEQ ID NO: 957; and Chr21_Y4, SEQ ID NO: 958 were used in the 3' probes.

[0192] Similar to the design in **Example 2**, each 5' probe included a locus specific hybridization sequence (LSHS) in the 3' portion followed by a stuffer L1 sequence and a primer binding sequence X in the 5' portion. In this example, four primer binding sequence X: Chr21_X1, SEQ ID NO: 951; Chr21_X2, SEQ ID NO: 952; Chr21_X3, SEQ ID NO: 953; and Chr21_X4, SEQ ID NO: 954 were used in the 5' probes.

[0193] In addition, similar to the design in **Example 2**, four forward primers and four reverse primers were designed for amplifying the ligation products. The four forward primers (Chr21_F1, SEQ ID NO: 943; Chr21_F2, SEQ ID NO: 944; Chr21_F3, SEQ ID NO: 945; and Chr21_F4, SEQ ID NO: 946) had unique sequences that were consistent with the four primer binding sequence X (Chr21_X1, SEQ ID NO: 951; Chr21_X2, SEQ ID NO: 952; Chr21_X3, SEQ ID NO: 953; and Chr21_X4, SEQ ID NO: 954), respectively. The four forward primers: Chr21_F1, Chr21_F2, Chr21_F3 and Chr21_F4 were labeled on the 5' ends with four different fluorescent dyes: FAM-blue, VIC-green, NED-yellow, and PET-red, respectively. The four reverse primers (Chr21_R1, SEQ ID NO: 947; Chr21_R2, SEQ ID NO: 948; Chr21_R3, SEQ ID NO: 949; and Chr21_R4, SEQ ID NO: 950) had unique sequences that were reversely complementary to the four primer binding sequence Y (Chr21_Y1, SEQ ID NO: 955; Chr21_Y2, SEQ ID NO: 956; Chr21_Y3, SEQ ID NO: 957; and Chr21_Y4, SEQ ID NO: 958), respectively. The Chr21_R2 and Chr21_R4 reverse primers also had a stuffer sequence Chr21_R_Stuffer, SEQ ID NO: 959 in the 5' portion. All primers were synthesized by Life Technologies Corporation.

Table 5. Names, sequence ID numbers, primer binding sequences and stuffer sequences for the probes used in human chromosome 21 copy number change detection. X1, X2, X3 and X4 refer to Chr21_X1, SEQ ID NO: 951; Chr21_X2, SEQ ID NO: 952; Chr21_X3, SEQ ID NO: 953; and Chr21_X4, SEQ ID NO: 954, respectively. Y1, Y2, Y3 and Y4 refer to Chr21_Y1, SEQ ID NO: 955; Chr21_Y2, SEQ ID NO: 956; Chr21_Y3, SEQ ID NO: 957; and Chr21_Y4, SEQ ID NO: 958, respectively.

SEQ ID NO.	Probe Name	Sequence X	Stuffer L1	Stuffer L2	Sequence Y
559	Chr21_01_3			ATT	Y1
560	Chr21_01_5	X2			
561	Chr21_02_3			ATTACGCGAT	Y1
562	Chr21_02_5	X3			
563	Chr21_03_3			ATTACGCG	Y1
564	Chr21_03_5	X3	ATTACGCGA		
565	Chr21_04_3			ATTACGCGATTACG	Y2
566	Chr21_04_5	X1	ATTAC		
567	Chr21_05_3			ATTACGCGATTA	Y1
568	Chr21_05_5	X2	ATT		
569	Chr21_06_3			ATTACGCGATTA	Y2
570	Chr21_06_5	X1	ATTACG		
571	Chr21_07_3			ATTACG	Y2
572	Chr21_07_5	X3			
573	Chr21_08_3			ATTACGCGATT	Y2
574	Chr21_08_5	X1	AT		
575	Chr21_09_3			ATTACGCGAT	Y2
576	Chr21_09_5	X3			
577	Chr21_10_3			ATTACG	Y3
578	Chr21_10_5	X3			
579	Chr21_11_3			ATTACGCGAT	Y3
580	Chr21_11_5	X3			
581	Chr21_12_3			ATTACGCGATTA	Y3
582	Chr21_12_5	X1	ATTACGCG		
583	Chr21_13_3			A	Y4

EP 2 893 034 B9

(continued)

	SEQ ID NO.	Probe Name	Sequence X	Stuffer L1	Stuffer L2	Sequence Y
5	584	Chr21_13_5	X4			
	585	Chr21_14_3			ATTACGCGAT	Y4
	586	Chr21_14_5	X2			
10	587	Chr21_15_3			ATTACGCGAT	Y2
	588	Chr21_15_5	X2	ATTAC		
	589	Chr21_16_3			ATTACGCGA	Y2
	590	Chr21_16_5	X3	ATTACGCG		
	591	Chr21_17_3			ATTACGCGATTAC	Y2
15	592	Chr21_17_5	X2	ATTAC		
	593	Chr21_18_3			ATTACG	Y4
	594	Chr21_18_5	X1			
	595	Chr21_19_3			ATTACGCGATT	Y3
20	596	Chr21_19_5	X1			
	597	Chr21_20_3			ATTACGCGAT	Y1
	598	Chr21_20_5	X4			
	599	Chr21_21_3			ATTACGCGATTA	Y3
	600	Chr21_21_5	X3	ATT		
25	601	Chr21_22_3			ATTACGCGA	Y4
	602	Chr21_22_5	X2	ATTACGC		
	603	Chr21_23_3			ATTACGCGATTA	Y3
	604	Chr21_23_5	X3	ATTACGC		
	605	Chr21_24_3			ATTACGCGA	Y1
30	606	Chr21_24_5	X4			
	607	Chr21_25_3			ATTA	Y1
	608	Chr21_25_5	X4			
	609	Chr21_26_3			ATTA	Y2
35	610	Chr21_26_5	X4			
	611	Chr21_27_3			ATTACGCGATTA	Y1
	612	Chr21_27_5	X4	ATTAC		
	613	Chr21_28_3			AT	Y2
	614	Chr21_28_5	X4			
40	615	Chr21_29_3			ATT	Y2
	616	Chr21_29_5	X2			
	617	Chr21_30_3			ATTACGCGA	Y2
	618	Chr21_30_5	X4			
45	619	Chr21_31_3			ATTACGCGATT	Y3
	620	Chr21_31_5	X2	ATTACGCG		
	621	Chr21_32_3			ATTA	Y3
	622	Chr21_32_5	X4			
	623	Chr21_33_3			ATTA	Y4
50	624	Chr21_33_5	X2			
	625	Chr21_34_3			ATTACGCG	Y4
	626	Chr21_34_5	X4	AT		
	627	Chr21_35_3			ATTACGCG	Y3
55	628	Chr21_35_5	X2	ATTACGCGAT		
	629	Chr21_36_3			ATTACGCGAT	Y4
	630	Chr21_36_5	X3	AT		
	631	Chr21_37_3			ATTACGCGATT	Y1

EP 2 893 034 B9

(continued)

	SEQ ID NO.	Probe Name	Sequence X	Stuffer L1	Stuffer L2	Sequence Y
5	632	Chr21_37_5	X2			
	633	Chr21_38_3			ATTACGC	Y1
	634	Chr21_38_5	X1			
10	635	Chr21_39_3			ATTACGCGATT	Y2
	636	Chr21_39_5	X2			
	637	Chr21_40_3			ATTACGCGATTAC	Y1
	638	Chr21_40_5	X3	AT		
	639	Chr21_41_3			ATTACGC	Y2
15	640	Chr21_41_5	X1			
	641	Chr21_42_3			ATTACGCGA	Y3
	642	Chr21_42_5	X4			
	643	Chr21_43_3			ATTACGC	Y3
20	644	Chr21_43_5	X1			
	645	Chr21_44_3			ATTACGCGAT	Y2
	646	Chr21_44_5	X4			
	647	Chr21_45_3			ATTACGCGATTAC	Y3
	648	Chr21_45_5	X1			
25	649	Chr21_46_3			AT	Y4
	650	Chr21_46_5	X2			
	651	Chr21_47_3			ATTACGCGATTACGC	Y4
	652	Chr21_47_5	X3	ATTACGCGATTACGCGATT		
30	653	Chr21_48_3			ATTACGCGATTA	Y1
	654	Chr21_48_5	X3	ATTACGCGATTAC		
	655	Chr21_49_3			ATTACGCGATTACG	Y2
	656	Chr21_49_5	X3	ATTACGCGAT		
	657	Chr21_50_3			ATTACGCGATTAC	Y4
35	658	Chr21_50_5	X1	ATTAC		
	659	Chr21_51_3			ATTACGCGATT	Y3
	660	Chr21_51_5	X2			
	661	Chr21_52_3			ATTACGCGATTA	Y4
40	662	Chr21_52_5	X4	ATTA		
	663	Chr21_53_3			ATTACGCGATTA	Y1
	664	Chr21_53_5	X2	ATTAC		
	665	Chr21_54_3			ATTA	Y1
	666	Chr21_54_5	X1			
45	667	Chr21_55_3			ATTACGCGATT	Y2
	668	Chr21_55_5	X4	ATTAC		
	669	Chr21_56_3			ATTACGCGATTAC	Y3
	670	Chr21_56_5	X4	ATTAC		
	671	Chr21_57_3			ATTA	Y4
50	672	Chr21_57_5	X3			
	673	Chr21_58_3			ATTACGCGAT	Y3
	674	Chr21_58_5	X1	ATTACGCGA		
	675	Chr21_59_3			ATTACGCGATTAC	Y2
55	676	Chr21_59_5	X3			
	677	Chr21_60_3			AT	Y1
	678	Chr21_60_5	X3			
	679	Chr21_61_3			ATTA	Y2

EP 2 893 034 B9

(continued)

	SEQ ID NO.	Probe Name	Sequence X	Stuffer L1	Stuffer L2	Sequence Y
5	680	Chr21_61_5	X1			
	681	Chr21_62_3			ATTACGCGA	Y2
	682	Chr21_62_5	X2	ATTACGCG		
10	683	Chr21_63_3			ATTACGCGATTAC	Y3
	684	Chr21_63_5	X2			
	685	Chr21_64_3			AT	Y2
	686	Chr21_64_5	X3			
	687	Chr21_65_3			ATTACGCGATTA	Y4
15	688	Chr21_65_5	X4			
	689	Chr21_66_3			ATTACGCGATT	Y3
	690	Chr21_66_5	X4	ATTACG		
	691	Chr21_67_3			ATTACGCGATTAC	Y4
	692	Chr21_67_5	X1			
20	693	Chr21_68_3			ATTA	Y3
	694	Chr21_68_5	X1			
	695	Chr21_69_3			ATTACGCGATTACG	Y4
	696	Chr21_69_5	X2	ATTACGCGATTAC		
25	697	Chr21_70_3			ATTACGCGATTAC	Y4
	698	Chr21_70_5	X3	ATT		
	699	Chr21_71_3			ATTACGC	Y1
	700	Chr21_71_5	X2			
	701	Chr21_72_3			ATTACGC	Y2
30	702	Chr21_72_5	X2			
	703	Chr21_73_3			ATTACGCGATTA	Y4
	704	Chr21_73_5	X3	ATTACGCGATT		
	705	Chr21_74_3			ATTACGC	Y3
35	706	Chr21_74_5	X2			
	707	Chr21_75_3			ATTACGCGATTAC	Y1
	708	Chr21_75_5	X2	ATTACGCG		
	709	Chr21_76_3			ATTACGC	Y3
	710	Chr21_76_5	X3	ATTACGCGAT		
40	711	Chr21_77_3			ATTACGCGATTAC	Y1
	712	Chr21_77_5	X1	ATTACG		
	713	Chr21_78_3			ATT	Y3
	714	Chr21_78_5	X2			
45	715	Chr21_79_3			ATTACGCGATTA	Y1
	716	Chr21_79_5	X1			
	717	Chr21_80_3			ATTACGCGATTA	Y4
	718	Chr21_80_5	X4	ATT		
	719	Chr21_81_3			ATTACGCGATTAC	Y1
50	720	Chr21_81_5	X1	ATT		
	721	Chr21_82_3			ATTACGCGATTACGC	Y2
	722	Chr21_82_5	X1	A		
	723	Chr21_83_3			ATTA	Y4
55	724	Chr21_83_5	X1			
	725	Chr21_84_3			ATTACGCGATTAC	Y3
	726	Chr21_84_5	X4			
	727	Chr21_85_3			AT	Y3

EP 2 893 034 B9

(continued)

	SEQ ID NO.	Probe Name	Sequence X	Stuffer L1	Stuffer L2	Sequence Y
5	728	Chr21_85_5	X3			
	729	Chr21_86_3			AT	Y4
	730	Chr21_86_5	X3			
10	731	Chr21_87_3			ATTACG	Y4
	732	Chr21_87_5	X1	ATTACGCGATT		
	733	Chr21_88_3			AT	Y3
	734	Chr21_88_5	X4	A		
	735	Chr21_89_3			ATTA	Y4
15	736	Chr21_89_5	X4			
	737	Chr21_90_3			ATTACGCGATT	Y1
	738	Chr21_90_5	X4	ATTACGC		
	739	Chr21_91_3			ATTACGC	Y1
20	740	Chr21_91_5	X3			
	741	Chr21_92_3			ATTACGCGATTAC	Y4
	742	Chr21_92_5	X2			
	743	Chr21_93_3			ATT	Y1
	744	Chr21_93_5	X4			
25	745	Chr21_94_3			ATTACGCGATTAC	Y1
	746	Chr21_94_5	X1	ATTACGC		
	747	Chr21_95_3			ATTACGCGATT	Y4
	748	Chr21_95_5	X1			
	749	Chr21_96_3			ATTACGCGATTACGC	Y2
30	750	Chr21_96_5	X4	ATTA		
	751	REF10p_A_3				Y2
	752	REF10p_A_5	X1			
35	753	REF10p_B_3			ATTAC	Y1
	754	REF10p_B_5	X4			
40	755	REF10p_C_3			ATTAC	Y4
	756	REF10p_C_5	X4			
45	757	REF10p_D_3			ATTACGCGATT	Y3
	758	REF10p_D_5	X4			
	759	REF10q_A_3			ATTACGCG	Y1
50	760	REF10q_A_5	X2	ATTAC		
	761	REF10q_B_3				Y3
55	762	REF10q_B_5	X2			
	763	REF10q_C_3			ATTACGCGATTA	Y1

EP 2 893 034 B9

(continued)

	SEQ ID NO.	Probe Name	Sequence X	Stuffer L1	Stuffer L2	Sequence Y
5	764	REF10q_C_5	X3	ATTACGCGATTACGCG		
	765	REF11p_A_3			ATTACGC	Y1
10	766	REF11p_A_5	X4			
	767	REF11p_B_3			ATTACGCGA	Y3
15	768	REF11p_B_5	X1	ATTAC		
	769	REF11p_C_3			ATTACGCGATTAC	Y3
	770	REF11p_C_5	X2	AT		
20	771	REF11p_D_3			ATTA	Y1
	772	REF11p_D_5	X1			
25	773	REF11q_A_3				Y4
	774	REF11q_A_5	X3	ATTACGCGATTA		
30	775	REF11q_B_3			ATTACGCGATTAC	Y2
	776	REF11q_B_5	X4	ATTACGCGATTA		
	777	REF11q_C_3			ATTACG	Y3
35	778	REF11q_C_5	X4	A		
	779	REF12p_A_3			AT	Y4
40	780	REF12p_A_5	X4	ATTACGCGAT		
	781	REF12p_B_3			ATTACGCGATTAC	Y4
45	782	REF12p_B_5	X3	ATTACGCGATTACGC		
	783	REF12p_C_3			ATTACGCGATTACG	Y2
	784	REF12p_C_5	X4	ATT		
50	785	REF12q_A_3			ATTACGC	Y1
	786	REF12q_A_5	X3			
55	787	REF12q_B_3			ATTACGCGAT	Y4
	788	REF12q_B_5	X2	ATTAC		

EP 2 893 034 B9

(continued)

	SEQ ID NO.	Probe Name	Sequence X	Stuffer L1	Stuffer L2	Sequence Y
5	789	REF12q_C_3			ATTACGCGA	Y4
	790	REF12q_C_5	X1			
10	791	REF14q_A_3				Y2
	792	REF14q_A_5	X2			
15	793	REF14q_B_3				Y3
	794	REF14q_B_5	X1			
	795	REF16p_A_3				Y2
20	796	REF16p_A_5	X4			
	797	REF16p_B_3				Y3
25	798	REF16p_B_5	X2	ATTACGCGATTACGCGA		
	799	REF16p_C_3			ATTACGCGATTAC	Y2
30	800	REF16p_C_5	X2	ATTACG		
	801	REF16p_D_3			ATTACGCGATTACG	Y4
	802	REF16p_D_5	X2	ATTACG		
35	803	REF16q_A_3			ATTACGCGAT	Y3
	804	REF16q_A_5	X1	ATTACG		
40	805	REF16q_B_3			ATTACGCGATTA	Y3
	806	REF16q_B_5	X4	ATTACGCGA		
45	807	REF16q_C_3			ATTAC	Y1
	808	REF16q_C_5	X3	ATTACGCG		
	809	REF16q_D_3				Y2
50	810	REF16q_D_5	X3			
	811	REF17q_A_3				Y4
55	812	REF17q_A_5	X3	AT		
	813	REF17q_B_3			ATTACGCGATTA	Y2

EP 2 893 034 B9

(continued)

	SEQ ID NO.	Probe Name	Sequence X	Stuffer L1	Stuffer L2	Sequence Y
5	814	REF17q_B_5	X1	ATTACGCGATTACG		
	815	REF18p_A_3			ATTACGCGATTA	Y1
10	816	REF18p_A_3	X1	AT		
	817	REF18p_B_3			ATTACGC	Y3
15	818	REF18p_B_3	X2			
	819	REF19p_A_3				Y4
20	820	REF19p_A_3	X4			
	821	REF19p_B_3			AT	Y4
	822	REF19p_B_5	X2			
25	823	REF19q_A_3			ATT	Y2
	824	REF19q_A_5	X1			
30	825	REF19q_B_3			ATTACGCGATTAC	Y2
	826	REF19q_B_5	X3	ATTACGC		
	827	REF19q_C_3				Y4
35	828	REF19q_C_5	X1			
	829	REF19q_D_3				Y3
40	830	REF19q_D_5	X4			
	831	REF20p_A_3			ATTACGCGATT	Y1
45	832	REF20p_A_3	X2	ATTA		
	833	REF20p_B_3			A	Y2
	834	REF20p_B_3	X4	ATTACGCGAT		
50	835	REF20p_C_3			ATTACGCGA	Y4
	836	REF20p_C_3	X2	A		
55	837	REF20q_A_3			ATTACGCGATTAC	Y2
	838	REF20q_A_5	X3			

EP 2 893 034 B9

(continued)

	SEQ ID NO.	Probe Name	Sequence X	Stuffer L1	Stuffer L2	Sequence Y
5	839	REF20q_B_3			ATTACGCGATTA	Y2
	840	REF20q_B_5	X1	ATTACGC		
10	841	REF20q_C_3			ATTACGCGATTACGCGA	Y4
	842	REF20q_C_3	X4	ATTAC		
15	843	REF20q_D_3			ATTAC	Y2
	844	REF20q_D_5	X2			
20	845	REF20q_E_3			ATT	Y3
	846	REF20q_E_3	X3			
	847	REF2p_A_3			ATTACGCGAT	Y1
	848	REF2p_A_5	X3	ATTACGCGA		
25	849	REF2p_B_3			ATTACGCGATTAC	Y3
	850	REF2p_B_5	X3	ATTACGCGATT		
	851	REF2p_C_3				Y4
	852	REF2p_C_5	X2			
30	853	REF2p_D_3			ATT	Y1
	854	REF2p_D_5	X2			
	855	REF2q_A_3			ATTACGCGATTACG	Y4
	856	REF2q_A_3	X3	ATTACG		
	857	REF2q_B_3			ATTACGCGATTA	Y2
35	858	REF2q_B_3	X2	ATTACGCGAT		
	859	REF3p_A_3			ATTACG	Y3
	860	REF3p_A_5	X1			
	861	REF3p_B_3			ATTACGCGATTAC	Y4
40	862	REF3p_B_5	X4	ATTACGCGATTAC		
	863	REF3p_C_3			ATTA	Y2
	864	REF3p_C_5	X4			
	865	REF3p_D_3			ATTAC	Y1
	866	REF3p_D_5	X3			
45	867	REF3p_E_3			ATTACGCGA	Y1
	868	REF3p_E_5	X1			
	869	REF3q_A_3			ATTACGCGATTACG	Y1
	870	REF3q_A_5	X4	ATTACGCGATTAC		
50	871	REF3q_B_3			ATTACGCGA	Y4
	872	REF3q_B_5	X3	ATT		
	873	REF3q_C_3			A	Y2
	874	REF3q_C_5	X3			
	875	REF3q_D_3			ATTAC	Y4
55	876	REF3q_D_5	X1			
	877	REF4q_A_3				Y4
	878	REF4q_A_5	X3			

EP 2 893 034 B9

(continued)

	SEQ ID NO.	Probe Name	Sequence X	Stuffer L1	Stuffer L2	Sequence Y
5	879	REF4q_B_3				Y2
	880	REF4q_B_5	X1	ATTACGCGATTAC		
	881	REF4q_C_3			ATTACGC	Y1
10	882	REF4q_C_5	X2			
	883	REF5p_A_3			ATTACGCGATTAC	Y3
	884	REF5p_A_5	X1	ATTACGCGATTACGC		
	885	REF5p_B_3			ATTACGCGA	Y1
	886	REF5p_B_5	X4	ATT		
15	887	REF5p_C_3			A	Y1
	888	REF5p_C_5	X2			
	889	REF5q_A_3			ATTAC	Y3
	890	REF5q_A_5	X2			
	891	REF5q_B_3			ATTACGCGATTA	Y4
20	892	REF5q_B_5	X1	ATTACGCGATTACGCGA		
	893	REF5q_C_3			ATTACGCGATTACGCG	Y1
	894	REF5q_C_5	X1	ATTACGCGATTACG		
	895	REF6p_A_3			ATT	Y3
25	896	REF6p_A_5	X3			
	897	REF6p_B_3				Y1
	898	REF6p_B_5	X3			
	899	REF6p_C_3				Y3
	900	REF6p_C_5	X1			
30	901	REF6q_A_3			ATTACGCGATTACG	Y1
	902	REF6q_A_5	X2	ATTACGCGATT		
	903	REF6q_B_3			ATTACGCGATTACGCG	Y4
	904	REF6q_B_5	X1	ATTACGC		
35	905	REF6q_C_3			ATTACGCG	Y3
	906	REF6q_C_5	X4			
	907	REF7p_A_3			ATTACGC	Y2
	908	REF7p_A_5	X4			
	909	REF7p_B_3			ATTACGCGATT	Y3
40	910	REF7p_B_5	X3	ATTA		
	911	REF7p_C_3			ATTAC	Y2
	912	REF7p_C_5	X3			
	913	REF7p_D_3			AT	Y4
45	914	REF7p_D_5	X1	ATTACGCGATTACGCG		
	915	REF8p_A_3			ATTACGCGA	Y1
	916	REF8p_A_5	X4	ATTACG		
	917	REF8p_B_3			ATTACGCGATTACG	Y3
	918	REF8p_B_5	X2	ATTACGCGATTACG		
50	919	REF8p_C_3			ATTAC	Y2
	920	REF8p_C_5	X1			
	921	REF8p_D_3			AT	Y2
	922	REF8p_D_5	X2	ATTACGCGATT		
55	923	REF8q_A_3			A	Y3
	924	REF8q_A_5	X3			
	925	REF8q_B_3			A	Y1
	926	REF8q_B_5	X1			

(continued)

SEQ ID NO.	Probe Name	Sequence X	Stuffer L1	Stuffer L2	Sequence Y
927	REF8q_C_3			ATTACGC	Y3
928	REF8q_C_5	X4	ATTAC		
929	REF9p_A_3			ATTACGCGATTACGCG	Y4
930	REF9p_A_5	X2	ATTACGCGATTACGC		
931	REF9p_B_3			ATTACGCGATTAC	Y2
932	REF9p_B_5	X3	ATTACGCGATT		
933	REF9p_C_3			ATTACGC	Y1
934	REF9p_C_5	X1	ATTACGC		
935	REF9p_D_3				Y1
936	REF9p_D_5	X4			
937	REF9q_A_3			A	Y3
938	REF9q_A_5	X3	ATTACGCGATTA		
939	REF9q_B_3				Y4
940	REF9q_B_5	X4			
941	REF9q_C_3			ATTACGCGA	Y2
942	REF9q C 5	X2			

[0194] In the example, five DNA samples with different human chromosome 21 copy numbers were prepared. Specifically, the five DNA samples (M0, M2, M4, M8, and M16) were prepared by mixing an aliquot of genomic DNA extract from a healthy volunteer with an aliquot of genomic DNA extract from a Down's Syndrome patient at a DNA quantity ratio of 100:0, 98:2, 96:4, 92:8, and 84:16 so that the copy numbers of human chromosome 21 in the five DNA samples increased 0%, 1%, 2%, 4%, and 8%, respectively. The copy numbers of chromosome 21 in M0, M2, M4, M8, and M16 DNA samples were designed to be 2.00, 2.02, 2.04, 2.08, and 2.16, respectively. This example is set out to detect the copy numbers of human chromosome 21 in M0, M2, M4, M8, and M16 DNA samples using an exemplary method according to this invention.

[0195] To detect copy numbers of chromosome 21 in the five DNA samples, the procedure of probe hybridization, probe ligation, ligation product amplification, and capillary electrophoresis as detailed below was performed for each sample and repeated three times.

[0196] For each DNA sample a ligation product was first generated with the 384 probes listed in **Table 5**. Briefly, about 100-200 microgram (μ g) genomic DNA was dissolved in 10 microliter (μ l) 1xTE buffer (10mM Tris.Cl, pH8.0, 1mM EDTA from Sigma-Aldrich). The dissolved genomic DNA was denatured at 98°C for 5 minutes and then immediately cooled down on ice. A 2xligation premix solution was prepared according to the following formula: a 10 μ l 2x ligation premix was made by mixing 2 μ l 10xTaq ligase buffer, 1 μ l 40U/ μ l Taq Ligase from NewEngland Biolabs, Inc., 1 μ l ProbeMix (the 384 probes with a final concentration of 0.005 micromolar for each probe in 1xTE), and 6 μ l ddH₂O (Distilled Milli-Q water from Milli-Q Advantage A10, Millipore). 10 μ l 2xligation premix was mixed with the denatured 10 μ l genomic DNA and the mixture was allowed to undergo 4 cycles of denaturation, hybridization, and ligation under the following conditions: 95°C for 30 seconds, and then 58°C for 4 hours. The ligation product thus obtained can be stored on ice for same day use or freezed in -20 °C for future use.

[0197] With the ligation product, an amplification step was then performed to obtain an amplification product. Briefly, two PCR reactions were performed using an aliquot from the same ligation product as the template. One PCR reaction had Chr21_F1, Chr21_F2, Chr21_F3 and Chr21_F4, Chr21_R1 and Chr21_R2 as primers. The other PCR reaction had Chr21_F1, Chr21_F2, Chr21_F3, Chr21_F4, Chr21_R3 and Chr21_R4 as primers. The PCR reaction mixture was prepared as follows: a 20 μ l reaction system was made by mixing 2 μ l 10x PCR buffer (Qiagen, Germany), 22 μ l 2.5mM dNTP mix (2.5mM each of dATP, dTTP, dCTP and dGTP from Takara Bio Inc.), 2 μ l primer mix (Chr21_F1, Chr21_F2, Chr21_F3 and Chr21_F4, Chr21_R1 and Chr21_R2 at final concentrations of 1 μ M, 1 μ M, 1 μ M, 1 μ M, 2 μ M and 2 μ M, respectively; or Chr21_F1, Chr21_F2, Chr21_F3, Chr21_F4, Chr21_R3 and Chr21_R4 at final concentrations of 1 μ M, 1 μ M, 1 μ M, 1 μ M, 2 μ M and 2 μ M, respectively), 1 μ l ligation product, 0.2 μ l 5U/ μ l HotStarTaq Plus Taq DNA polymerase (Qiagen, Germany), and 12.8 μ l ddH₂O. The PCR mixture was allowed to undergo a polymerase chain reaction under the following conditions: 95 °C for 2 minutes; followed by 35 cycles of 94 °C for 20 second, 57°C for 40 second, and 72 °C for 1.5 minutes; and after the 35th cycle, the reaction mixture was kept in 60 °C for 1 hour.

[0198] To analyze the amplification product, 1 μ l of the amplification product was first diluted with ddH₂O 10 times into

10 μ l. Then 1 μ l was taken out of the 10 μ l diluted amplification product and mixed with 0.1 μ l GeneScan™ 500 LIZ® size standard (Life Technologies, Inc.) and 8.9 μ l Hi-Di formamide (Life Technologies, Inc.). The mixture was denatured at 95°C for 5 minutes and run through capillary electrophoresis by ABI3130XL according to manufacturer's manual. The capillary electrophoresis data was processed using Genemapper 4.0 to obtain peak intensity values for each amplification product.

[0199] The chromatograms from the two PCR reactions were designated as Panel A and Panel B, respectively. In each panel, amplification products were categorized into eight groups. The amplification products in each group were amplified with the same primer pair. As such, in panel A, the eight groups corresponded to the eight primer pairs: F1/R1, F1/R2, F2/R1, F2/R2, F3/R1, F3/R2, F4/R1, and F4/R2; and in panel B, the eight groups corresponded to the eight primer pairs: F1/R3, F1/R4, F2/R3, F2/R4, F3/R3, F3/R4, F4/R3, and F4/R4. Each primer pair was designed to amplify 12 target sites including 6 target sites from human chromosome 21 and 6 reference target sites.

[0200] The amplification products obtained for each DNA sample could be separated and the peaks corresponding to each target site could be individually identified by capillary electrophoresis. For example, **Figures 13A and 13F** showed the chromatograms of all amplification products from the healthy control sample, i.e., the M0 sample with 0% increase of human chromosome 21 DNA in panel A and panel B, respectively. **Figures 13B, 13C, 13D, and 13E**, which were derived from the control sample panel A, showed the chromatograms of the amplification products labeled with blue, green, yellow, and red fluorescent dyes, respectively. Similarly, **Figures 13G, 13H, 13I, and 13J**, which were derived from the control sample panel B, show the chromatograms of the amplification products labeled with blue, green, yellow, and red fluorescent dyes, respectively. As seen in **Figures 13B-13E and 13H-13J**, the peaks from the amplification products labeled with the same fluorescent dye could be identified on the basis of fragment sizes. See **Table 6** for the sizes of amplification products for the 192 target sites in the control sample. The fluorescent intensity for each peak could be obtained. These peak intensity values were used to determine copy number changes of chromosome 21 in the samples.

Table 6. Fragment sizes of the amplification products for the 192 target sites in the control sample. LP SIZE refers to Ligation Product Size; REF refers to Reference; CE refers to Capillary Electrophoresis.

PANEL	GROUP	PROBE LOCUS	TARGET REGION	PCR Primers	LP SIZE	CE REF SIZE
A	1	Chr21_38	Chromosome 21	Chr21_F1/Chr21_R1	104	101.27
A	1	Chr21_54	Chromosome 21	Chr21_F1/Chr21_R1	98	94.89
A	1	Chr21_77	Chromosome 21	Chr21_F1/Chr21_R1	125	121.88
A	1	Chr21_79	Chromosome 21	Chr21_F1/Chr21_R1	110	107.53
A	1	Chr21_81	Chromosome 21	Chr21_F1/Chr21_R1	116	113.63
A	1	Chr21_94	Chromosome 21	Chr21_F1/Chr21_R1	122	119.37
A	1	REF11p_D	REFERENCE	Chr21_F1/Chr21_R1	101	99.32
A	1	REF18p_A	REFERENCE	Chr21_F1/Chr21_R1	119	117.63
A	1	REF3p_E	REFERENCE	Chr21_F1/Chr21_R1	107	104.76
A	1	REF5q_C	REFERENCE	Chr21_F1/Chr21_R1	128	124.95
A	1	REF8q_B	REFERENCE	Chr21_F1/Chr21_R1	95	92.79
A	1	REF9p_C	REFERENCE	Chr21_F1/Chr21_R1	113	111.16
A	5	Chr21_04	Chromosome 21	Chr21_F1/Chr21_R2	160	159.78
A	5	Chr21_06	Chromosome 21	Chr21_F1/Chr21_R2	163	163.46
A	5	Chr21_08	Chromosome 21	Chr21_F1/Chr21_R2	150	148.61
A	5	Chr21_41	Chromosome 21	Chr21_F1/Chr21_R2	142	140.76

EP 2 893 034 B9

(continued)

	PANEL	GROUP	PROBE LOCUS	TARGET REGION	PCR Primers	LP SIZE	CE REF SIZE
5	A	5	Chr21_61	Chromosome 21	Chr21_F1/Chr21_R2	136	134.16
	A	5	Chr21_82	Chromosome 21	Chr21_F1/Chr21_R2	154	154.32
10	A	5	REF10p_A	REFERENCE	Chr21_F1/Chr21_R2	133	130.3
	A	5	REF17q_B	REFERENCE	Chr21_F1/Chr21_R2	166	166.18
	A	5	REF19q_A	REFERENCE	Chr21_F1/Chr21_R2	139	137.98
	A	5	REF20q_B	REFERENCE	Chr21_F1/Chr21_R2	157	157.52
	A	5	REF4q_B	REFERENCE	Chr21_F1/Chr21_R2	151	151.76
15	A	5	REF8p_C	REFERENCE	Chr21_F1/Chr21_R2	145	144.39
	B	9	Chr21_12	Chromosome 21	Chr21_F1/Chr21_R3	122	120.82
	B	9	Chr21_19	Chromosome 21	Chr21_F1/Chr21_R3	110	108.88
20	B	9	Chr21_43	Chromosome 21	Chr21_F1/Chr21_R3	104	102.51
	B	9	Chr21_45	Chromosome 21	Chr21_F1/Chr21_R3	113	111.65
25	B	9	Chr21_58	Chromosome 21	Chr21_F1/Chr21_R3	125	123.11
	B	9	Chr21_68	Chromosome 21	Chr21_F1/Chr21_R3	98	96.6
	B	9	REF11p_B	REFERENCE	Chr21_F1/Chr21_R3	116	115.19
30	B	9	REF14q_B	REFERENCE	Chr21_F1/Chr21_R3	101	99.44
	B	9	REF16q_A	REFERENCE	Chr21_F1/Chr21_R3	120	117.57
	B	9	REF3p_A	REFERENCE	Chr21_F1/Chr21_R3	107	105.06
	B	9	REF5p_A	REFERENCE	Chr21_F1/Chr21_R3	128	125.52
	B	9	REF6p_C	REFERENCE	Chr21_F1/Chr21_R3	95	92.74
35	B	13	Chr21_18	Chromosome 21	Chr21_F1/Chr21_R4	142	139.76
	B	13	Chr21_50	Chromosome 21	Chr21_F1/Chr21_R4	160	160.98
40	B	13	Chr21_67	Chromosome 21	Chr21_F1/Chr21_R4	151	150.71
	B	13	Chr21_83	Chromosome 21	Chr21_F1/Chr21_R4	136	133.94
	B	13	Chr21_87	Chromosome 21	Chr21_F1/Chr21_R4	163	163
45	B	13	Chr21_95	Chromosome 21	Chr21_F1/Chr21_R4	148	146.89
	B	13	REF12q_C	REFERENCE	Chr21_F1/Chr21_R4	145	142.39
	B	13	REF19q_C	REFERENCE	Chr21_F1/Chr21_R4	133	131.04
50	B	13	REF3q_D	REFERENCE	Chr21_F1/Chr21_R4	139	137.19
	B	13	REF5q_B	REFERENCE	Chr21_F1/Chr21_R4	166	166.06
	B	13	REF6q_B	REFERENCE	Chr21_F1/Chr21_R4	159	156.41
	B	13	REF7p_D	REFERENCE	Chr21_F1/Chr21_R4	154	154.2
55	A	2	Chr21_01	Chromosome 21	Chr21_F2/Chr21_R1	98	97.28
	A	2	Chr21_05	Chromosome 21	Chr21_F2/Chr21_R1	116	114.32

EP 2 893 034 B9

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	PANEL	GROUP	PROBE LOCUS	TARGET REGION	PCR Primers	LP SIZE	CE REF SIZE
5	A	2	Chr21_37	Chromosome 21	Chr21_F2/Chr21_R1	110	108.49
	A	2	Chr21_53	Chromosome 21	Chr21_F2/Chr21_R1	125	123.41
10	A	2	Chr21_71	Chromosome 21	Chr21_F2/Chr21_R1	104	103.17
	A	2	Chr21_75	Chromosome 21	Chr21_F2/Chr21_R1	124	121.91
	A	2	REF10q_A	REFERENCE	Chr21_F2/Chr21_R1	113	111.9
15	A	2	REF20p_A	REFERENCE	Chr21_F1/Chr21_R1	119	118.5
	A	2	REF2p_D	REFERENCE	Chr21_F2/Chr21_R1	101	100.11
	A	2	REF4q_C	REFERENCE	Chr21_F1/Chr21_R1	107	105.82
	A	2	REF5p_C	REFERENCE	Chr21_F2/Chr21_R1	95	92.94
	A	2	REF6q_A	REFERENCE	Chr21_F1/Chr21_R1	128	126.71
20	A	6	Chr21_15	Chromosome 21	Chr21_F2/Chr21_R2	154	155.7
	A	6	Chr21_17	Chromosome 21	Chr21_F2/Chr21_R2	160	160.33
25	A	6	Chr21_29	Chromosome 21	Chr21_F2/Chr21_R2	136	135.05
	A	6	Chr21_39	Chromosome 21	Chr21_F2/Chr21_R2	148	148.76
	A	6	Chr21_62	Chromosome 21	Chr21_F2/Chr21_R2	163	163.8
30	A	6	Chr21_72	Chromosome 21	Chr21_F2/Chr21_R2	142	141.14
	A	6	REF14q_A	REFERENCE	Chr21_F2/Chr21_R2	133	131.48
	A	6	REF16p_C	REFERENCE	Chr21_F2/Chr21_R2	157	158.2
35	A	6	REF20q_D	REFERENCE	Chr21_F2/Chr21_R2	139	138.1
	A	6	REF2q_B	REFERENCE	Chr21_F2/Chr21_R2	166	167.35
	A	6	REF8p_D	REFERENCE	Chr21_F2/Chr21_R2	151	152
	A	6	REF9q_C	REFERENCE	Chr21_F2/Chr21_R2	145	145.32
40	B	10	Chr21_31	Chromosome 21	Chr21_F2/Chr21_R3	122	120.6
	B	10	Chr21_35	Chromosome 21	Chr21_F2/Chr21_R3	125	123.44
	B	10	Chr21_51	Chromosome 21	Chr21_F2/Chr21_R3	110	108.56
45	B	10	Chr21_63	Chromosome 21	Chr21_F2/Chr21_R3	113	112.4
	B	10	Chr21_74	Chromosome 21	Chr21_F2/Chr21_R3	104	102.84
50	B	10	Chr21_78	Chromosome 21	Chr21_F2/Chr21_R3	98	97.74
	B	10	REF10q_B	REFERENCE	Chr21_F2/Chr21_R3	95	94.16
	B	10	REF11p_C	REFERENCE	Chr21_F2/Chr21_R3	119	118.21
	B	10	REF16p_B	REFERENCE	Chr21_F2/Chr21_R3	117	115.11
55	B	10	REF18p_B	REFERENCE	Chr21_F2/Chr21_R3	107	106.22
	B	10	REF5q_A	REFERENCE	Chr21_F2/Chr21_R3	101	99.55
	B	10	REF8p_B	REFERENCE	Chr21_F2/Chr21_R3	128	127.17

EP 2 893 034 B9

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	PANEL	GROUP	PROBE LOCUS	TARGET REGION	PCR Primers	LP SIZE	CE REF SIZE
5	B	14	Chr21_14	Chromosome 21	Chr21_F2/Chr21_R4	148	146.39
	B	14	Chr21_22	Chromosome 21	Chr21_F2/Chr21_R4	160	159.1
10	B	14	Chr21_33	Chromosome 21	Chr21_F2/Chr21_R4	139	137.76
	B	14	Chr21_46	Chromosome 21	Chr21_F2/Chr21_R4	136	136.18
	B	14	Chr21_69	Chromosome 21	Chr21_F2/Chr21_R4	163	164.44
15	B	14	Chr21_92	Chromosome 21	Chr21_F2/Chr21_R4	151	151.88
	B	14	REF12q_B	REFERENCE	Chr21_F2/Chr21_R4	155	154.32
	B	14	REF16p_D	REFERENCE	Chr21_F2/Chr21_R4	157	157.18
20	B	14	REF19p_B	REFERENCE	Chr21_F2/Chr21_R4	142	140.51
	B	14	REF20p_C	REFERENCE	Chr21_F2/Chr21_R4	146	143.27
	B	14	REF2p_C	REFERENCE	Chr21_F2/Chr21_R4	133	132.04
	B	14	REF9p_A	REFERENCE	Chr21_F2/Chr21_R4	168	167.19
25	A	3	Chr21_02	Chromosome 21	Chr21_F3/Chr21_R1	110	107.31
	A	3	Chr21_03	Chromosome 21	Chr21_F3/Chr21_R1	122	119.34
	A	3	Chr21_40	Chromosome 21	Chr21_F3/Chr21_R1	116	113.52
30	A	3	Chr21_48	Chromosome 21	Chr21_F3/Chr21_R1	125	122.97
	A	3	Chr21_60	Chromosome 21	Chr21_F3/Chr21_R1	98	95.46
35	A	3	Chr21_91	Chromosome 21	Chr21_F3/Chr21_R1	104	101.27
	A	3	REF10q_C	REFERENCE	Chr21_F3/Chr21_R1	128	124.84
	A	3	REF12q_A	REFERENCE	Chr21_F3/Chr21_R1	107	104.55
	A	3	REF16q_C	REFERENCE	Chr21_F3/Chr21_R1	113	109.55
40	A	3	REF2p_A	REFERENCE	Chr21_F3/Chr21_R1	119	115.79
	A	3	REF3p_D	REFERENCE	Chr21_F3/Chr21_R1	101	98.87
	A	3	REF6p_B	REFERENCE	Chr21_F3/Chr21_R1	95	92.83
	A	7	Chr21_07	Chromosome 21	Chr21_F3/Chr21_R2	142	141.01
45	A	7	Chr21_09	Chromosome 21	Chr21_F3/Chr21_R2	148	147.51
	A	7	Chr21_16	Chromosome 21	Chr21_F3/Chr21_R2	160	159.66
50	A	7	Chr21_49	Chromosome 21	Chr21_F3/Chr21_R2	163	164.13
	A	7	Chr21_59	Chromosome 21	Chr21_F3/Chr21_R2	151	150.82
55	A	7	Chr21_64	Chromosome 21	Chr21_F3/Chr21_R2	136	133.71
	A	7	REF16q_D	REFERENCE	Chr21_F3/Chr21_R2	133	130.03
	A	7	REF19q_B	REFERENCE	Chr21_F3/Chr21_R2	157	157.18

EP 2 893 034 B9

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	PANEL	GROUP	PROBE LOCUS	TARGET REGION	PCR Primers	LP SIZE	CE REF SIZE
5	A	7	REF20q_A	REFERENCE	Chr21_F3/Chr21_R2	154	153.74
	A	7	REF3q_C	REFERENCE	Chr21_F3/Chr21_R2	145	143.89
	A	7	REF7p_C	REFERENCE	Chr21_F3/Chr21_R2	139	136.52
	A	7	REF9p_B	REFERENCE	Chr21_F3/Chr21_R2	166	166.29
10	B	11	Chr21_10	Chromosome 21	Chr21_F3/Chr21_R3	104	102.63
	B	11	Chr21_11	Chromosome 21	Chr21_F3/Chr21_R3	110	107.92
	B	11	Chr21_21	Chromosome 21	Chr21_F3/Chr21_R3	116	113.79
15	B	11	Chr21_23	Chromosome 21	Chr21_F3/Chr21_R3	123	119.9
	B	11	Chr21_76	Chromosome 21	Chr21_F3/Chr21_R3	125	122.24
20	B	11	Chr21_85	Chromosome 21	Chr21_F3/Chr21_R3	98	95.93
	B	11	REF20q_E	REFERENCE	Chr21_F3/Chr21_R3	107	104.63
	B	11	REF2p_B	REFERENCE	Chr21_F3/Chr21_R3	128	125.85
	B	11	REF6p_A	REFERENCE	Chr21_F3/Chr21_R3	101	97.85
25	B	11	REF7p_B	REFERENCE	Chr21_F3/Chr21_R3	118	117.13
	B	11	REF8q_A	REFERENCE	Chr21_F3/Chr21_R3	95	93.51
	B	11	REF9q_A	REFERENCE	Chr21_F3/Chr21_R3	113	110.48
	B	15	Chr21_36	Chromosome 21	Chr21_F3/Chr21_R4	151	149.38
30	B	15	Chr21_47	Chromosome 21	Chr21_F3/Chr21_R4	171	169.07
	B	15	Chr21_57	Chromosome 21	Chr21_F3/Chr21_R4	139	137.19
35	B	15	Chr21_70	Chromosome 21	Chr21_F3/Chr21_R4	160	160.11
	B	15	Chr21_73	Chromosome 21	Chr21_F3/Chr21_R4	163	162.49
40	B	15	Chr21_86	Chromosome 21	Chr21_F3/Chr21_R4	136	133.49
	B	15	REF11q_A	REFERENCE	Chr21_F3/Chr21_R4	154	153.63
	B	15	REF12p_B	REFERENCE	Chr21_F3/Chr21_R4	166	165.95
	B	15	REF17q_A	REFERENCE	Chr21_F3/Chr21_R4	142	140.51
45	B	15	REF2q_A	REFERENCE	Chr21_F3/Chr21_R4	157	157.07
	B	15	REF3q_B	REFERENCE	Chr21_F3/Chr21_R4	148	145.34
	B	15	REF4q_A	REFERENCE	Chr21_F3/Chr21_R4	133	130.93
	A	4	Chr21_20	Chromosome 21	Chr21_F4/Chr21_R1	110	111.27
50	A	4	Chr21_24	Chromosome 21	Chr21_F4/Chr21_R1	107	108.17
	A	4	Chr21_25	Chromosome 21	Chr21_F4/Chr21_R1	101	102.32
55	A	4	Chr21_27	Chromosome 21	Chr21_F4/Chr21_R1	123	123.56
	A	4	Chr21_90	Chromosome 21	Chr21_F4/Chr21_R1	119	121.11

EP 2 893 034 B9

(continued)

	PANEL	GROUP	PROBE LOCUS	TARGET REGION	PCR Primers	LP SIZE	CE REF SIZE
5	A	4	Chr21_93	Chromosome 21	Chr21_F4/Chr21_R1	95	97.84
	A	4	REF10p_B	REFERENCE	Chr21_F4/Chr21_R1	98	99.66
	A	4	REF11p_A	REFERENCE	Chr21_F4/Chr21_R1	104	105.4
	A	4	REF3q_A	REFERENCE	Chr21_F4/Chr21_R1	125	126.71
10	A	4	REF5p_B	REFERENCE	Chr21_F4/Chr21_R1	113	113.89
	A	4	REF8p_A	REFERENCE	Chr21_F4/Chr21_R1	116	117.2
	A	4	REF9p_D	REFERENCE	Chr21_F4/Chr21_R1	92	94.54
15	A	8	Chr21_26	Chromosome 21	Chr21_F4/Chr21_R2	139	139.76
	A	8	Chr21_28	Chromosome 21	Chr21_F4/Chr21_R2	133	132.57
	A	8	Chr21_30	Chromosome 21	Chr21_F4/Chr21_R2	145	147.39
20	A	8	Chr21_44	Chromosome 21	Chr21_F4/Chr21_R2	148	150.35
	A	8	Chr21_55	Chromosome 21	Chr21_F4/Chr21_R2	160	162.72
25	A	8	Chr21_96	Chromosome 21	Chr21_F4/Chr21_R2	158	160.55
	A	8	REF11q_B	REFERENCE	Chr21_F4/Chr21_R2	163	167.59
	A	8	REF12p_C	REFERENCE	Chr21_F4/Chr21_R2	154	158.31
	A	8	REF16p_A	REFERENCE	Chr21_F4/Chr21_R2	136	137.87
30	A	8	REF20p_B	REFERENCE	Chr21_F4/Chr21_R2	151	154.55
	A	8	REF3p_C	REFERENCE	Chr21_F4/Chr21_R2	133	134.83
	A	8	REF7p_A	REFERENCE	Chr21_F4/Chr21_R2	142	144.39
	B	12	Chr21_32	Chromosome 21	Chr21_F4/Chr21_R3	101	105.27
35	B	12	Chr21_42	Chromosome 21	Chr21_F4/Chr21_R3	107	109.94
	B	12	Chr21_56	Chromosome 21	Chr21_F4/Chr21_R3	119	121.47
40	B	12	Chr21_66	Chromosome 21	Chr21_F4/Chr21_R3	122	124.86
	B	12	Chr21_84	Chromosome 21	Chr21_F4/Chr21_R3	113	114.87
	B	12	Chr21_88	Chromosome 21	Chr21_F4/Chr21_R3	96	97.26
45	B	12	REF10p_D	REFERENCE	Chr21_F4/Chr21_R3	116	118.32
	B	12	REF11q_C	REFERENCE	Chr21_F4/Chr21_R3	105	107.24
	B	12	REF16q_B	REFERENCE	Chr21_F4/Chr21_R3	125	126.62
	B	12	REF19q_D	REFERENCE	Chr21_F4/Chr21_R3	92	94.79
50	B	12	REF6q_C	REFERENCE	Chr21_F4/Chr21_R3	98	99.55
	B	12	REF8q_C	REFERENCE	Chr21_F4/Chr21_R3	110	110.86
	B	16	Chr21_13	Chromosome 21	Chr21_F4/Chr21_R4	133	134.61
55	B	16	Chr21_34	Chromosome 21	Chr21_F4/Chr21_R4	147	147.73
	B	16	Chr21_52	Chromosome 21	Chr21_F4/Chr21_R4	157	160.54

(continued)

PANEL	GROUP	PROBE LOCUS	TARGET REGION	PCR Primers	LP SIZE	CE REF SIZE
B	16	Chr21_65	Chromosome 21	Chr21_F4/Chr21_R4	151	154.67
B	16	Chr21_80	Chromosome 21	Chr21_F4/Chr21_R4	160	163.37
B	16	Chr21_89	Chromosome 21	Chr21_F4/Chr21_R4	139	141.64
B	16	REF10p_C	REFERENCE	Chr21_F4/Chr21_R4	142	144.27
B	16	REF12p_A	REFERENCE	Chr21_F4/Chr21_R4	148	151.53
B	16	REF19p_A	REFERENCE	Chr21_F4/Chr21_R4	136	137.42
B	16	REF20q_C	REFERENCE	Chr21_F4/Chr21_R4	157	158.24
B	16	REF3p_B	REFERENCE	Chr21_F4/Chr21_R4	163	166.72
B	16	REF9q_B	REFERENCE	Chr21_F4/Chr21_R4	130	131.49

[0201] To determine the copy number changes of chromosome 21, the following exemplary statistical method was applied. The copy number for each of the 96 target sites on chromosome 21 was calculated. A ratio for a chromosome 21 target site in the test sample (R_{test}) was obtained by dividing the peak intensity value of the chromosome 21 target site with the peak intensity value of each of the six reference target sites in the same group. As such, six ratios (R_{test}) were obtained for the chromosome 21 target site. Similarly, a ratio for the chromosome 21 target site was obtained in the control sample (R_{control}). It was known that the copy number of chromosome 21 in the control sample M0 (C_{control}) was 2. The copy number of chromosome 21 in the test sample (C_{test}) was calculated according to the formula: $C_{\text{test}} = C_{\text{control}} \times R_{\text{test}} / R_{\text{control}}$. Because there were six reference target sites for each chromosome 21 target site, six C_{test} values were derived for each chromosome 21 target site. The median of the six C_{test} was then deemed copy number for the chromosome 21 target site in the test sample.

[0202] For example, referring to **Table 6**, R_{test} and R_{control} for the target site Chr21_01 were obtained as follows. Chr21_01 was in the group consisting of twelve chromosome 21 target sites: Chr21_01, Chr21_05, Chr21_37, Chr21_53, Chr21_71, and Chr21_75 and six reference target sites: REF10q_A, REF20p_A, REF2p_D, REF4q_C, REF5p_C, and REF6q_A. Six R_{test} values for Chr21_01 target site in the test sample were obtained by dividing the peak intensity of the Chr21_01 target site with the peak intensity of each of the six reference target sites: REF10q_A, REF20p_A, REF2p_D, REF4q_C, REF5p_C, and REF6q_A. Similarly, six R_{test} values for Chr21_01 target site in the control sample (i.e., the M0 DNA sample) were obtained. For each of the six reference target sites: REF10q_A, REF20p_A, REF2p_D, REF4q_C, REF5p_C, and REF6q_A, a C_{test} was derived for Chr21_01 target site. Therefore, a total of six C_{test} were derived for Chr21_01 target site in the test sample. The median of the six C_{test} values was deemed as the copy number for Chr21_01 target site in the test sample.

[0203] As such the copy number for each of the 96 chromosome 21 target sites was calculated. Because the testing was repeated three times for each DNA sample, three R values for each of the 96 chromosome 21 target sites could be derived from the testing results for each sample. See **Table 7** for the R values. The median copy number value in each testing for the 96 chromosome 21 target sites was considered the copy number of chromosome 21 in the testing and listed in the last row in **Table 7**. In addition, the copy number of chromosome 21 in each DNA sample was derived by averaging the three median values in the three repeated tests. For example, the copy number for M4 DNA sample was 2.037, which is the average of 2.04, 2.03 and 2.04.

Table 7. Copy number measurement of human chromosome 21. R1, R2, and R3 refer to calculated copy numbers in the three repeated testing for each DNA sample.

PROBE LOCUS	M0			M2			M4			M8			M16		
	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3
Chr21_01	2.00	2.12	1.99	1.86	1.95	1.95	1.95	1.98	1.97	2.05	1.95	2.00	2.23	2.09	2.04
Chr21_02	2.08	2.07	1.96	1.98	1.99	1.91	1.88	1.96	1.83	1.95	2.02	1.82	2.18	2.02	2.22
Chr21_03	2.04	2.00	1.96	1.90	1.95	2.09	1.93	2.04	1.99	2.12	2.07	1.91	2.11	2.18	2.14
Chr21_04	2.06	1.97	2.01	1.93	2.06	2.02	2.07	2.18	2.09	2.05	2.02	2.13	2.19	2.24	1.94
Chr21_05	2.08	1.94	2.00	2.06	1.95	1.88	1.99	2.08	2.14	2.08	1.97	2.13	2.19	2.08	2.10
Chr21_06	2.03	2.11	2.00	1.93	2.08	2.00	1.91	2.12	1.98	2.16	2.05	2.06	2.18	2.16	2.14
Chr21_07	2.04	2.02	1.99	2.03	1.95	2.03	2.07	1.90	2.07	2.18	1.98	2.02	2.10	2.11	2.07
Chr21_08	1.93	2.00	2.17	2.00	2.00	1.97	2.06	2.06	2.07	1.97	1.92	2.25	2.18	2.30	2.20
Chr21_09	1.92	2.17	1.95	2.12	2.26	2.17	2.18	2.10	2.06	2.21	2.21	2.06	2.29	2.34	2.12
Chr21_10	2.05	2.00	1.94	2.06	2.10	1.93	2.06	1.96	2.11	2.17	2.02	2.22	2.10	2.17	2.12
Chr21_11	2.12	1.97	1.96	2.15	2.05	1.93	2.00	1.98	2.13	2.21	2.16	2.10	2.22	2.11	2.24
Chr21_12	1.92	2.09	2.09	2.16	2.31	2.11	1.96	1.97	2.12	2.18	2.04	2.20	2.29	2.19	2.31
Chr21_13	1.90	2.17	1.96	2.24	2.07	2.10	2.00	1.95	1.99	2.05	1.98	1.94	2.23	1.96	2.17
Chr21_14	1.94	2.09	2.06	1.99	2.00	2.01	2.07	2.05	2.02	2.00	1.97	2.09	2.22	2.19	2.18
Chr21_15	2.00	2.11	1.94	2.01	2.13	2.17	2.09	1.96	2.08	2.18	2.27	2.20	2.23	2.16	2.17
Chr21_16	1.98	1.98	1.97	2.00	1.93	2.03	1.98	1.94	2.24	2.08	2.11	2.18	2.05	2.15	2.19
Chr21_17	2.00	2.08	1.94	1.69	2.08	2.02	1.99	2.05	2.01	2.24	1.94	2.04	1.91	2.06	2.04
Chr21_18	1.97	2.06	2.03	1.99	1.92	2.04	2.04	2.03	2.02	2.08	2.11	2.08	2.09	2.10	2.03
Chr21_19	2.00	2.06	1.97	2.22	2.16	2.06	2.14	2.08	2.03	2.15	2.16	2.17	2.16	2.17	2.21
Chr21_20	2.13	2.01	2.03	1.92	1.86	2.13	2.06	2.17	2.01	2.11	2.12	2.17	2.19	2.01	2.14
Chr21_21	2.07	2.00	2.00	1.95	1.91	2.18	1.98	2.07	2.00	2.25	1.99	2.09	1.95	2.08	1.97
Chr21_22	2.04	1.97	2.04	2.09	2.05	2.14	2.08	2.07	2.13	2.06	1.95	2.14	2.09	2.21	2.20
Chr21_23	2.02	2.06	1.93	2.09	2.09	1.99	2.16	2.17	2.10	2.28	2.16	2.28	2.23	2.15	2.20
Chr21_24	1.95	2.00	2.08	1.92	2.00	1.96	2.05	2.04	2.04	2.04	1.97	2.09	2.13	2.18	2.19
Chr21_25	2.08	2.01	2.04	1.89	2.08	1.99	1.98	2.03	1.97	2.05	2.15	1.99	2.35	2.06	1.97
Chr21_26	2.01	1.95	2.09	2.23	1.95	2.09	2.01	1.99	1.94	2.02	2.08	1.99	2.07	2.10	2.04
Chr21_27	2.08	1.90	2.10	1.89	1.82	1.99	2.06	2.31	2.33	2.06	1.80	2.07	2.11	2.04	2.40
Chr21_28	2.09	1.92	2.00	1.87	2.02	1.99	2.04	2.00	1.97	2.11	1.98	2.06	2.06	2.09	2.09
Chr21_29	1.99	1.99	1.98	2.00	2.01	1.97	1.94	1.98	1.95	2.03	2.00	2.06	2.18	2.00	2.19
Chr21_30	2.05	1.98	1.98	1.90	1.97	1.95	2.05	1.94	1.98	2.16	2.02	2.01	2.11	2.02	2.05
Chr21_31	1.99	2.02	1.86	1.83	1.87	1.93	1.95	1.96	1.88	1.98	2.08	1.94	2.11	1.94	1.98
Chr21_32	1.98	2.01	1.93	2.08	1.97	2.01	2.13	2.01	2.00	2.02	2.03	2.05	2.19	2.02	2.11

(continued)

PROBE LOCUS	M0			M2			M4			M8			M16		
	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3
Chr21_33	2.03	1.99	2.01	2.01	1.99	2.02	1.93	1.95	2.10	2.17	2.12	2.07	2.12	2.10	2.17
Chr21_34	1.89	1.97	2.06	2.04	2.03	2.02	2.10	2.14	2.11	2.09	2.00	2.00	1.99	2.01	2.12
Chr21_35	2.01	2.01	1.83	1.96	2.02	1.94	1.91	1.97	1.99	1.93	1.99	1.98	2.11	2.06	2.07
Chr21_36	2.07	1.86	2.03	1.99	2.10	2.01	2.05	2.03	2.07	2.11	1.96	2.22	2.12	2.31	2.32
Chr21_37	2.01	1.98	1.95	1.92	1.91	1.87	1.91	1.97	2.05	2.06	2.07	2.00	2.17	1.99	1.97
Chr21_38	1.95	1.95	2.21	2.18	2.07	2.06	2.01	1.89	2.01	2.10	2.04	1.95	2.21	2.19	2.17
Chr21_39	2.02	2.10	1.90	2.06	2.10	2.22	2.25	2.05	2.04	2.19	2.13	2.15	2.28	2.09	2.27
Chr21_40	1.90	1.93	2.09	1.96	1.91	1.96	1.97	2.02	1.89	1.95	2.10	1.99	1.93	2.15	2.17
Chr21_41	2.10	2.06	1.97	2.16	2.19	2.11	1.99	2.06	2.05	2.25	1.99	2.27	2.31	2.16	2.33
Chr21_42	2.06	1.93	2.04	2.22	1.96	2.13	2.12	2.02	2.01	2.20	2.28	2.05	2.23	2.23	2.37
Chr21_43	2.04	2.07	1.98	2.19	2.04	2.10	2.08	2.14	2.12	2.22	2.10	2.12	2.28	2.23	2.26
Chr21_44	2.08	1.91	2.00	2.02	1.92	1.87	2.14	2.03	2.04	2.15	2.05	1.90	2.05	2.18	2.14
Chr21_45	1.97	1.94	2.01	1.88	1.83	1.83	1.86	2.00	1.92	1.84	1.81	1.96	1.97	1.93	1.99
Chr21_46	2.00	2.13	2.01	2.21	2.19	2.32	2.14	2.09	2.20	2.20	2.27	2.11	2.45	2.47	2.84
Chr21_47	2.20	1.98	2.02	2.18	2.11	2.02	2.06	2.04	2.02	2.22	2.06	2.10	2.15	2.06	2.31
Chr21_48	2.03	1.97	1.85	1.78	1.76	1.86	2.02	2.07	1.99	2.08	1.91	2.13	2.20	2.22	2.18
Chr21_49	1.96	2.09	1.98	1.95	2.08	1.99	2.07	1.97	2.10	2.10	2.06	2.24	1.98	2.18	2.03
Chr21_50	1.96	1.96	2.03	2.02	2.13	2.08	2.11	2.09	1.99	2.21	2.15	2.09	2.09	2.27	2.11
Chr21_51	2.01	2.02	2.03	2.14	2.00	2.04	1.97	1.97	1.96	2.26	2.13	2.05	2.31	2.13	2.14
Chr21_52	2.04	2.04	1.99	2.20	2.06	2.01	2.11	2.11	2.21	2.10	2.00	2.25	2.25	2.39	2.06
Chr21_53	1.95	2.07	1.83	1.81	1.93	1.82	2.03	2.11	1.92	1.85	2.01	2.12	2.13	2.05	2.01
Chr21_54	2.05	1.91	2.04	2.07	2.07	2.03	2.01	1.91	2.02	2.03	2.20	2.09	2.32	2.22	2.09
Chr21_55	2.06	1.97	2.00	1.96	1.97	1.95	1.97	2.08	1.99	1.92	2.02	2.11	2.05	2.08	1.96
Chr21_56	2.10	2.01	1.91	2.10	1.98	1.96	1.91	1.88	1.93	2.03	1.93	2.03	2.18	1.99	1.94
Chr21_57	2.03	1.97	2.14	2.13	2.14	2.17	1.99	1.98	1.96	2.17	2.16	1.88	2.39	1.93	2.35
Chr21_58	1.91	1.99	2.01	2.00	2.02	1.97	2.06	2.00	2.13	1.97	1.93	2.12	2.06	2.17	2.21
Chr21_59	1.97	2.03	2.01	2.13	2.00	2.03	2.06	2.07	2.06	1.99	2.15	2.09	2.09	2.28	2.12
Chr21_60	1.98	2.00	2.16	1.96	1.98	2.07	2.03	2.07	2.16	2.05	2.08	2.07	1.98	2.16	2.16
Chr21_61	2.02	1.99	2.11	2.23	1.99	2.09	1.93	2.01	2.07	2.11	2.09	2.06	2.20	2.17	2.05
Chr21_62	2.10	2.01	1.92	1.84	2.21	2.18	2.13	2.05	2.08	2.21	2.08	2.18	2.23	2.24	2.14
Chr21_63	1.95	2.00	2.01	2.12	1.97	2.02	2.07	1.96	1.98	2.04	2.11	2.09	2.22	2.06	2.15
Chr21_64	1.91	2.15	2.05	2.19	2.07	2.12	2.14	1.96	2.15	2.22	2.20	2.14	2.15	2.14	2.26

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PROBE LOCUS	M0			M2			M4			M8			M16		
	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3
Chr21_65	1.99	2.02	2.05	2.01	1.97	2.16	1.94	1.94	1.97	1.98	2.02	2.03	2.16	2.12	2.30
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Chr21_68	1.98	1.93	2.03	2.04	2.07	1.90	2.07	2.02	2.05	2.10	2.06	2.11	2.18	2.12	2.15
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Chr21_70	2.20	1.91	2.09	1.93	1.90	2.11	2.11	2.11	2.02	2.04	1.87	2.08	2.21	2.21	2.10
Chr21_71	2.12	2.07	1.97	2.06	2.08	2.07	2.10	2.09	2.08	2.09	2.10	1.99	2.19	2.13	2.15
Chr21_72	2.01	2.01	1.97	1.83	1.96	1.92	2.02	1.97	1.98	2.03	1.92	2.09	2.11	2.11	2.05
Chr21_73	2.23	1.98	2.00	2.04	2.04	1.95	2.03	1.96	2.22	2.00	1.96	2.16	1.99	2.21	2.17
Chr21_74	2.05	1.89	2.04	2.09	2.08	1.99	2.05	2.00	2.07	2.13	2.17	2.04	2.20	2.17	2.34
Chr21_75	2.10	2.09	1.98	2.04	2.23	1.79	2.01	2.04	2.08	2.17	2.00	1.96	2.03	2.16	2.18
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Chr21_90	2.03	1.98	1.94	2.08	2.31	2.00	2.00	2.16	2.11	2.11	2.08	1.98	2.16	2.25	2.22
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Chr21_94	2.01	1.92	2.03	1.93	1.97	2.17	2.09	1.77	2.03	2.00	2.12	2.12	2.24	2.13	2.15
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(continued)

PROBE LOCUS	M0			M2			M4			M8			M16		
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Median->	2.01	2.00	2.00	2.02	2.01	2.02	2.04	2.03	2.04	2.09	2.06	2.08	2.17	2.14	2.16

[0204] This exemplary method was sensitive enough to detect a chromosome 21 copy number increase as small as about 2%. The copy number obtained for the five DNA samples were statistically analyzed by performing Student's *t* test using M0 as the control sample. As shown in **Figure 14**, there was no statistically significant difference ($P=0.1008<0.01$) between the copy number in M2 versus M0, suggesting that the exemplary method was not sensitive enough to detect the copy number difference between sample M2 and sample M0. However, there was statistically significant difference ($P=0.0065<0.01$) between the copy number in M4 versus M0, suggesting that the exemplary method was sensitive enough to detect the copy number difference between sample M4 and sample M0. The same is true between M8 and M0, and between M16 and M0 because the P values were 0.0028 and 0.0001, respectively. Because the designed copy number of Chromosome 21 in M4 sample was 2.04, which was a 2% increase from the normal copy number of 2.00, the exemplary method was able to detect an increase as small as 2% in a statistically significant manner.

[0205] Indeed, there was a strong correlation between the calculated or measured copy number and the designed copy number in the five DNA samples. By plotting the measured copy number over the designed copy number in the five DNA samples, a strong correlation could be seen. As shown in **Figure 15**, the linear correlation between the two corresponding values gave rise to a R^2 of 0.9977.

[0206] Therefore, the exemplary method according to the present invention could be used to detect small copy number changes. The exemplary method was sensitive enough to detect a 2% copy number change. The sensitivity may be increased if more regions on chromosome 21 are used as target sites, if more reference target sites are used for some of the target sites on chromosome 21, or if the testing in a sample was repeated for additional times. As such, a person skilled in the art may design and use probes for more target sites on chromosome 21, e.g., 100-500 target sites on chromosome 21, design and use probes for more reference target sites, e.g., 50 reference target sites, and/or repeat the testing in the sample for additional time, e.g., 6-10 times, and by doing so the sensitivity for detecting small copy number changes of chromosome 21 may be increased so that a change less than 2% can be achieved.

SEQUENCE LISTING

[0207]

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EP 2 893 034 B9

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EP 2 893 034 B9

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EP 2 893 034 B9

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EP 2 893 034 B9

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EP 2 893 034 B9

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EP 2 893 034 B9

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EP 2 893 034 B9

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Claims

45 1. A method of assaying nucleic acids in a sample, comprising the steps of:

a) adding multiple sets of probes into the sample to form a mixture, each set of probes comprising:

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i. a first probe having a first portion at least partially complementary to a first region of a target nucleic acid in the sample and a second portion forming a first primer binding site;

ii. a second probe having a first portion at least partially complementary to a second region of the target nucleic acid in the sample and a second portion forming a second primer binding site, wherein the 5' end of the first probe is adjacent to the 3' end of the second probe when both probes are hybridized to the target nucleic acid;

55

b) denaturing nucleic acids in the mixture;

c) hybridizing the set of probes to the complementary regions of the target nucleic acid;

d) performing a ligation reaction with a ligase enzyme on the set of hybridized probes to connect the adjacent

5' end of the first probe and the 3' end of the second probe to form a third probe, wherein steps b-d are repeated 1-100 times;

e) amplifying the third probe with multiple sets of primers to obtain an amplification product, each set of primers comprising:

- i. a first primer at least partially complementary to the first primer binding site in one or more first probes of the multiple sets of probes;
- ii. a second primer at least partially complementary to the second primer binding site in one or more second probes of the multiple sets of probes;

f) assaying the presence, absence or quantity of the target nucleic acid in the sample by determining the presence, absence or quantity of the third probe in the amplification product; and

wherein at least one primer of each set of primers is labeled with a detectable moiety; and at least one primer of the multiple sets of primers includes a stuffer sequence; and at least one probe of the multiple sets of probes includes a stuffer sequence, wherein the measurement is carried out using capillary electrophoresis.

2. The method of claim 1, wherein the stuffer sequence in the at least one primer of the multiple sets of primers has about 10 to about 500 nucleotides, preferably about 10 to about 60 nucleotides; and/or the stuffer sequence in the at least one probe of the multiple sets of probes has about 1 to about 200 nucleotides, preferably about 1 to about 55 nucleotides.

3. The method of claim 1 or 2, wherein at least one primer of each set of primers includes an oligonucleotide comprising a sequence GTTCTT or a functional equivalent variant of the oligonucleotide comprising a sequence GTTCTT.

4. The method of any one of preceding claims, wherein the determination of the presence, absence or quantity of the third probe in the amplification product is carried out by measuring the presence, absence or quantity of the third probe in the amplification product on the basis of detectable moieties, fragment sizes, or both.

5. The method of any one of preceding claims, wherein the moiety is a fluorescent dye; preferably the moiety is a fluorescent dye selected from the group consisting of FAM (5-or 6-carboxyfluorescein), VIC, NED, PET, Fluorescein, FITC, IRD-700/800, CY3, CY5, CY3.5, CY5.5, HEX, TET, TAMRA, JOE, ROX, BODIPY TMR, Oregon Green, Rhodamine Green, Rhodamine Red, Texas Red, and Yakima Yellow.

6. The method of any one of preceding claims, wherein the target nucleic acid is the dystrophin gene having a deletion of one or more exons and the sets of probes for assaying the dystrophin gene comprise one or more probe pairs selected from SEQ ID NOs: 158-541; or the target nucleic acid corresponds to a part of human chromosome 21 and the sets of probes for assaying the part of human chromosome 21 comprise probe pairs selected from SEQ ID NOs: 559-942.

7. The method of any one of preceding claims, wherein the denaturing step is carried at about 90°C to about 99°C for about 5 seconds to about 30 minutes, and the hybridization and the ligation steps are carried out simultaneously at about 4°C to about 70°C for about 1 minute to about 48 hours, preferably the denaturing step is carried at about 95°C for about 30 seconds, and the hybridization and the ligation steps are carried out simultaneously at about 58°C for about 4 hours, and the steps of denaturing, hybridization and ligation are repeated 4 times.

8. The method of any one of preceding claims, wherein two or more sets of probes are used to hybridize to two or more target sites in the target nucleic acid, with each set of probes hybridizing to a different target site.

9. The method of any one of preceding claims, wherein the target nucleic acid has a quantitative variation of about 0.1% to about 30% between two samples.

10. The method of any one of preceding claims, wherein one set of primers is used to amplify a group of the third probes, said group of the third probes comprises multiple third probes which are formed from multiple sets of probes hybridizing to multiple target sites and from multiple sets of reference probes hybridizing to multiple reference target sites.

11. The method of any one of preceding claims, wherein the multiple third probes in the group are formed from about 1 to about 100 sets of probes hybridizing to target sites and about 1 to about 100 sets of reference probes.

12. The method of any one of preceding claims, wherein about 50 to about 500 sets of probes are used to hybridize to about 50 to about 500 target sites on the target nucleic acid.

13. The method of any one of preceding claims, wherein the target nucleic acid corresponds to at least a part of human chromosome 21, human chromosome 18, human chromosome 13, human chromosome region 22q11.2, or the pseudoautosomal regions of human chromosomes X or Y in a maternal blood or urine sample.

14. The method of any one of preceding claims, wherein the copy number of each target site is determined by the following four steps: a) a ratio of the quantity of the third probe targeting the target site to the quantity of the third probe in the same group targeting one reference sites are calculated; b) a copy number value is calculated by this ratio value in a test sample divided by the corresponding ratio value in a control sample or the median value of the corresponding ratio values in all control samples or all test samples, and then times 2; c) more copy number values are calculated by repeating a-b by using the quantity of another third probe in the same group targeting another reference sites in step a; d) the copy number of each target site is calculated by taking the average or median of all the copy number values with or without abandoning egregious value.

15. The method of any one of preceding claims, further comprising a step of determining the copy number of the target nucleic acid in a sample by taking the average or median of the copy numbers of all target sites on the target nucleic acid or by taking the average or median of the copy numbers of all target sites on the target nucleic acid after abandoning egregious values.

16. A kit for assaying nucleic acids in a sample according to claims 1-15, comprising:

a) multiple sets of probes corresponding to a target nucleic acid, each set of probes comprising:

- i. a first probe having a first portion at least partially complementary to a first region of a target nucleic acid in the sample and a second portion forming a first primer binding site;
- ii. a second probe having a first portion at least partially complementary to a second region of the target nucleic acid in the sample and a second portion forming a second primer binding site, wherein the 5' end of the first probe is adjacent to the 3' end of the second probe when both probes are hybridized to the target nucleic acid and the first and the second probes may be ligated to form a third probe;

b) multiple sets of primers for amplifying the third probe, wherein each set of multiple sets of primers comprising:

- i. a first primer at least partially complementary to the first primer binding site in one or more first probes of the multiple sets of probes;
- ii. a second primer at least partially complementary to the second primer binding site in one or more second probes of the multiple sets of probes;

c) reagents including a ligase, a buffer for a ligation reaction, a DNA polymerase, a buffer for a polymerase chain reaction, or a combination thereof; and

d) optionally a brochure containing instructions of using the kit;

wherein at least one primer of each set of primers is labeled with a detectable moiety; and at least one primer of the multiple sets of primers includes a stuffer sequence; and at least one probe of the multiple sets of probes includes a stuffer sequence, wherein the measurement is carried out using capillary electrophoresis.

Patentansprüche

1. Verfahren zur Bestimmung von Nucleinsäuren in einer Probe, umfassend die Schritte:

a) Hinzufügen von mehreren Mengen von Sonden zu der Probe unter Bildung eines Gemischs, wobei jede Menge von Sonden Folgendes umfasst:

- i. eine erste Sonde mit einem ersten Teil, der wenigstens teilweise komplementär zu einem ersten Bereich einer Zielnucleinsäure in der Probe ist, und einem zweiten Teil, der eine erste Primerbindungsstelle bildet;
- ii. eine zweite Sonde mit einem ersten Teil, der wenigstens teilweise komplementär zu einem zweiten

Bereich der Zielnucleinsäure in der Probe ist, und einem zweiten Teil, der eine zweite Primerbindungsstelle bildet, wobei das 5'-Ende der ersten Sonde dem 3'-Ende der zweiten Sonde benachbart ist, wenn beide Sonden mit der Zielnucleinsäure hybridisiert sind;

- b) Denaturieren der Nucleinsäuren in dem Gemisch;
- c) Hybridisieren der Menge von Sonden mit den komplementären Bereichen der Zielnucleinsäure;
- d) Durchführen einer Ligationsreaktion mit einem Ligaseenzym an der Menge der hybridisierten Sonden, um das benachbarte 5'-Ende der ersten Sonde und das 3'-Ende der zweiten Sonde unter Bildung einer dritten Sonde miteinander zu verbinden, wobei die Schritte b-d 1-100mal wiederholt werden;
- e) Amplifizieren der dritten Sonde mit mehreren Mengen von Primern unter Erhalt eines Amplifikationsprodukts, wobei jede Menge von Primern Folgendes umfasst:

- i. einen ersten Primer, der wenigstens teilweise zu der ersten Primerbindungsstelle in einer oder mehreren ersten Sonden der mehreren Mengen von Sonden komplementär ist;
- ii. einen zweiten Primer, der wenigstens teilweise zu der zweiten Primerbindungsstelle in einer oder mehreren zweiten Sonden der mehreren Mengen von Sonden komplementär ist;

- f) Bestimmen der Anwesenheit, Abwesenheit oder Menge der Zielnucleinsäure in der Probe durch Bestimmen der Anwesenheit, Abwesenheit oder Menge der dritten Sonde in dem Amplifikationsprodukt; und

wobei wenigstens ein Primer jeder Menge von Primern mit einer nachweisbaren Struktureinheit markiert ist und wenigstens ein Primer der mehreren Mengen von Primern eine Füllsequenz umfasst und wenigstens eine Sonde der mehreren Mengen von Sonden eine Füllsequenz umfasst, wobei die Messung durchgeführt wird, indem man Kapillarelektrophorese verwendet.

2. Verfahren gemäß Anspruch 1, wobei die Füllsequenz in dem wenigstens einen Primer der mehreren Mengen von Primern etwa 10 bis etwa 500 Nucleotide, vorzugsweise etwa 10 bis etwa 60 Nucleotide, aufweist; und/oder die Füllsequenz in der wenigstens einen Sonde der mehreren Mengen von Sonden etwa 1 bis etwa 200 Nucleotide, vorzugsweise etwa 1 bis etwa 55 Nucleotide, aufweist.
3. Verfahren gemäß Anspruch 1 oder 2, wobei wenigstens ein Primer jeder Menge von Primern ein Oligonucleotid, das eine Sequenz GTTCTT umfasst, oder eine funktionell äquivalente Variante des Oligonucleotids, das eine Sequenz GTTCTT umfasst, umfasst.
4. Verfahren gemäß einem der vorstehenden Ansprüche, wobei die Bestimmung der Anwesenheit, Abwesenheit oder Menge der dritten Sonde in dem Amplifikationsprodukt durchgeführt wird, indem man die Anwesenheit, Abwesenheit oder Menge der dritten Sonde in dem Amplifikationsprodukt auf der Basis von nachweisbaren Struktureinheiten, Fragmentgrößen oder beiden misst.
5. Verfahren gemäß einem der vorstehenden Ansprüche, wobei die Struktureinheit ein Fluoreszenzfarbstoff ist; wobei die Struktureinheit vorzugsweise ein Fluoreszenzfarbstoff ist, der aus der Gruppe ausgewählt ist, die aus FAM (5- oder 6-Carboxyfluorescein), VIC, NED, PET, Fluorescein, FITC, IRD-700/800, CY3, CY5, CY3.5, CY5.5, HEX, TET, TAMRA, JOE, ROX, BODIPY-TMR, Oregongrün, Rhodamingrün, Rhodaminrot, Texasrot und Yakima-gelb besteht.
6. Verfahren gemäß einem der vorstehenden Ansprüche, wobei die Zielnucleinsäure das Dystrophin-Gen ist, das eine Deletion von einem oder mehreren Exons aufweist, und die Mengen von Sonden zur Bestimmung des Dystrophin-Gens ein oder mehrere Sondenpaare umfassen, die aus SEQ ID Nr. 158-541 ausgewählt sind; oder die Zielnucleinsäure einem Teil des humanen Chromosoms 21 entspricht und die Mengen von Sonden zur Bestimmung des Teils des humanen Chromosoms 21 Sondenpaare umfassen, die aus SEQ ID Nr. 559-942 ausgewählt sind.
7. Verfahren gemäß einem der vorstehenden Ansprüche, wobei der Denaturierungsschritt während etwa 5 Sekunden bis etwa 30 Minuten bei etwa 90 °C bis etwa 99 °C durchgeführt wird und der Hybridisierungs- und der Ligationsschritt gleichzeitig während etwa 1 Minute bis etwa 48 Stunden bei etwa 4 °C bis etwa 70 °C durchgeführt werden, vorzugsweise der Denaturierungsschritt während etwa 30 Sekunden bei etwa 95 °C durchgeführt wird und der Hybridisierungs- und der Ligationsschritt gleichzeitig während etwa 4 Stunden bei etwa 58 °C durchgeführt werden, und die Schritte der Denaturierung, Hybridisierung und Ligation 4-mal wiederholt werden.

8. Verfahren gemäß einem der vorstehenden Ansprüche, wobei zwei oder mehr Mengen von Sonden verwendet werden, um an zwei oder mehr Zielstellen in der Zielnucleinsäure zu hybridisieren, wobei jede Menge von Sonden an eine andere Zielstelle hybridisiert.
- 5 9. Verfahren gemäß einem der vorstehenden Ansprüche, wobei die Zielnucleinsäure eine quantitative Variation von etwa 0,1% bis etwa 30% zwischen zwei Proben aufweist.
10. Verfahren gemäß einem der vorstehenden Ansprüche, wobei eine Menge von Primern verwendet wird, um eine Gruppe der dritten Sonden zu amplifizieren, wobei die Gruppe der dritten Sonden mehrere dritte Sonden umfasst,
10 die aus mehreren Mengen von Sonden, welche an mehrere Zielstellen hybridisieren, und aus mehreren Mengen von Referenzsonden, welche an mehrere Referenzzielstellen hybridisieren, gebildet werden.
11. Verfahren gemäß einem der vorstehenden Ansprüche, wobei die mehreren dritten Sonden in der Gruppe aus etwa
15 1 bis etwa 100 Mengen von Sonden, die an Zielstellen hybridisieren, und etwa 1 bis etwa 100 Mengen von Referenzsonden gebildet werden.
12. Verfahren gemäß einem der vorstehenden Ansprüche, wobei etwa 50 bis etwa 500 Mengen von Sonden verwendet werden, um an etwa 50 bis etwa 500 Zielstellen auf der Zielnucleinsäure zu hybridisieren.
- 20 13. Verfahren gemäß einem der vorstehenden Ansprüche, wobei die Zielnucleinsäure wenigstens einem Teil des humanen Chromosoms 21, des humanen Chromosoms 18, des humanen Chromosoms 13, des humanen Chromosomenbereichs 22q11.2 oder der pseudoautosomalen Bereiche der humanen Chromosomen X oder Y in einer mütterlichen Blut- oder Urinprobe entspricht.
- 25 14. Verfahren gemäß einem der vorstehenden Ansprüche, wobei die Kopienzahl jeder Zielstelle durch die folgenden vier Schritte bestimmt wird: a) das Verhältnis der Menge der dritten Sonde, die die Zielstelle ansteuert, zur Menge der dritten Sonde in derselben Gruppe, die eine der Referenzstellen ansteuern, wird berechnet; b) der Wert der Kopienzahl wird berechnet durch diesen Verhältniswert in einer Testprobe, dividiert durch den entsprechenden Verhältniswert in einer Kontrollprobe oder den Medianwert der entsprechenden Verhältniswerte in allen Kontrollproben oder allen Testproben, und dann mal zwei; c) weitere Werte der Kopienzahl werden berechnet, indem man
30 a-b wiederholt und dabei die Menge einer anderen, dritten Sonde in derselben Gruppe, die in Schritt a eine andere Referenzstelle ansteuert, verwendet; d) die Kopienzahl jeder Zielstelle wird dadurch berechnet, dass man den Durchschnitt oder Medianwert aller Werte der Kopienzahlen mit oder ohne Weglassen von Ausreißern nimmt.
- 35 15. Verfahren gemäß einem der vorstehenden Ansprüche, weiterhin umfassend einen Schritt des Bestimmens der Kopienzahl der Zielnucleinsäure in einer Probe, indem man den Durchschnitt oder Medianwert der Kopienzahlen aller Zielstellen auf der Zielnucleinsäure nimmt oder indem man den Durchschnitt oder Medianwert der Kopienzahlen aller Zielstellen auf der Zielnucleinsäure nach Weglassen von Ausreißern nimmt.
- 40 16. Kit zur Bestimmung von Nucleinsäuren in einer Probe gemäß den Ansprüchen 1 bis 15, umfassend:

a) mehrere Mengen von Sonden, die einer Zielnucleinsäure entsprechen, wobei jede Menge von Sonden Folgendes umfasst:

45 i. eine erste Sonde mit einem ersten Teil, der wenigstens teilweise komplementär zu einem ersten Bereich einer Zielnucleinsäure in der Probe ist, und einem zweiten Teil, der eine erste Primerbindungsstelle bildet;
ii. eine zweite Sonde mit einem ersten Teil, der wenigstens teilweise komplementär zu einem zweiten Bereich der Zielnucleinsäure in der Probe ist, und einem zweiten Teil, der eine zweite Primerbindungsstelle bildet, wobei das 5'-Ende der ersten Sonde dem 3'-Ende der zweiten Sonde benachbart ist, wenn beide
50 Sonden mit der Zielnucleinsäure hybridisiert sind, und die erste und die zweite Sonde unter Bildung einer dritten Sonde miteinander ligiert sein können;

b) mehrere Mengen von Primern zum Amplifizieren der dritten Sonde, wobei jede Menge von mehreren Mengen von Primern Folgendes umfasst:

55 i. einen ersten Primer, der wenigstens teilweise zu der ersten Primerbindungsstelle in einer oder mehreren ersten Sonden der mehreren Mengen von Sonden komplementär ist;
ii. einen zweiten Primer, der wenigstens teilweise zu der zweiten Primerbindungsstelle in einer oder meh-

renen zweiten Sonden der mehreren Mengen von Sonden komplementär ist;

- c) Reagentien einschließlich einer Ligase, eines Puffers für eine Ligationsreaktion, einer DNA-Polymerase, eines Puffers für eine Polymerase-Kettenreaktion oder einer Kombination davon; und
- d) gegebenenfalls eine Broschüre, die Anweisungen zur Verwendung des Kits enthält;

wobei wenigstens ein Primer jeder Menge von Primern mit einer nachweisbaren Struktureinheit markiert ist und wenigstens ein Primer der mehreren Mengen von Primern eine Füllsequenz umfasst und wenigstens eine Sonde der mehreren Mengen von Sonden eine Füllsequenz umfasst, wobei die Messung durchgeführt wird, indem man Kapillarelektrophorese verwendet.

Revendications

1. Procédé de dosage d'acides nucléiques dans un échantillon, comprenant les étapes de :

a) ajout de multiples jeux de sondes dans l'échantillon pour former un mélange, chaque jeu de sondes comprenant :

- i. une première sonde ayant une première portion au moins partiellement complémentaire d'une première région d'un acide nucléique cible dans l'échantillon et une seconde portion formant un premier site de liaison à une amorce ;
- ii. une deuxième sonde ayant une première portion au moins partiellement complémentaire d'une seconde région de l'acide nucléique cible dans l'échantillon et une seconde portion formant un second site de liaison à une amorce, dans lequel l'extrémité 5' de la première sonde est adjacente à l'extrémité 3' de la deuxième sonde lorsque les deux sondes sont hybridées avec l'acide nucléique cible ;

b) la dénaturation d'acides nucléiques dans le mélange ;

c) l'hybridation du jeu de sondes avec les régions complémentaires de l'acide nucléique cible ;

d) la réalisation d'une réaction de ligature avec une enzyme ligase sur le jeu de sondes hybridées pour raccorder l'extrémité 5' adjacente de la première sonde et l'extrémité 3' de la deuxième sonde pour former une troisième sonde, dans lequel les étapes b à d sont répétées 1 à 100 fois ;

e) l'amplification de la troisième sonde avec de multiples jeux d'amorces pour obtenir un produit d'amplification, chaque jeu d'amorces comprenant :

- i. une première amorce au moins partiellement complémentaire du premier site de liaison à une amorce dans une ou plusieurs premières sondes des multiples jeux de sondes ;
- ii. une seconde amorce au moins partiellement complémentaire du second site de liaison à une amorce dans une ou plusieurs secondes sondes des multiples jeux de sondes ;

f) le dosage de la présence, de l'absence ou de la quantité de l'acide nucléique cible dans l'échantillon par détermination de la présence, de l'absence ou de la quantité de la troisième sonde dans le produit d'amplification ; et

dans lequel au moins une amorce de chaque jeu d'amorces est marquée avec une fraction détectable ; et au moins une amorce des multiples jeux d'amorces inclut une séquence d'extension ; et au moins une sonde des multiples jeux de sondes inclut une séquence d'extension, dans lequel la mesure est réalisée à l'aide d'une électrophorèse capillaire.

2. Procédé selon la revendication 1, dans lequel la séquence d'extension dans la au moins une amorce des multiples jeux d'amorces a environ 10 à environ 500 nucléotides, de préférence environ 10 à environ 60 nucléotides ; et/ou

la séquence d'extension dans la au moins une sonde des multiples jeux de sondes a environ 1 à environ 200 nucléotides, de préférence environ 1 à environ 55 nucléotides.

3. Procédé selon la revendication 1 ou 2, dans lequel au moins une amorce de chaque jeu d'amorces inclut un oligonucléotide comprenant une séquence GTTCTT ou un variant équivalent fonctionnel de l'oligonucléotide comprenant une séquence GTTCTT.

4. Procédé selon l'une quelconque des revendications précédentes, dans lequel la détermination de la présence, de l'absence ou de la quantité de la troisième sonde dans le produit d'amplification est effectuée par mesure de la présence, de l'absence ou de la quantité de la troisième sonde dans le produit d'amplification sur la base de fractions détectables, de tailles de fragment, ou des deux.

5. Procédé selon l'une quelconque des revendications précédentes, dans lequel la fraction est une teinte fluorescente ;

de préférence la fraction est une teinte fluorescente choisie dans le groupe consistant en FAM (5- ou 6-carboxyfluorescéine), VIC, NED, PET, Fluorescéine, FITC, IRD-700/800, CY3, CY5, CY3.5, CY5.5, HEX, TET, TAMRA, JOE, ROX, BODIPY TMR, vert d'Orégon, vert de Rhodamine, rouge de Rhodamine, rouge de Texas, et jaune de Yakima.

6. Procédé selon l'une quelconque des revendications précédentes, dans lequel l'acide nucléique cible est le gène de dystrophine ayant une délétion d'un ou plusieurs exons et les jeux de sondes destinés à doser le gène de dystrophine comprennent une ou plusieurs paires de sondes choisies parmi SEQ ID N° 158 à 541 ;

ou bien l'acide nucléique cible correspond à une partie du chromosome humain 21 et les jeux de sondes destinés à doser la partie du chromosome humain 21 comprennent des paires de sondes choisies parmi les SEQ ID N° 559 à 942.

7. Procédé selon l'une quelconque des revendications précédentes, dans lequel l'étape de dénaturation est effectuée à environ 90 °C à environ 99 °C pendant environ 5 secondes à environ 30 minutes, et les étapes d'hybridation et de ligature sont effectuées simultanément à environ 4 °C à environ 70 °C pendant environ 1 minute à environ 48 heures, de préférence l'étape de dénaturation est effectuée à environ 95 °C pendant environ 30 secondes, et les étapes d'hybridation et de ligature sont effectuées simultanément à environ 58 °C pendant environ 4 heures, et les étapes de dénaturation, hybridation et ligature sont répétées 4 fois.

8. Procédé selon l'une quelconque des revendications précédentes, dans lequel deux ou plus de deux jeux de sondes sont utilisés pour hybrider les deux ou plus de deux sites cibles dans l'acide nucléique cible, chaque jeu de sondes s'hybridant avec un site cible différent.

9. Procédé selon l'une quelconque des revendications précédentes, dans lequel l'acide nucléique cible a une variation quantitative d'environ 0,1 % à environ 30 % entre deux échantillons.

10. Procédé selon l'une quelconque des revendications précédentes, dans lequel un jeu d'amorces est utilisé pour amplifier un groupe des troisièmes sondes, ledit groupe de troisièmes sondes comprend de multiples troisièmes sondes qui sont formées à partir de multiples jeux d'amorces s'hybridant avec de multiples sites cibles et à partir de multiples jeux de sondes de référence s'hybridant avec de multiples sites cibles de référence.

11. Procédé selon l'une quelconque des revendications précédentes, dans lequel les multiples troisièmes sondes dans le groupe sont formées d'environ 1 à environ 100 jeux de sondes s'hybridant avec des sites cibles et environ 1 à environ 100 jeux de sondes de référence.

12. Procédé selon l'une quelconque des revendications précédentes, dans lequel environ 50 à environ 500 jeux de sondes sont utilisés pour s'hybrider avec environ 50 à environ 500 sites cibles sur l'acide nucléique cible.

13. Procédé selon l'une quelconque des revendications précédentes, dans lequel l'acide nucléique cible correspond à au moins une partie du chromosome humain 21, du chromosome humain 18, du chromosome humain 13, de la région de chromosome humain 22q11.2, ou des régions pseudoautosomiques des chromosomes humains X ou Y dans un échantillon de sang ou d'urine maternel(le).

14. Procédé selon l'une quelconque des revendications précédentes, dans lequel le nombre de copies de chaque site cible est déterminé par les quatre étapes suivantes : a) un rapport de la quantité de la troisième sonde ciblant le site cible sur la quantité de la troisième sonde dans le même groupe ciblant un site de référence est calculé ; b) une valeur de nombre de copies est calculée par cette valeur de rapport dans un échantillon d'essai divisé par la valeur de rapport correspondante dans un échantillon témoin ou la valeur médiane des valeurs de rapport correspondantes dans tous les échantillons témoins ou tous les échantillons d'essai, puis fois 2 ; c) plus de valeurs de nombre de copies sont calculées en répétant les étapes a-b en utilisant la quantité d'une autre troisième sonde

dans le même groupe ciblant un autre site de référence dans l'étape a ; d) le nombre de copies de chaque site cible est calculé en prenant la moyenne ou la médiane de toutes les valeurs de nombre de copies avec ou sans abandon de valeur énorme.

5 **15.** Procédé selon l'une quelconque des revendications précédentes, comprenant en outre une étape de détermination du nombre de copies de l'acide nucléique cible dans un échantillon en prenant la moyenne ou la médiane des nombres de copies de tous les sites cibles sur l'acide nucléique cible ou en prenant la moyenne ou la médiane des nombres de copies de tous les sites cibles sur l'acide nucléique cible après abandon de valeurs aberrantes.

10 **16.** Nécessaire de dosage d'acides nucléiques dans un échantillon selon les revendications 1 à 15, comprenant :

a) de multiples jeux de sondes correspondant à un acide nucléique cible, chaque jeu de sondes comprenant :

15 i. une première sonde ayant une première portion au moins partiellement complémentaire d'une première région d'un acide nucléique cible dans l'échantillon et une seconde portion formant un premier site de liaison à une amorce ;

20 ii. une deuxième sonde ayant une première portion au moins partiellement complémentaire d'une seconde région de l'acide nucléique cible dans l'échantillon et une seconde portion formant un second site de liaison à une amorce, dans lequel l'extrémité 5' de la première sonde est adjacente à l'extrémité 3' de la deuxième sonde lorsque les deux sondes sont hybridées avec l'acide nucléique cible et les première et deuxième sondes peuvent être ligaturées pour former une troisième sonde ;

25 b) de multiples jeux d'amorces pour amplifier la troisième sonde, dans lequel chaque jeu de multiples jeux d'amorces comprend :

i. une première amorce au moins partiellement complémentaire du premier site de liaison à une amorce dans une ou plusieurs premières sondes des multiples jeux de sondes ;

30 ii. une seconde amorce au moins partiellement complémentaire du second site de liaison à une amorce dans une ou plusieurs secondes sondes des multiples jeux de sondes ;

c) des réactifs incluant une ligase, un tampon pour une réaction de ligature, une ADN polymérase, un tampon pour une amplification en chaîne par polymérase, ou l'une de leur combinaison ; et

d) facultativement une brochure contenant des instructions d'utilisation du nécessaire ;

35 dans lequel au moins une amorce de chaque jeu d'amorces est marquée avec une fraction détectable ; et au moins une amorce des multiples jeux d'amorces inclut une séquence d'extension ; et au moins une sonde des multiples jeux de sondes inclut une séquence d'extension, dans lequel la mesure est réalisée à l'aide d'une électrophorèse capillaire.

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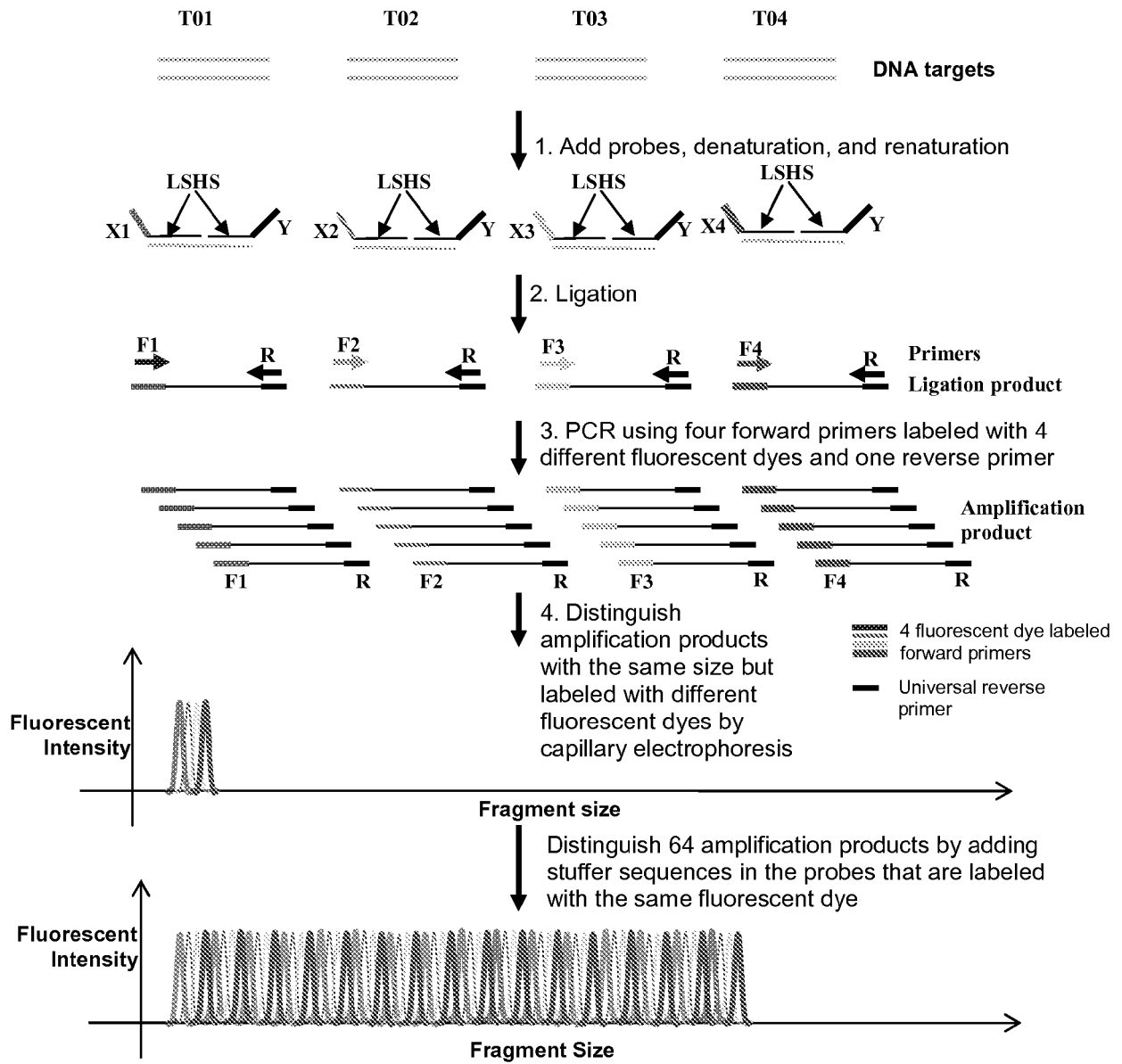


Figure 1

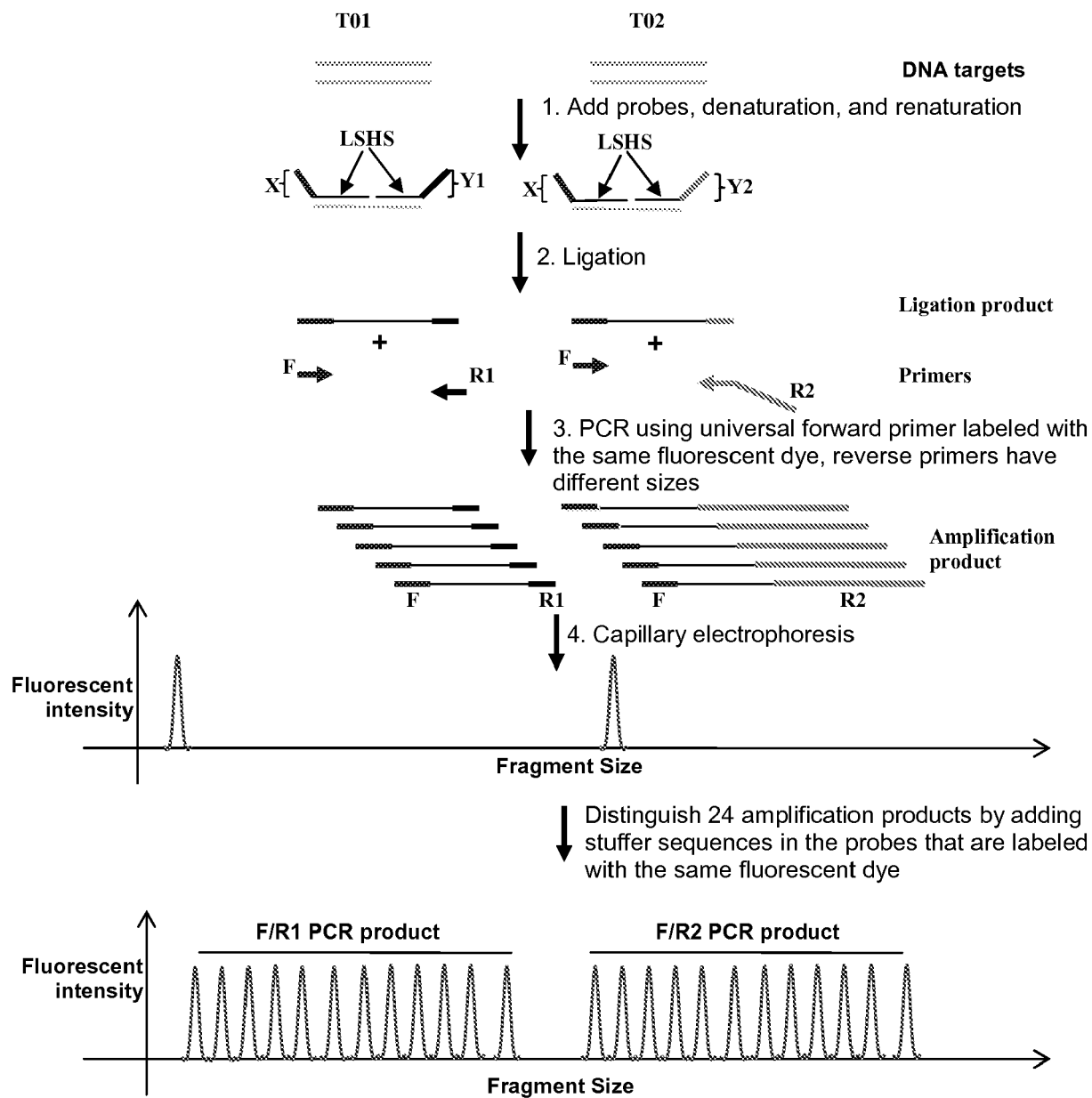


Figure 2

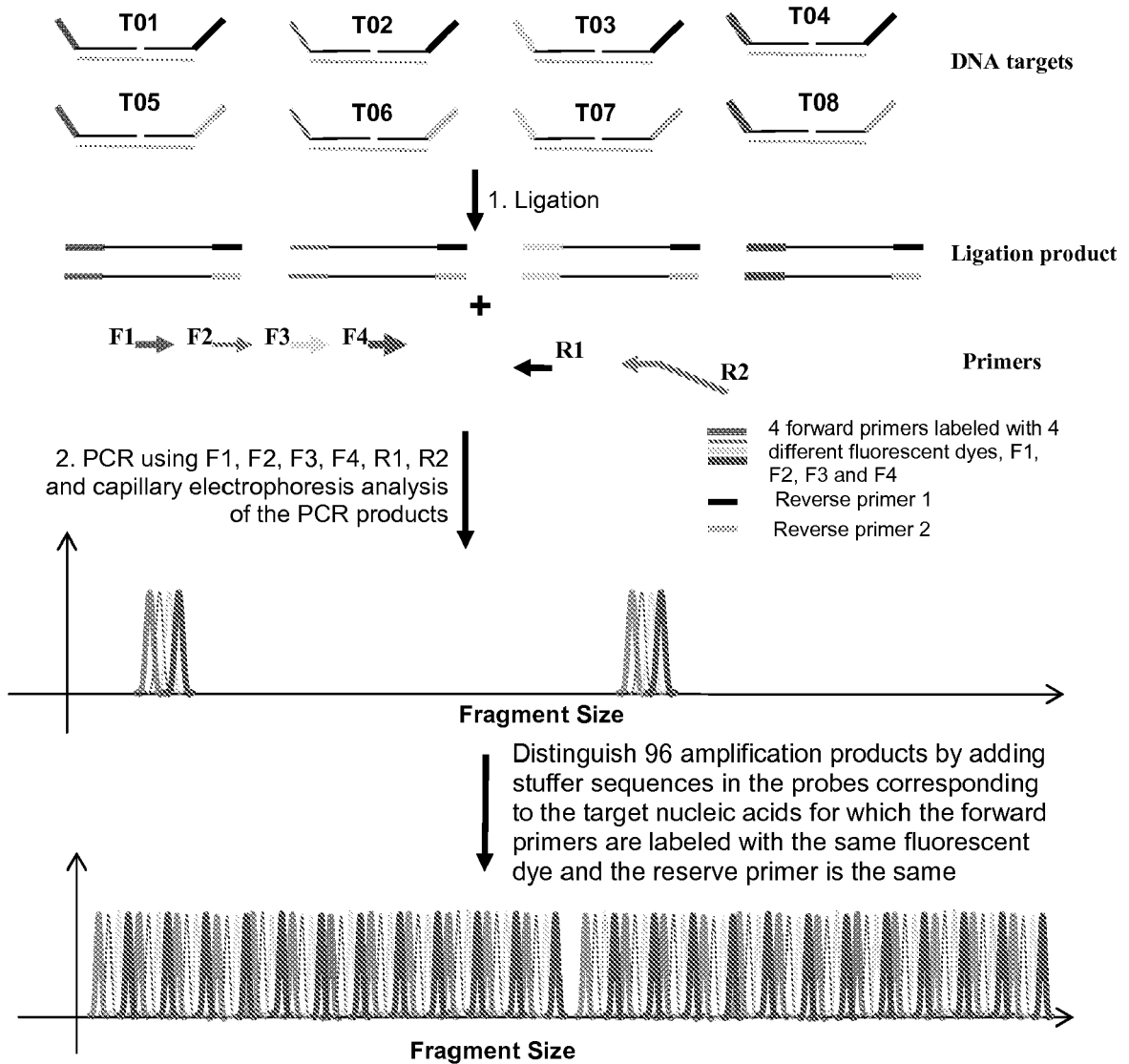


Figure 3

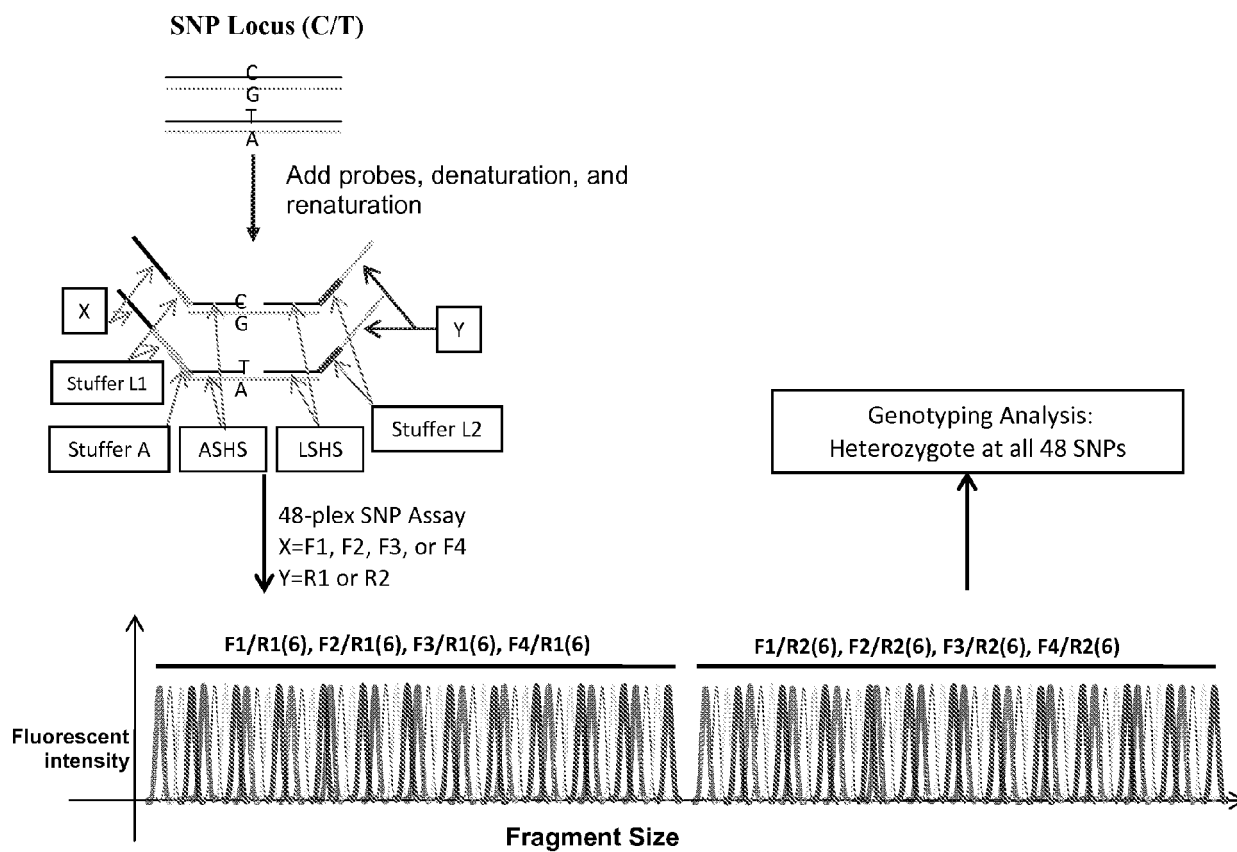


Figure 4

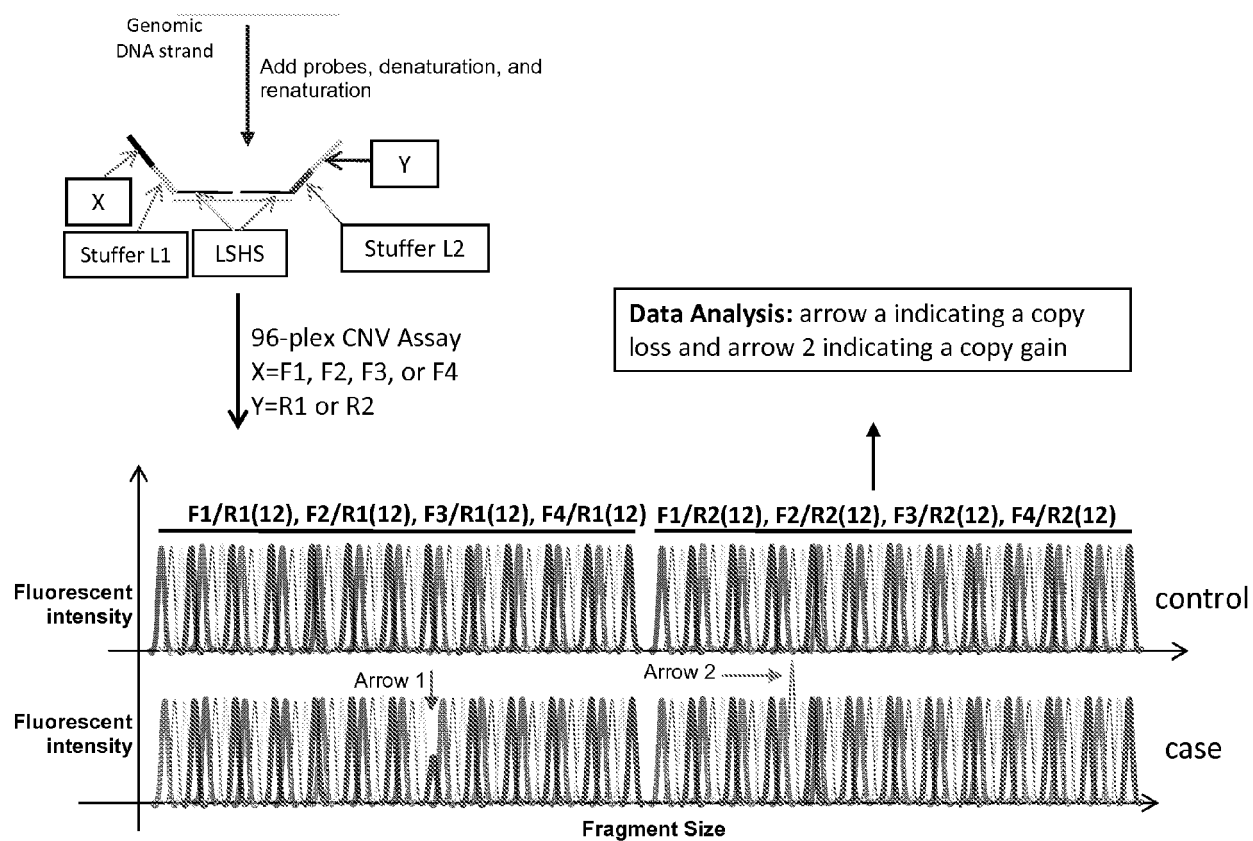


Figure 5

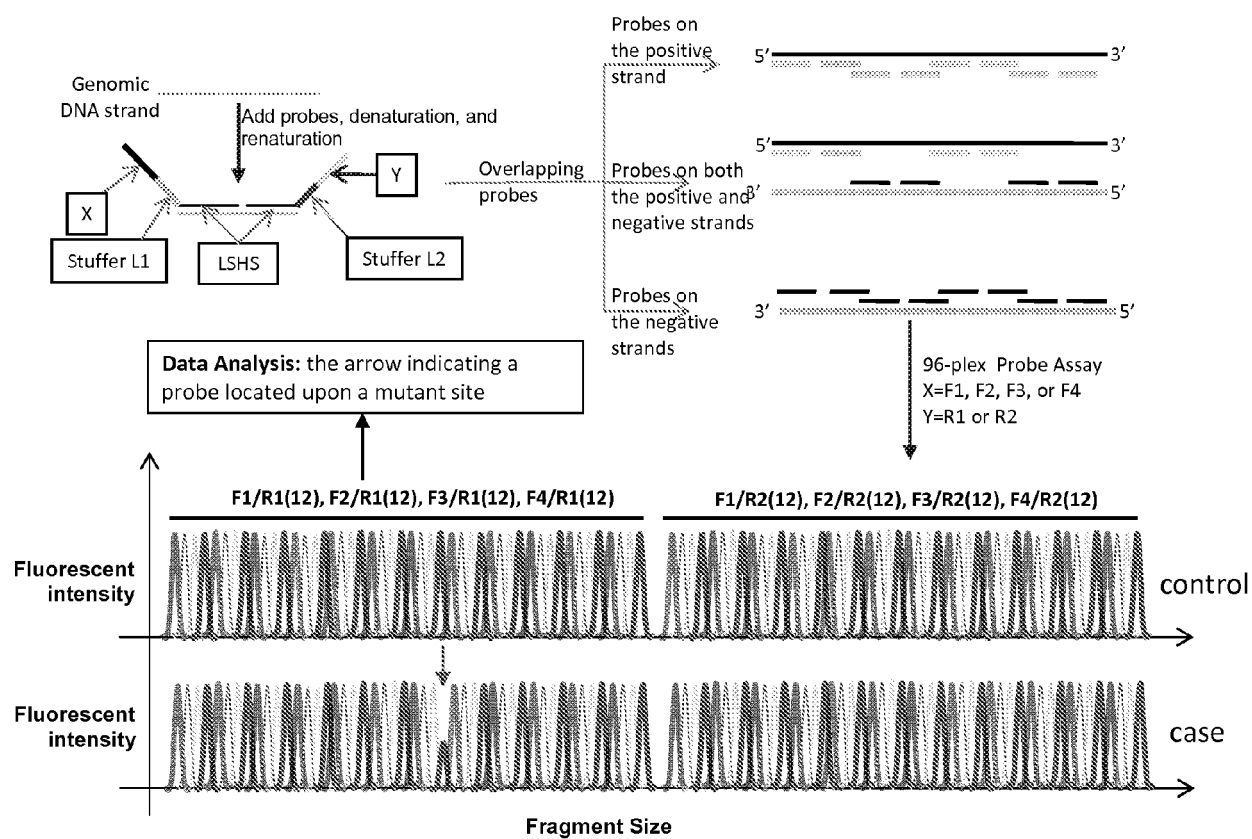


Figure 6

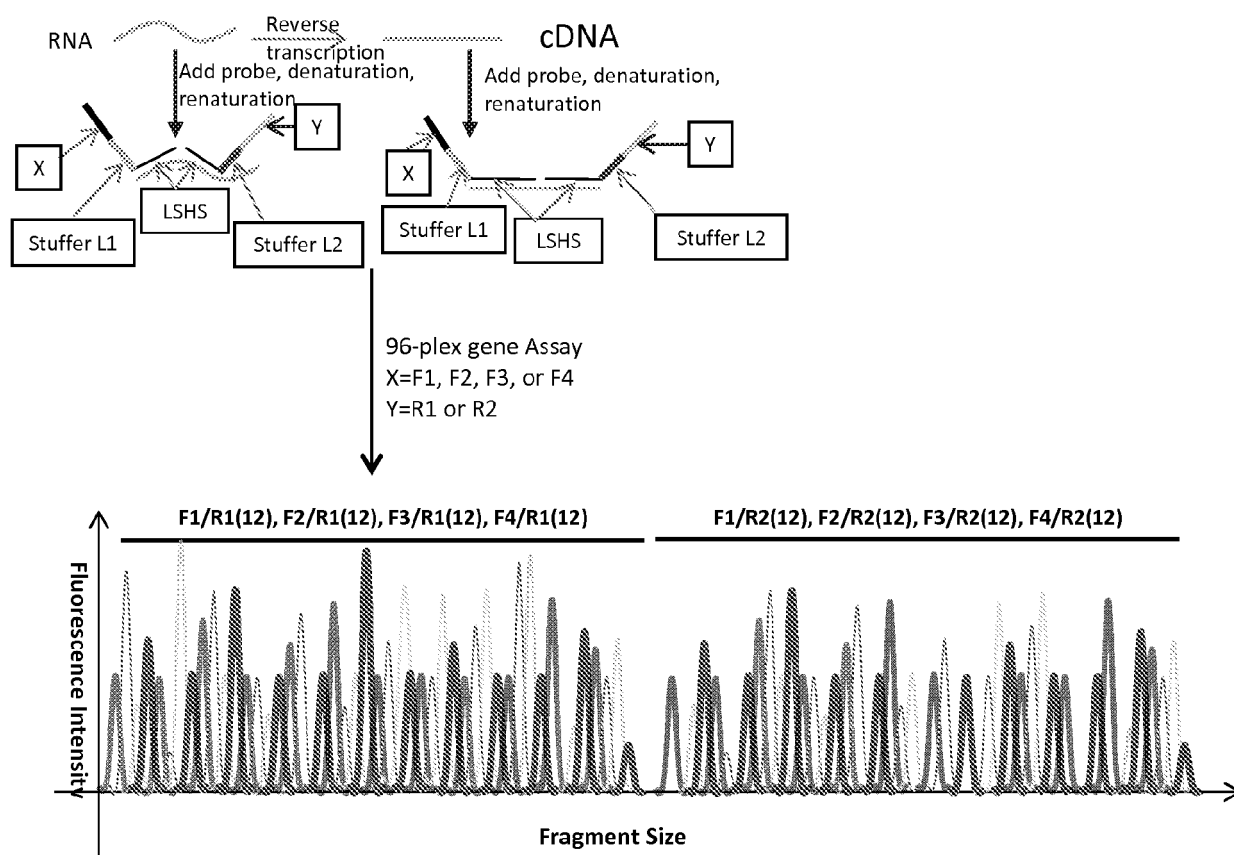


Figure 7

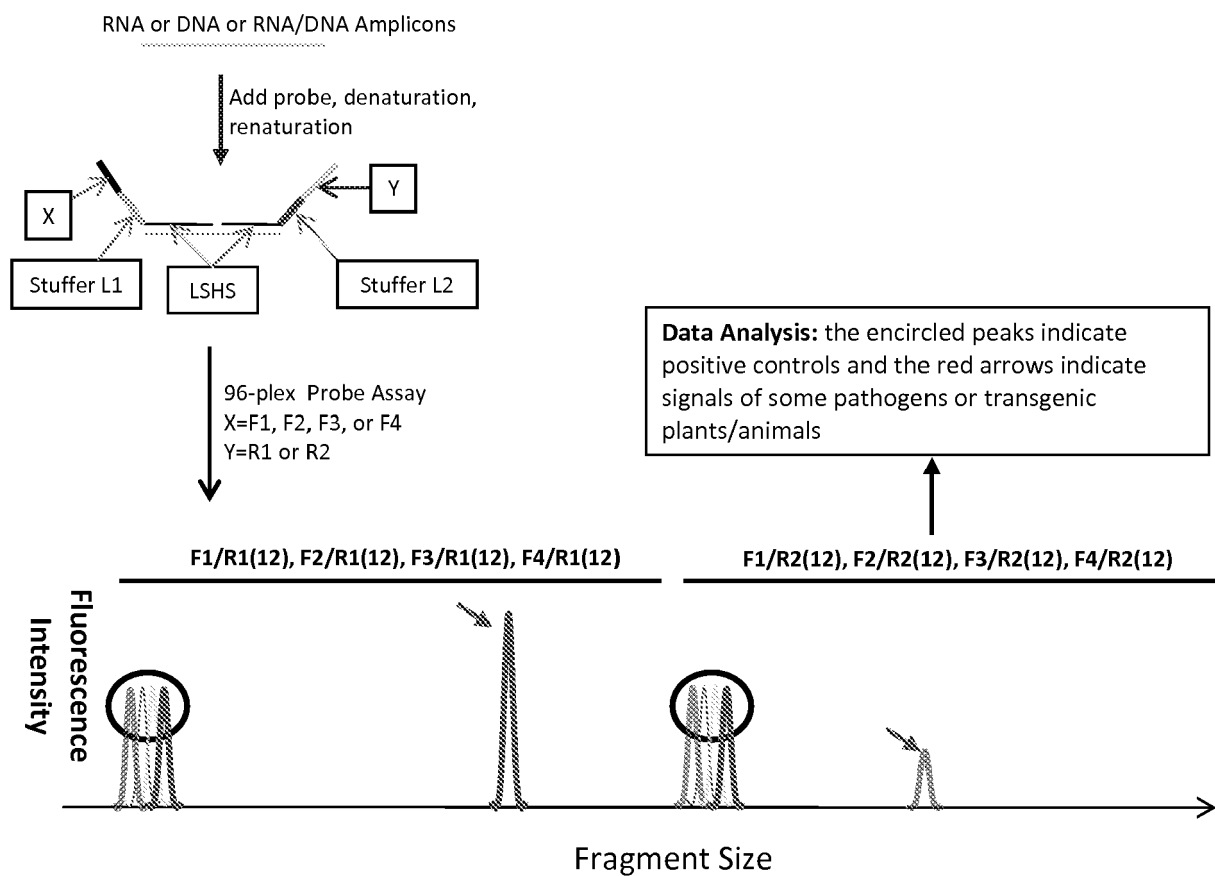


Figure 8

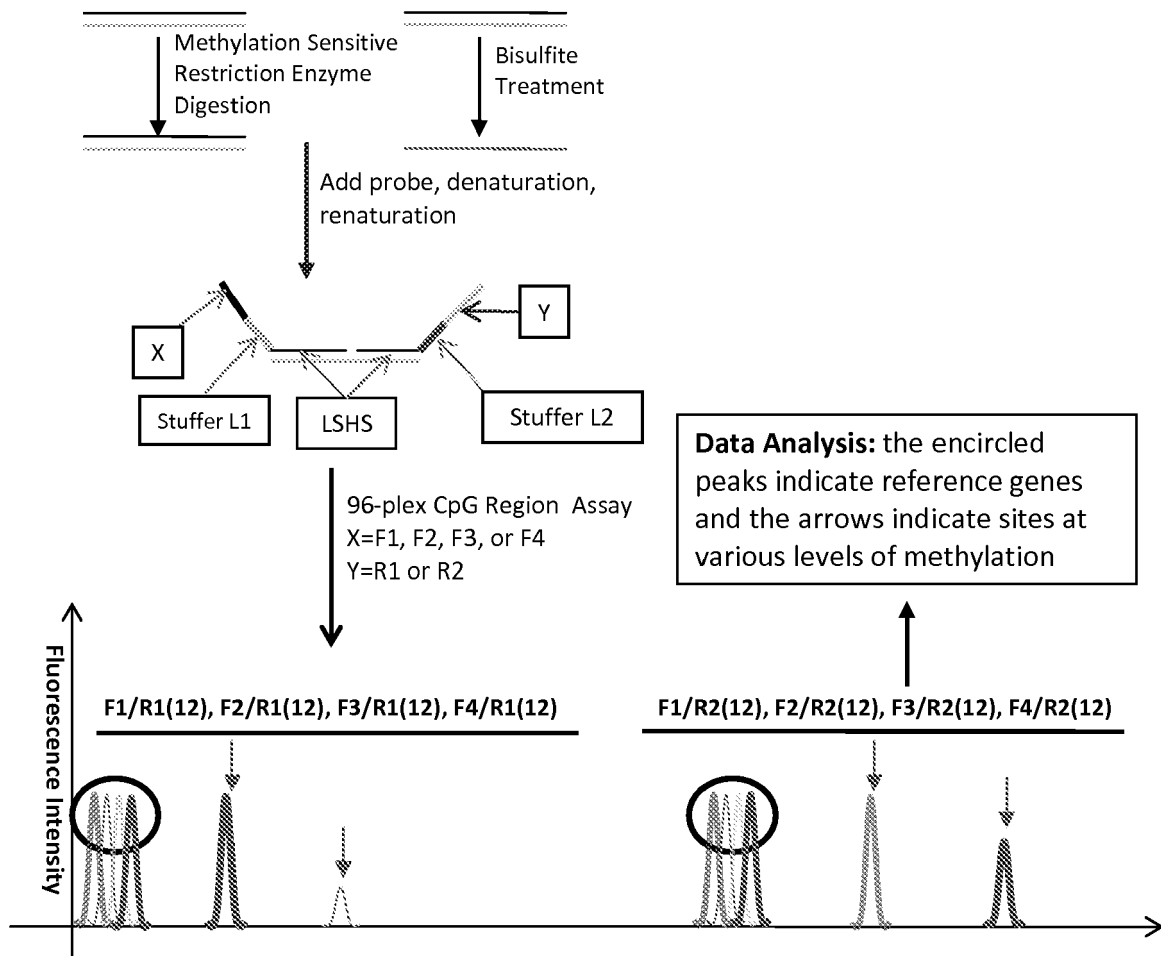


Figure 9

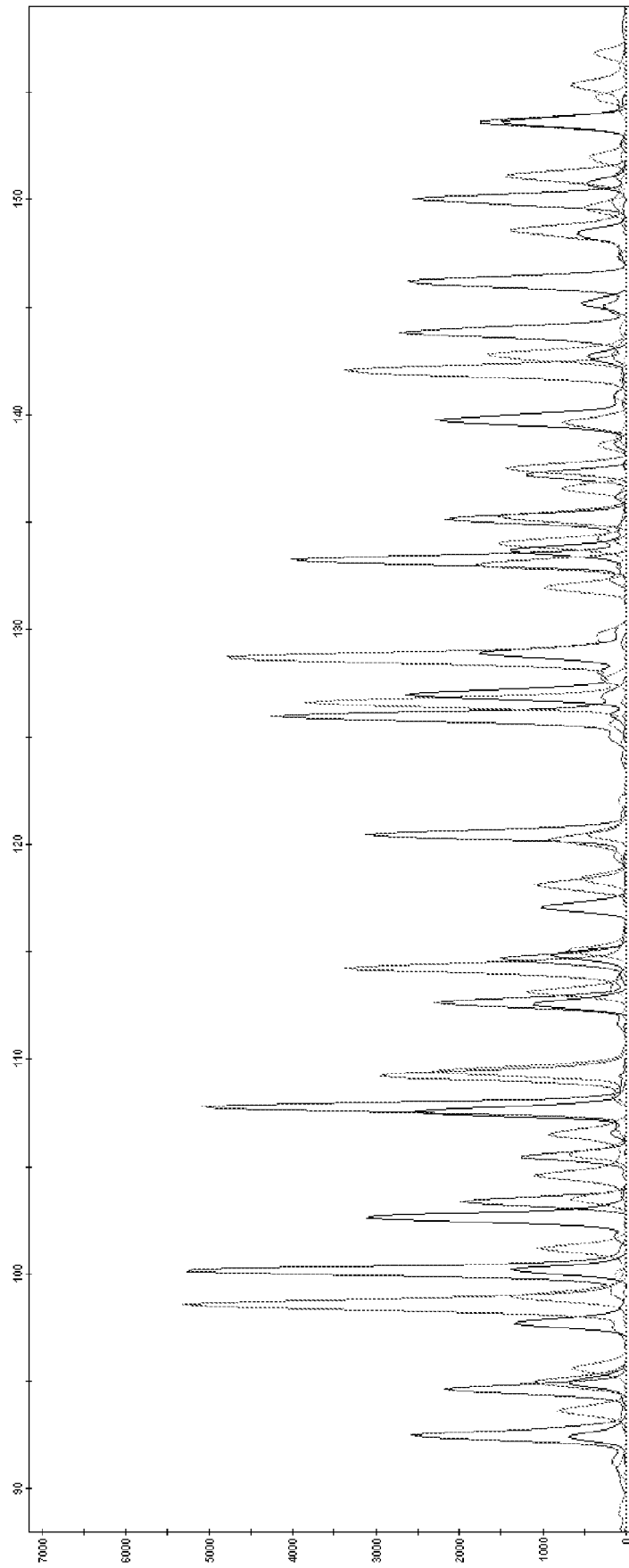


Figure 10A

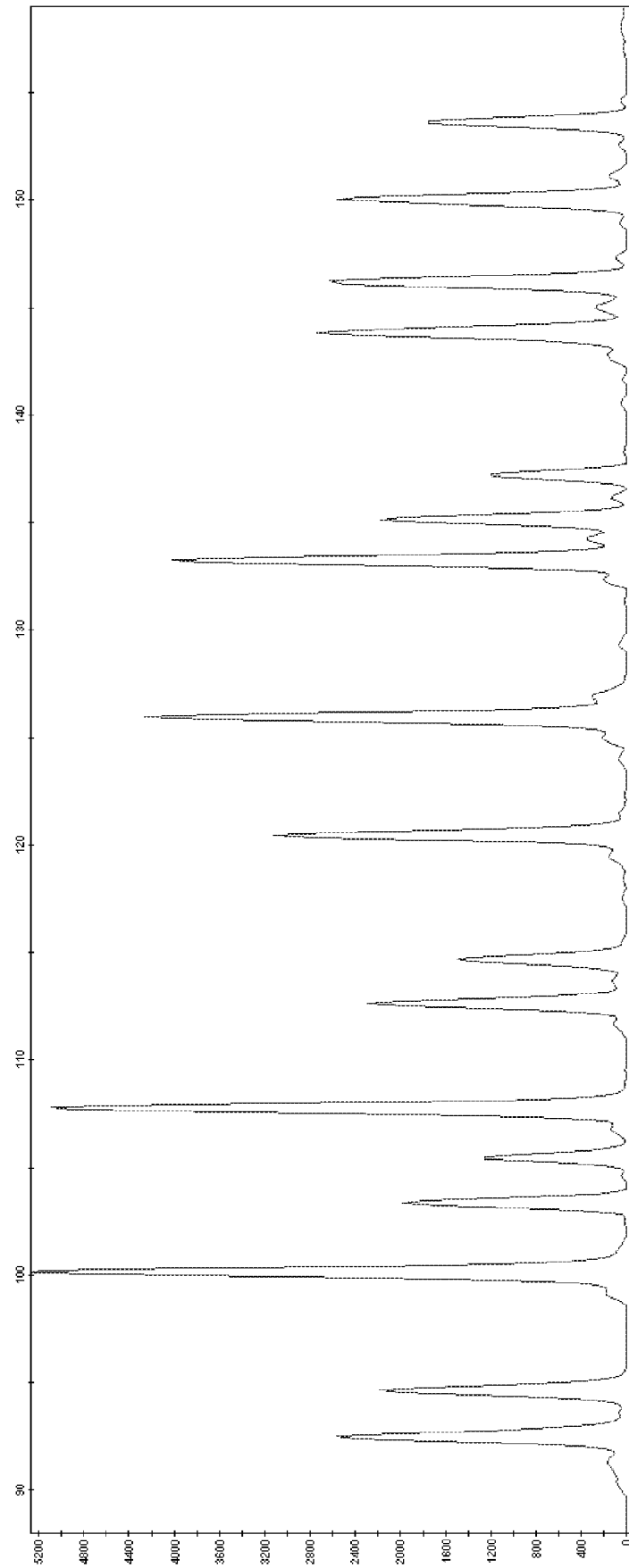


Figure 10B

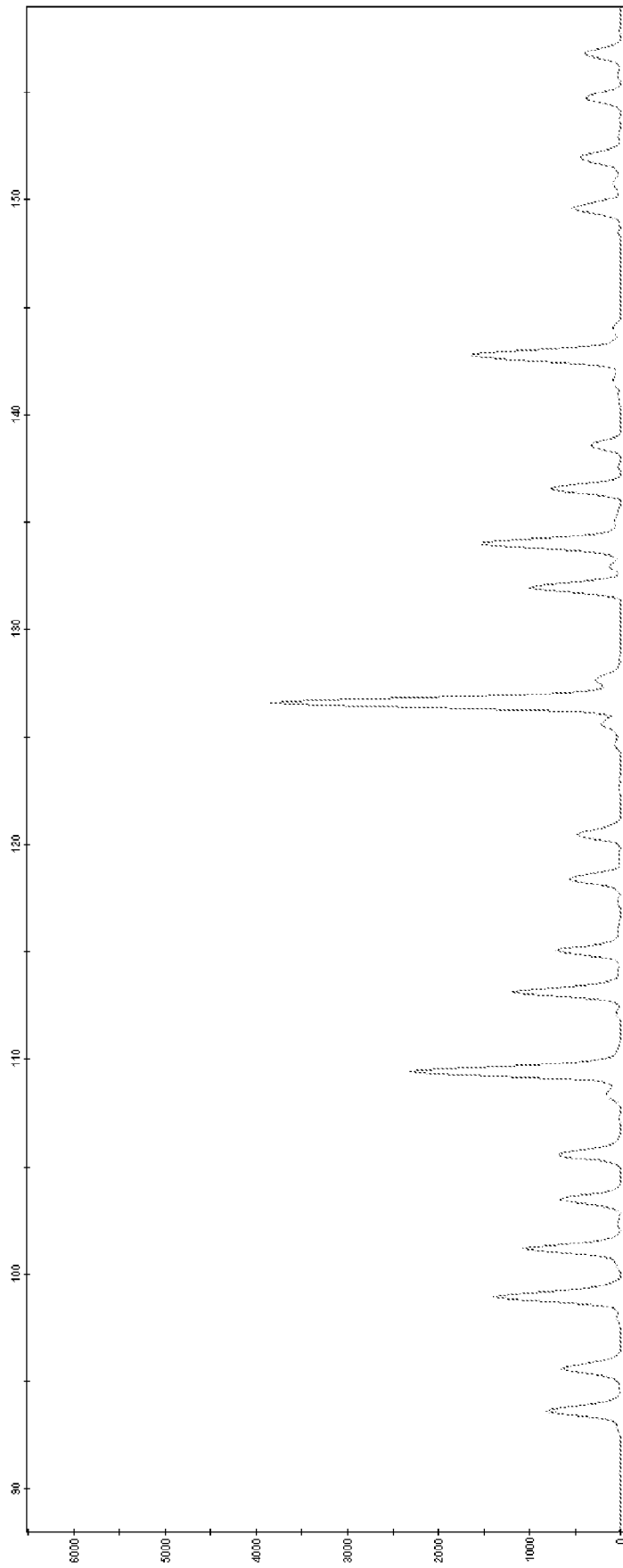


Figure 10C

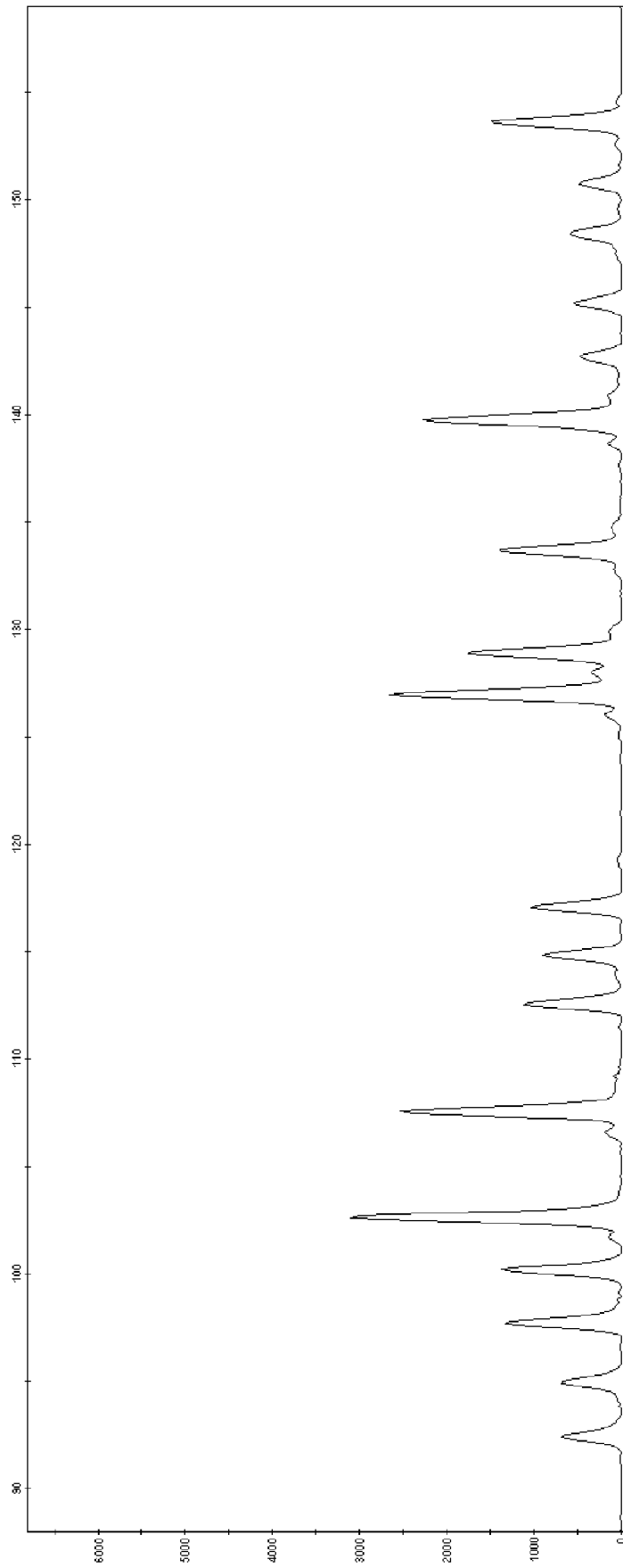


Figure 10D

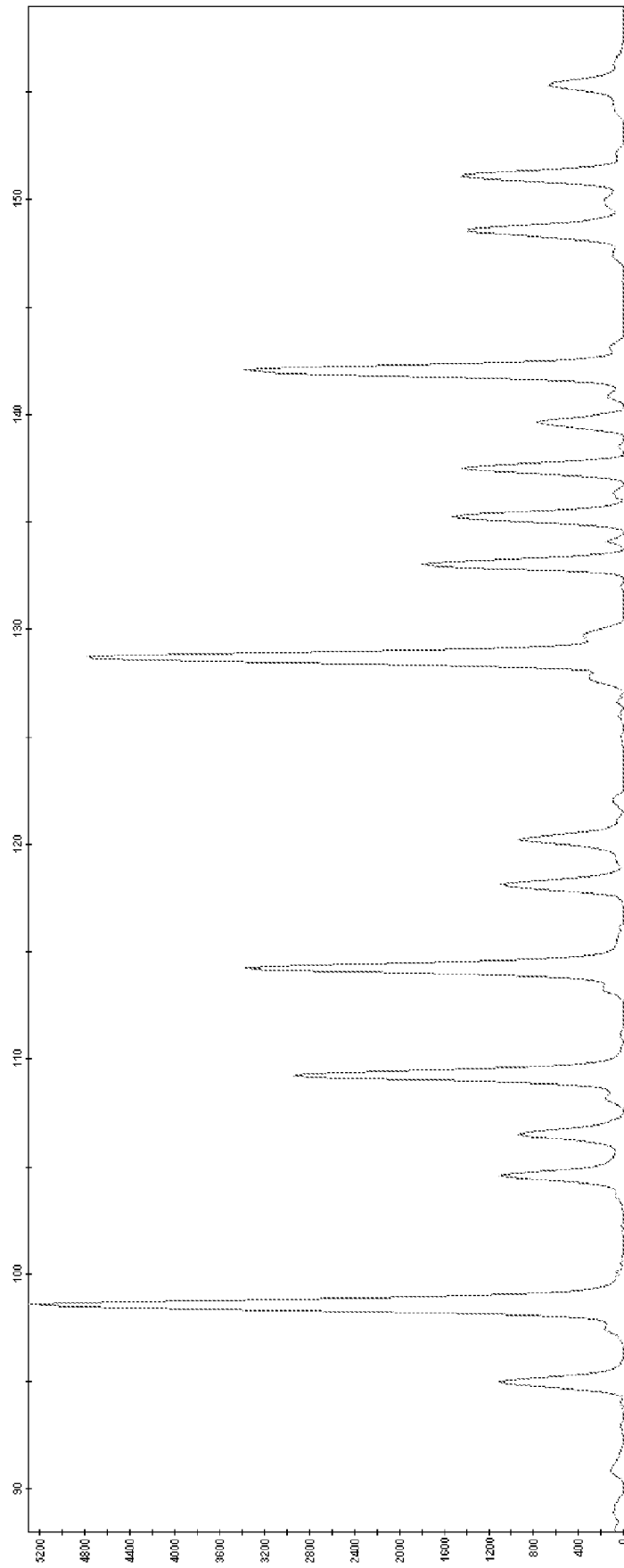


Figure 10E

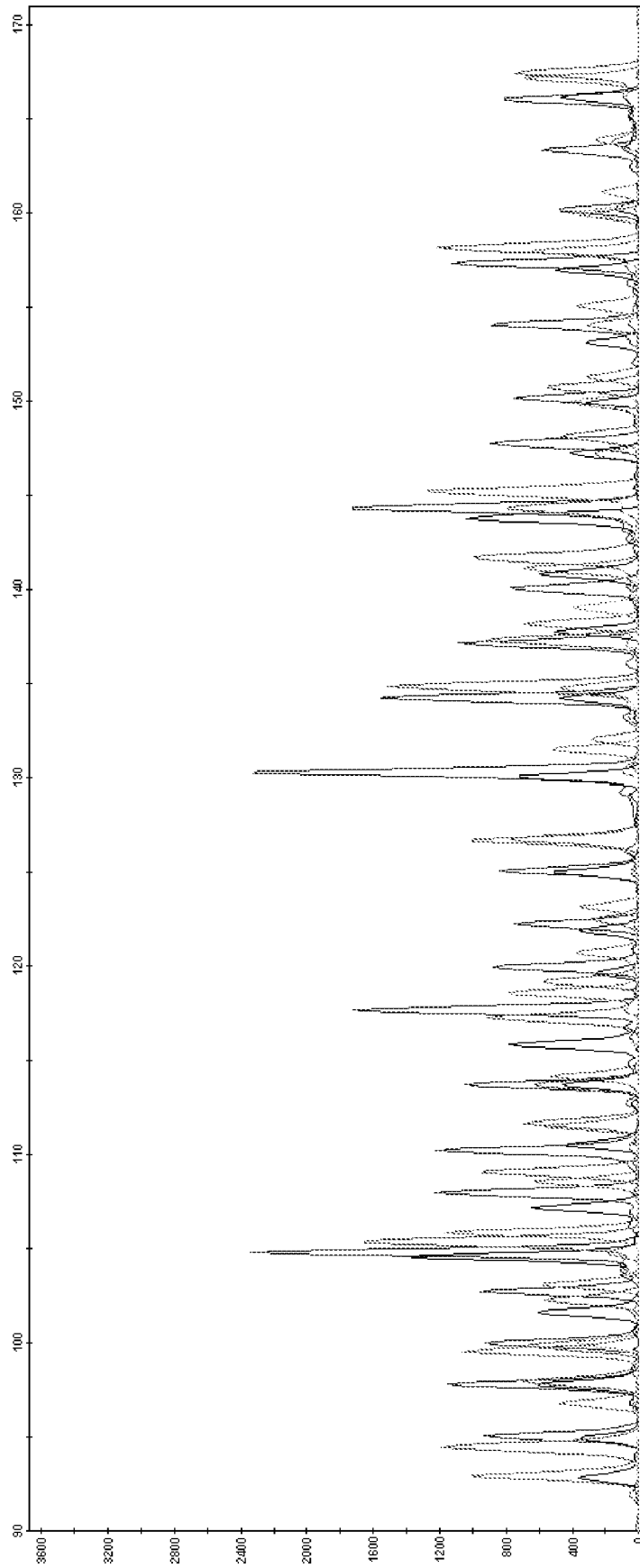


Figure 11A

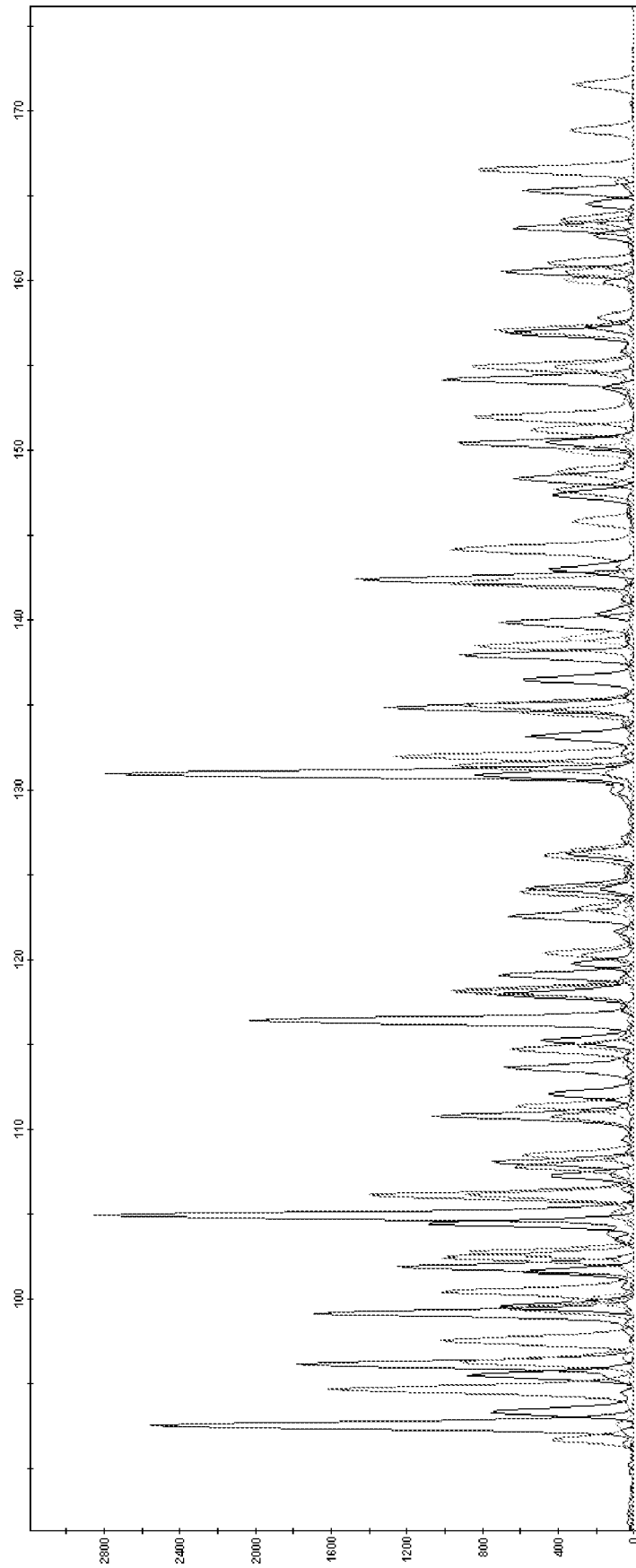


Figure 11B

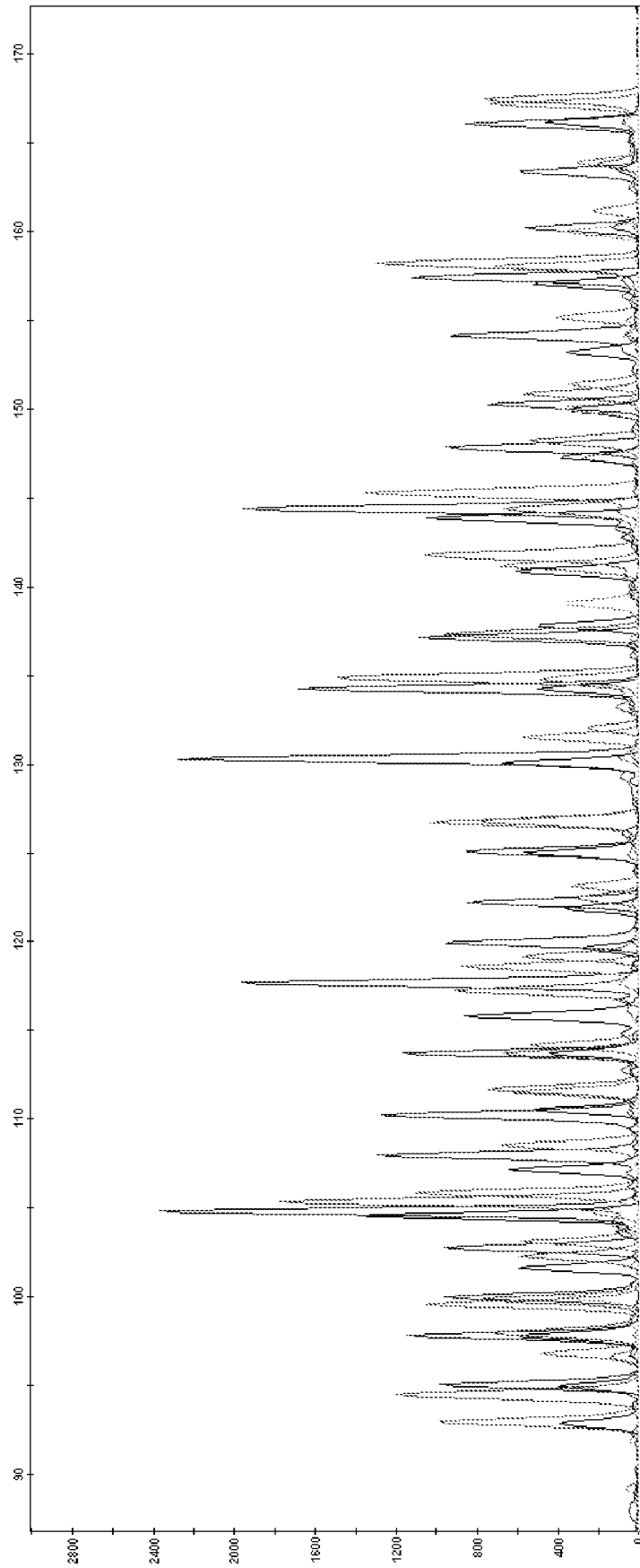


Figure 11C

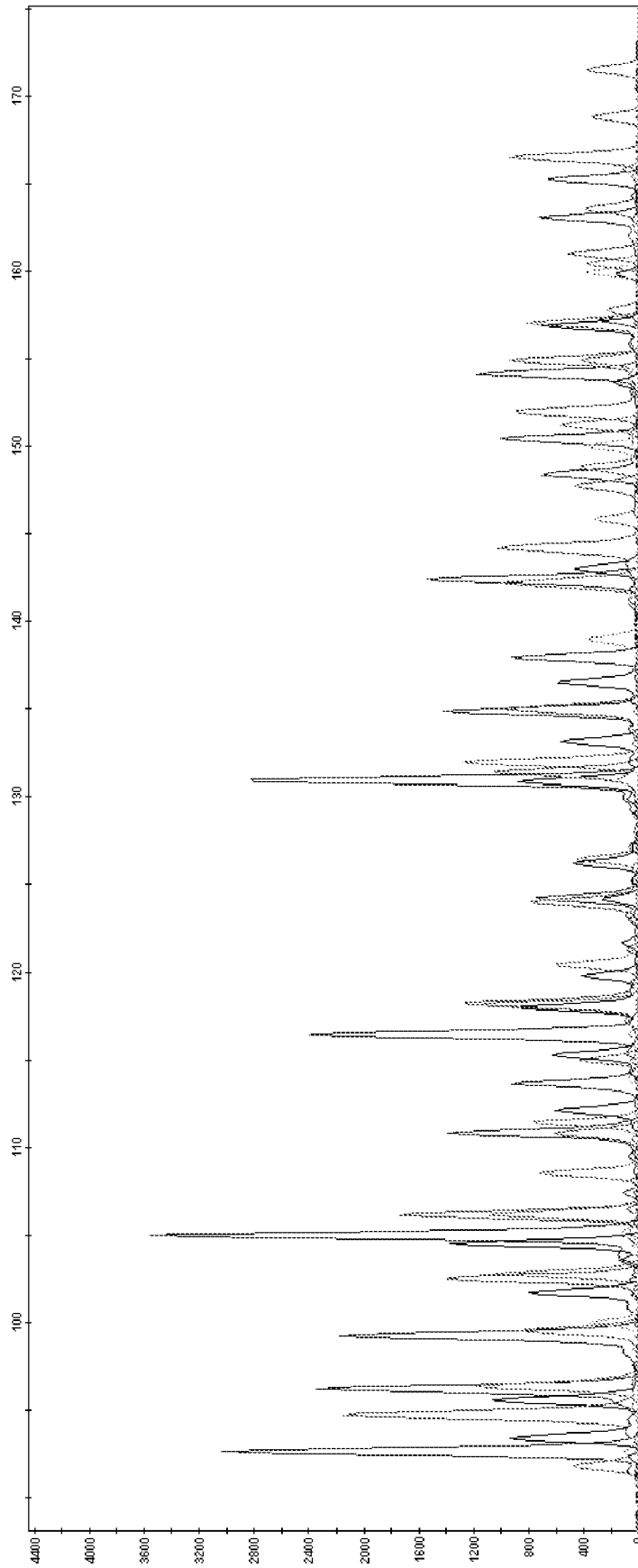


Figure 11D

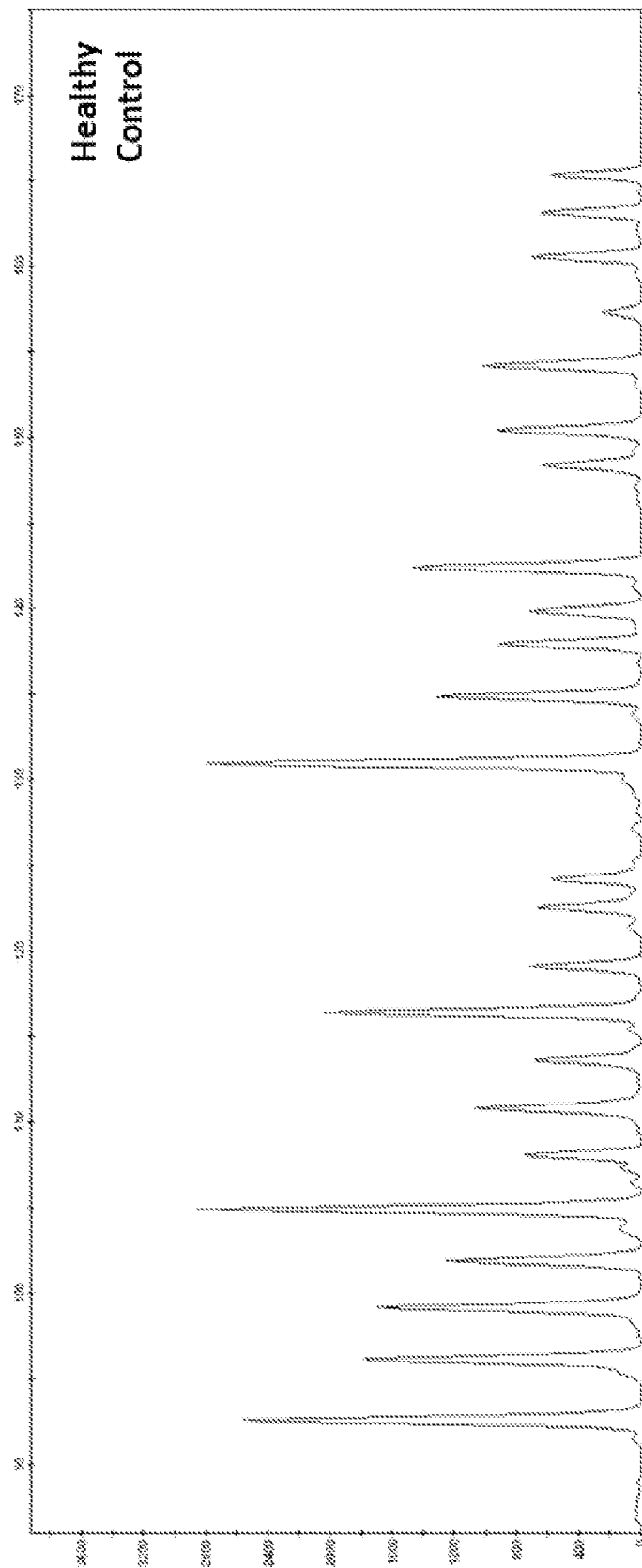


Figure 11E

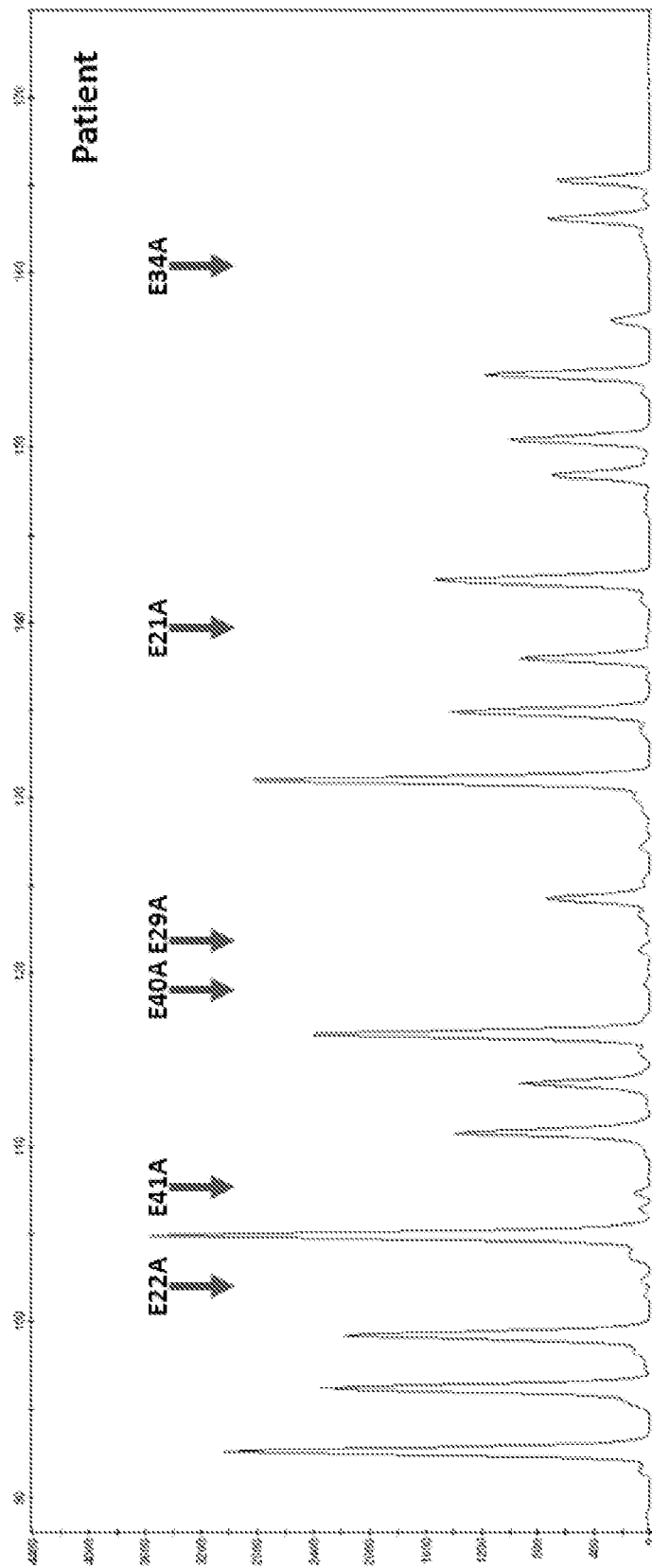


Figure 11F

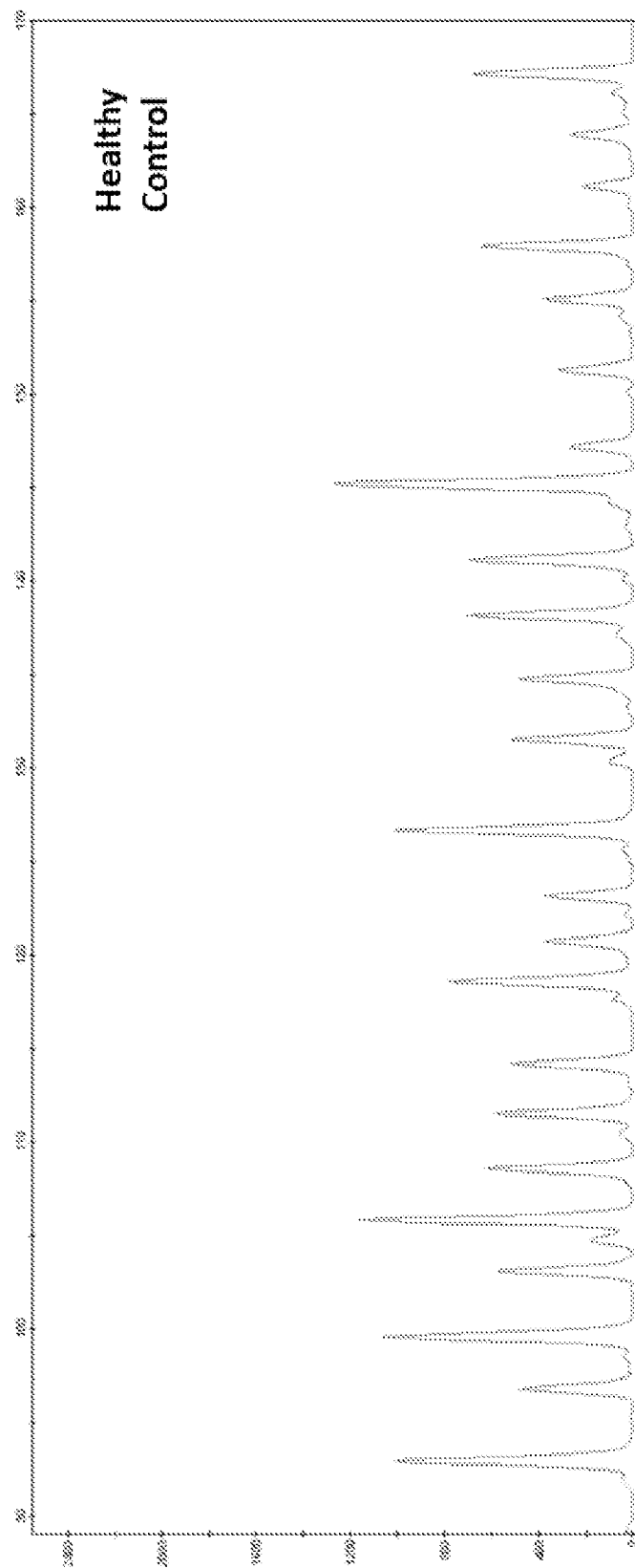


Figure 11G

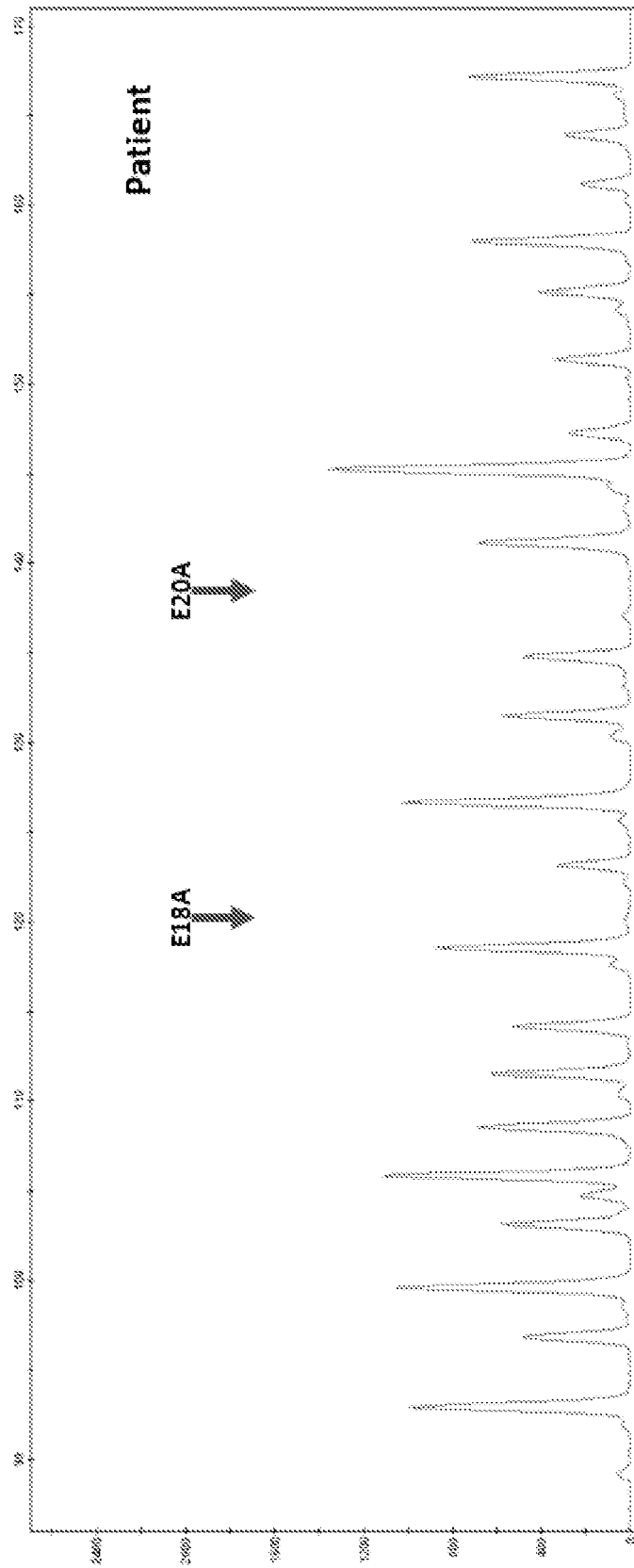


Figure 11H

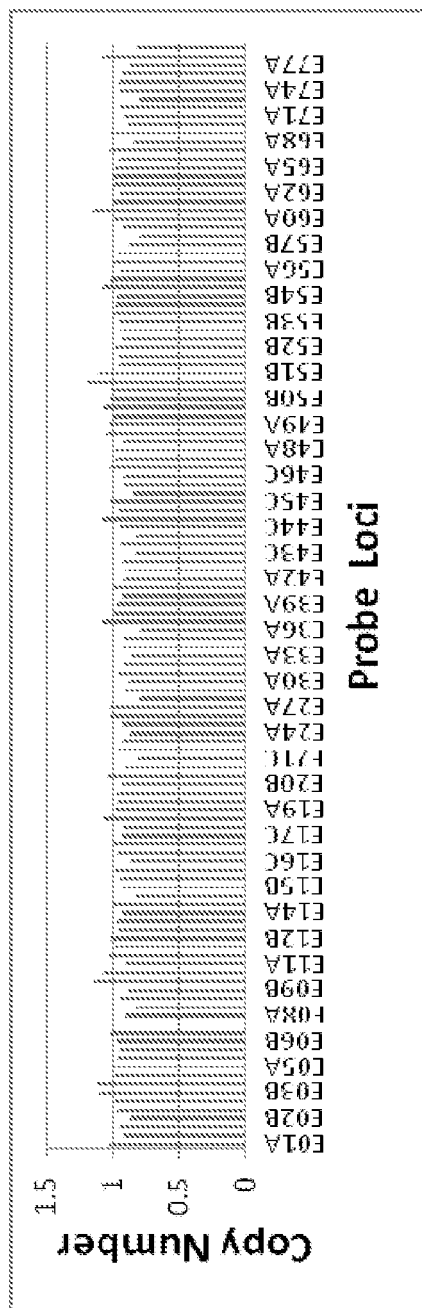


Figure 12A

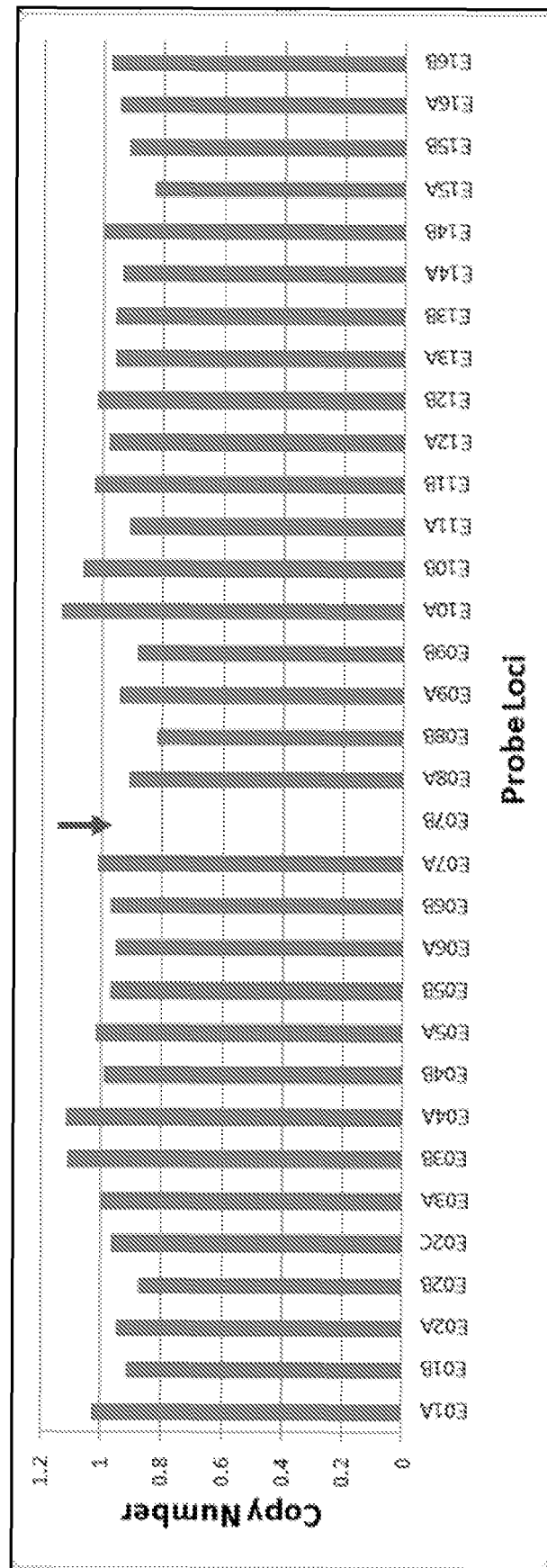
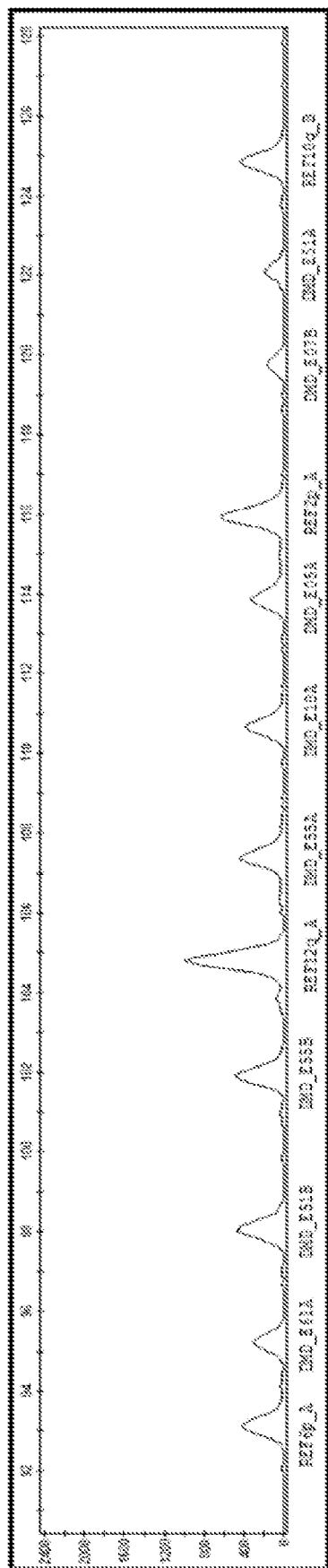


Figure 12B

Healthy Control



Male DMD Patient

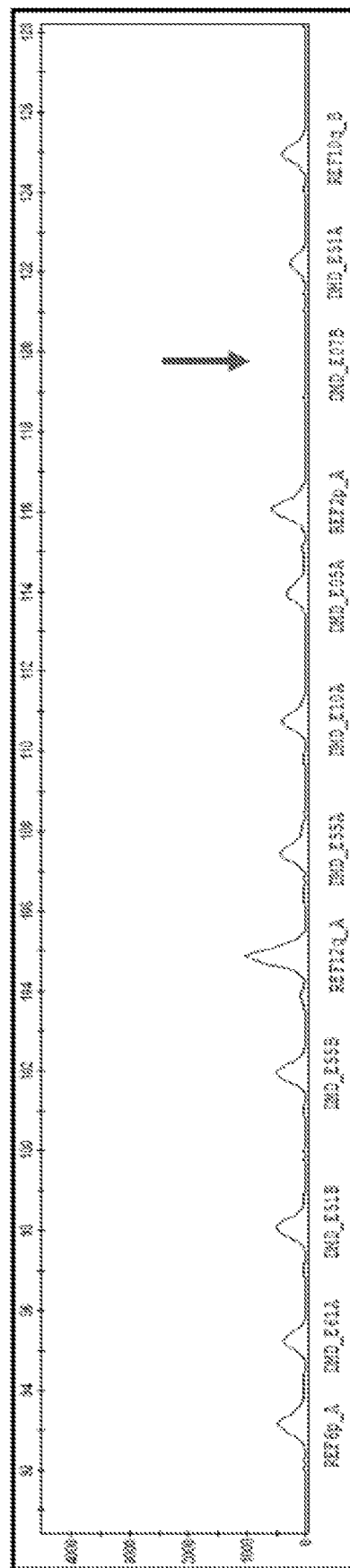


Figure 12C

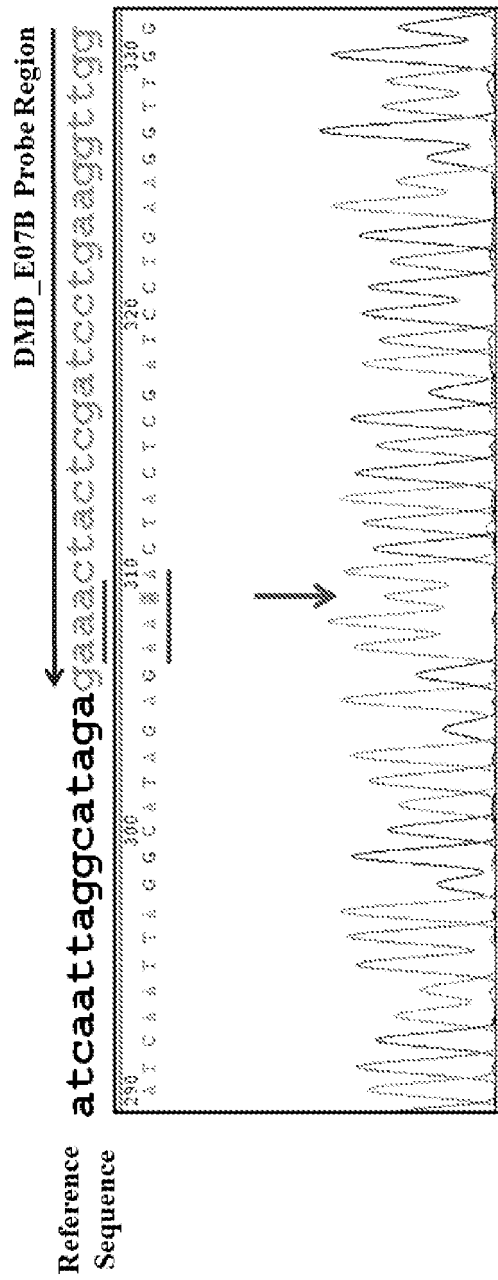


Figure 12D

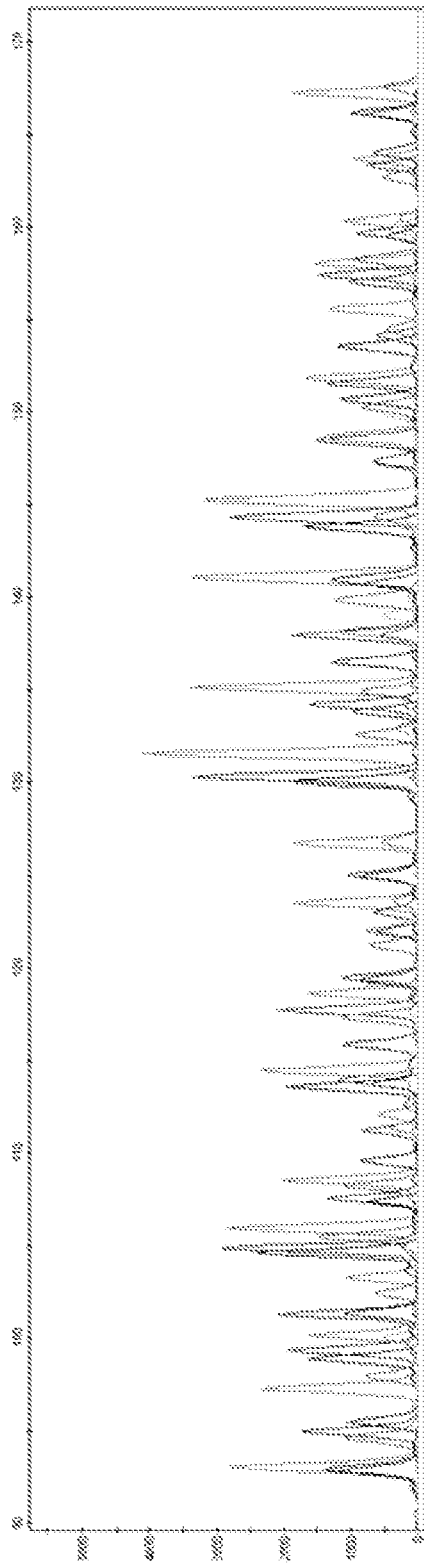


Figure 13A

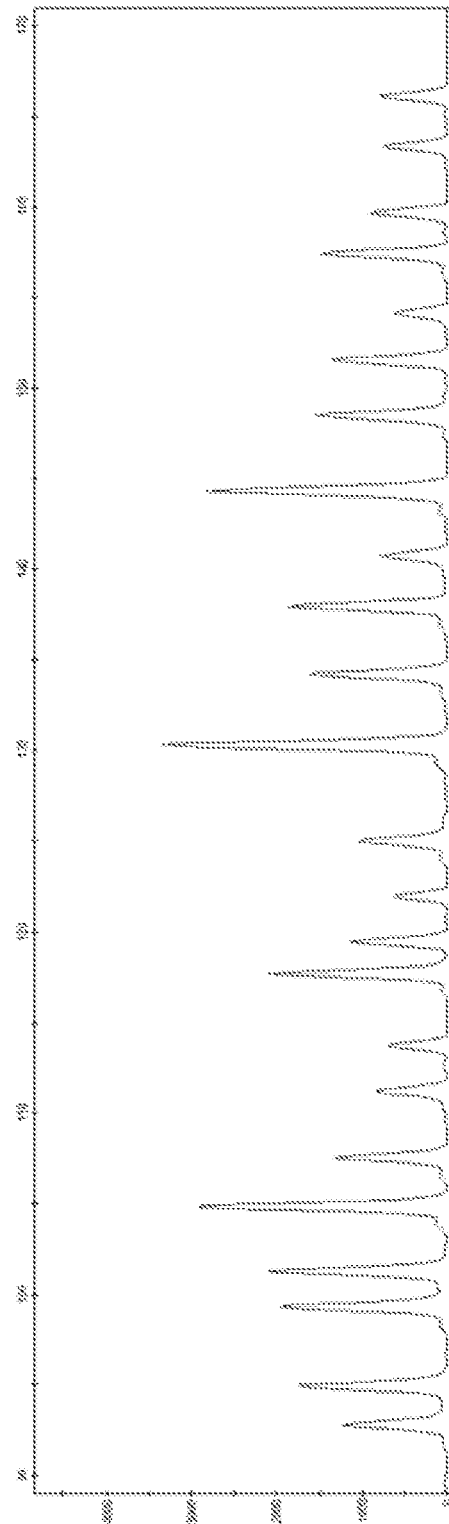


Figure 13B

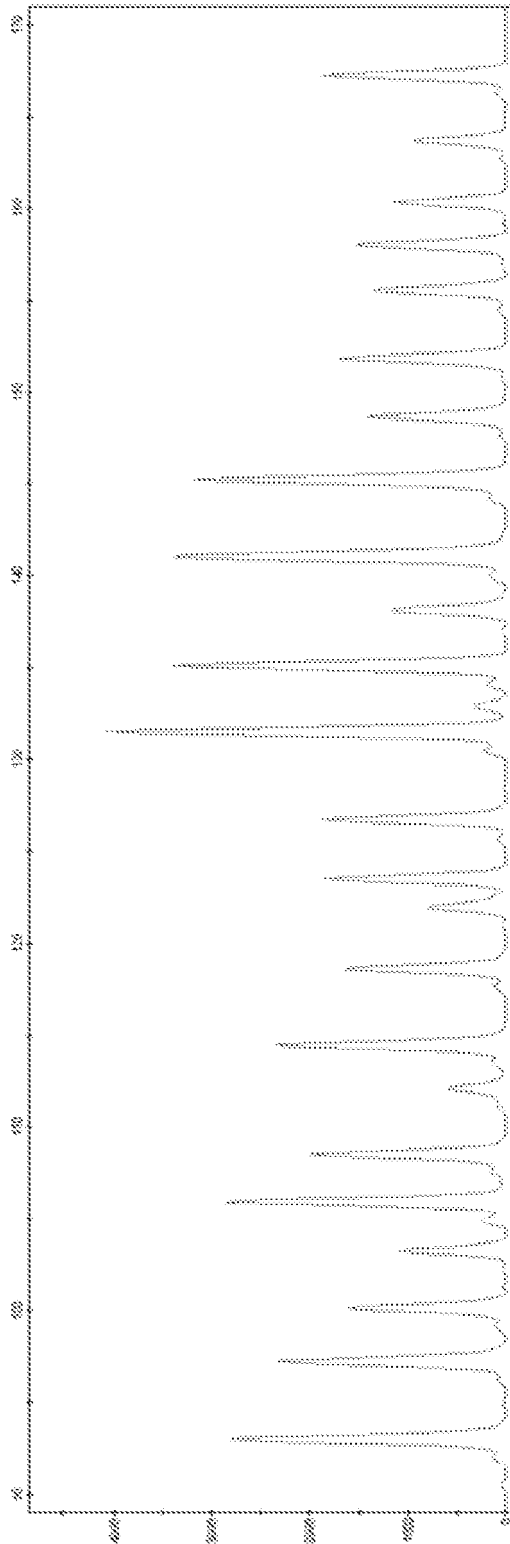


Figure 13C

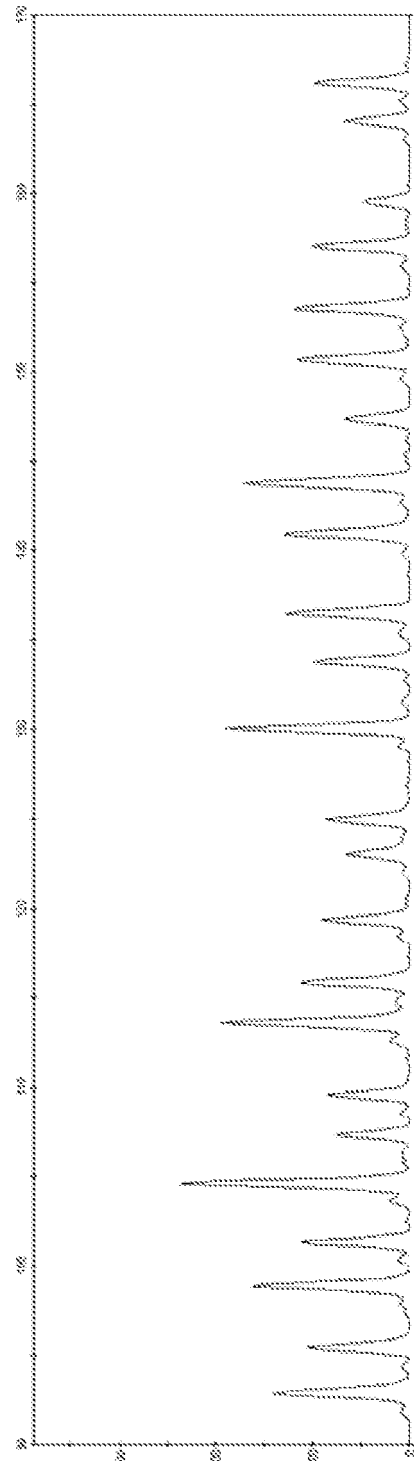


Figure 13D

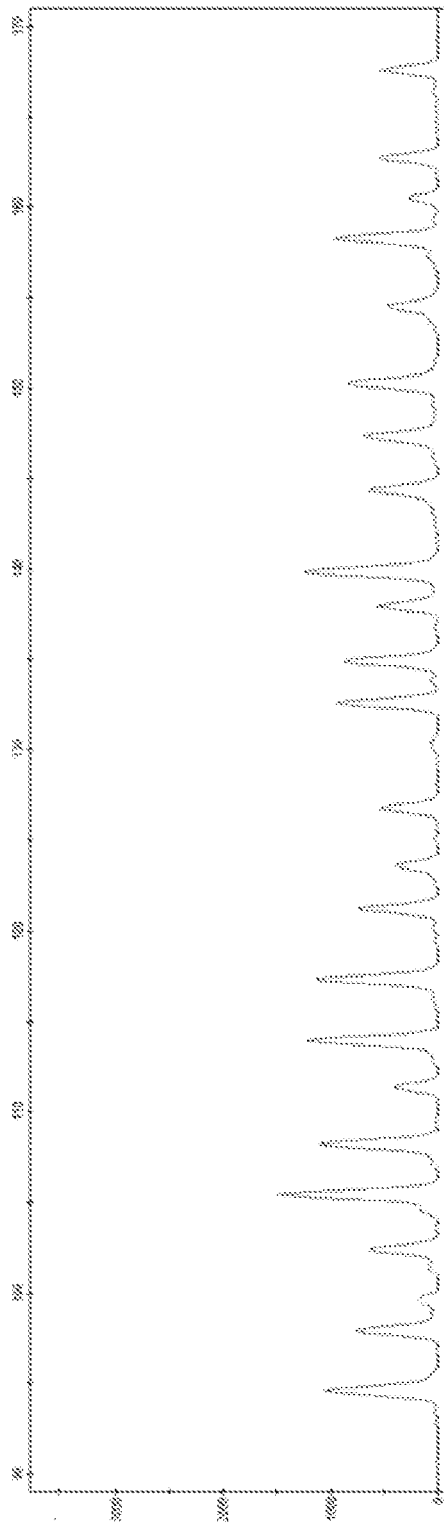


Figure 13E

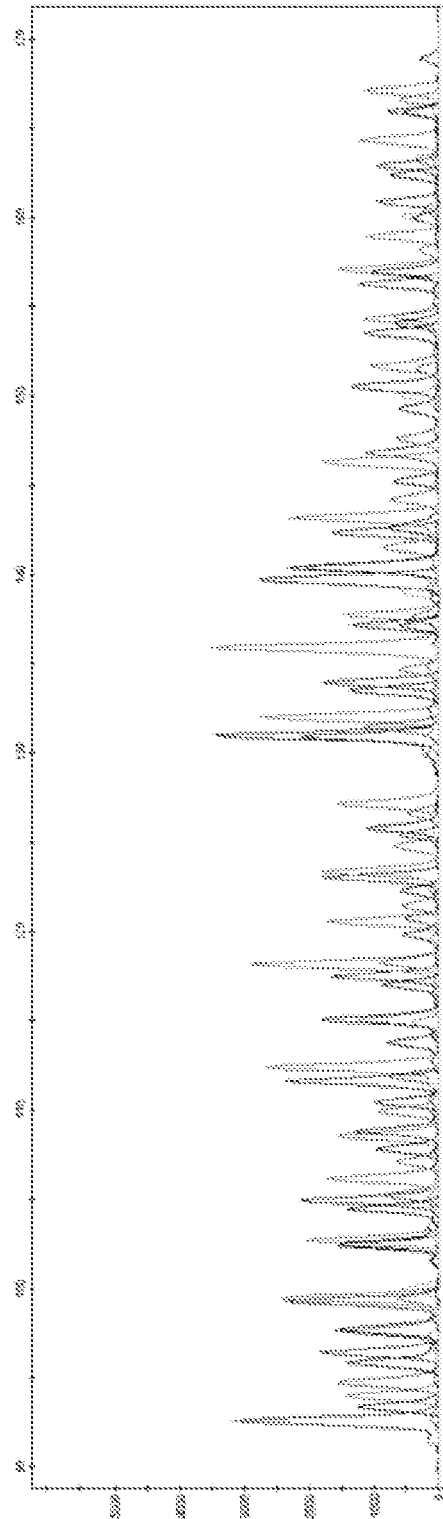


Figure 13F

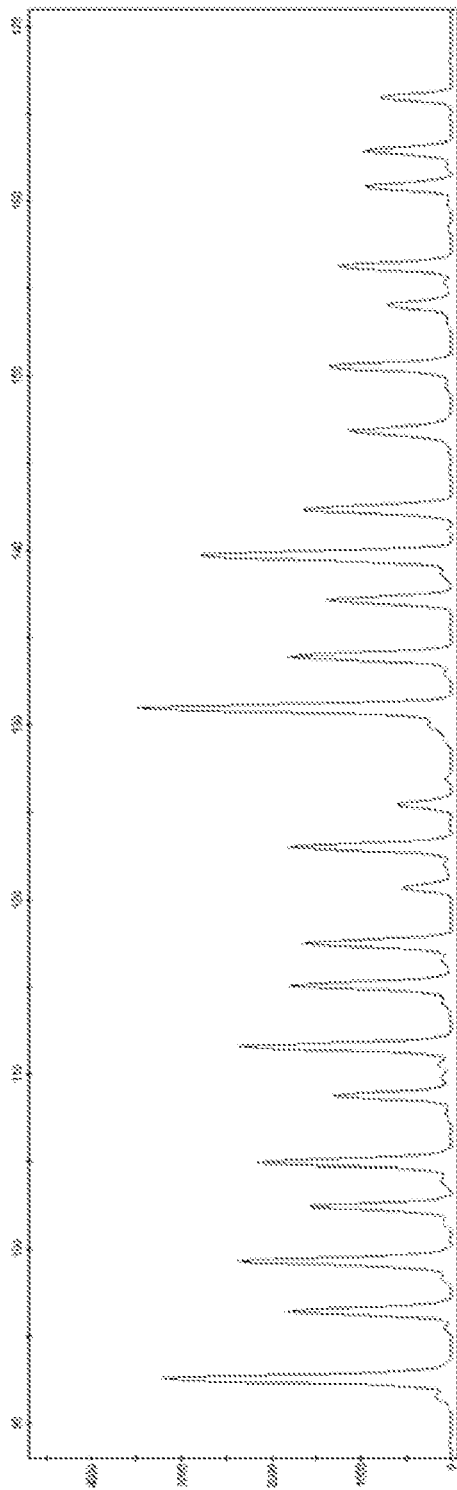


Figure 13G

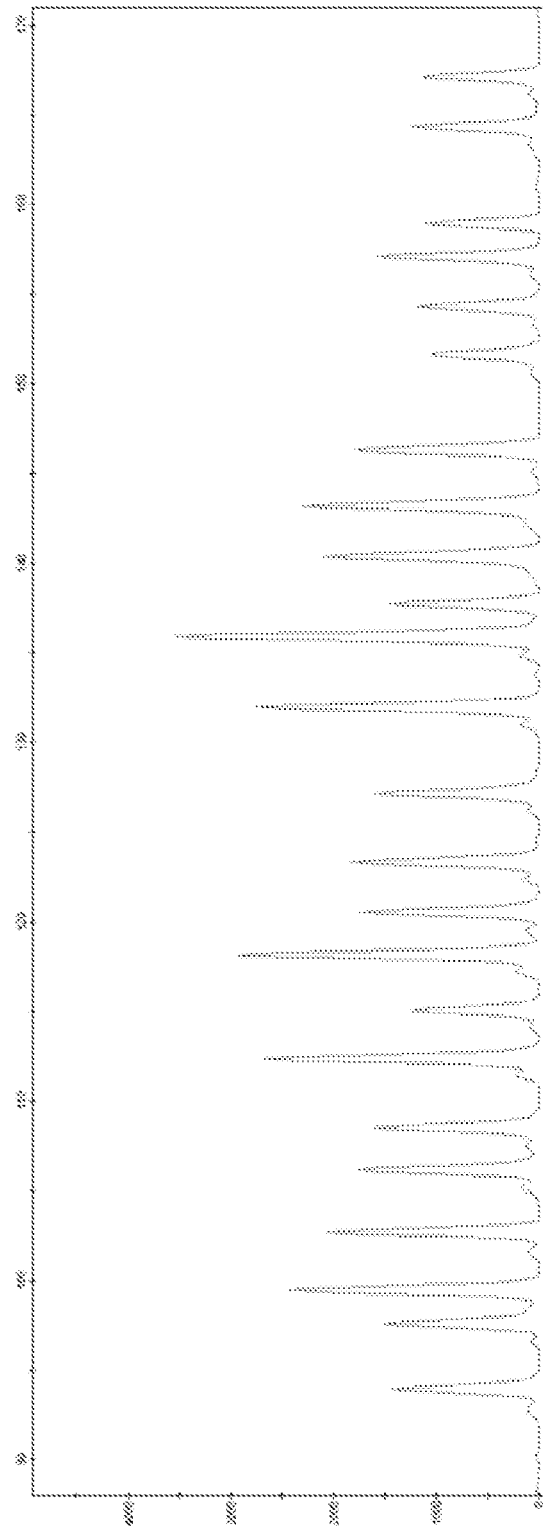


Figure 13H

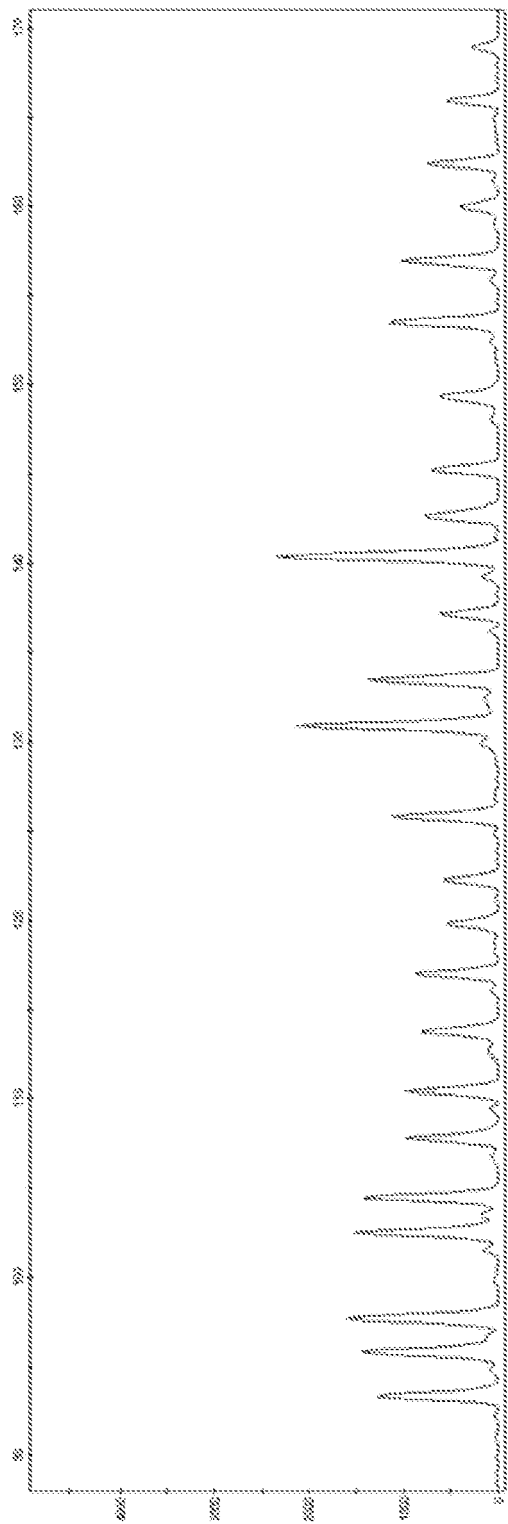


Figure 13I

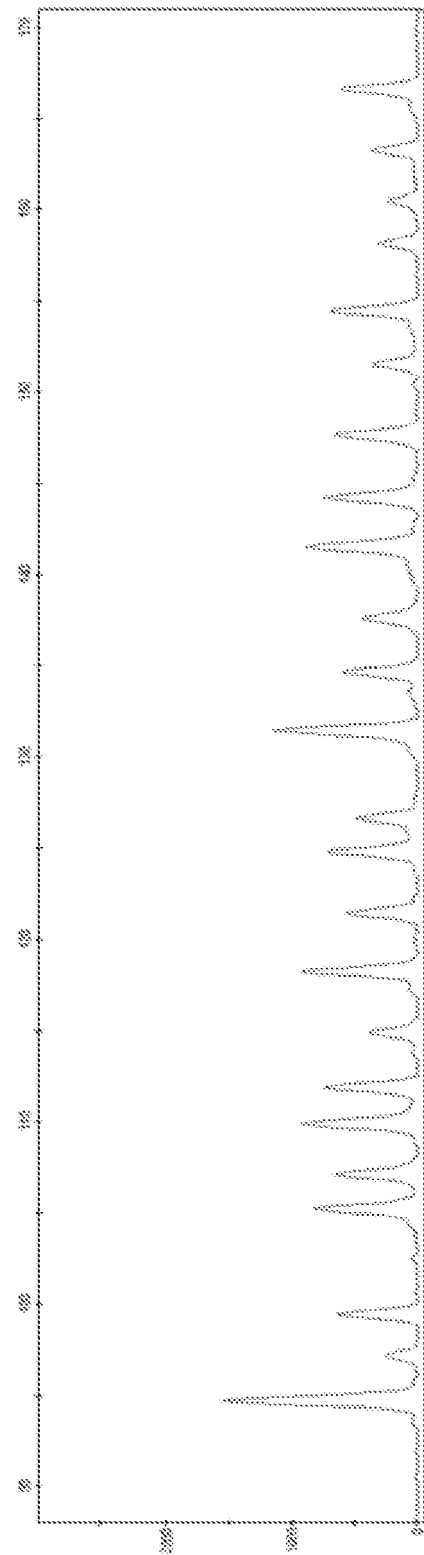


Figure 13J

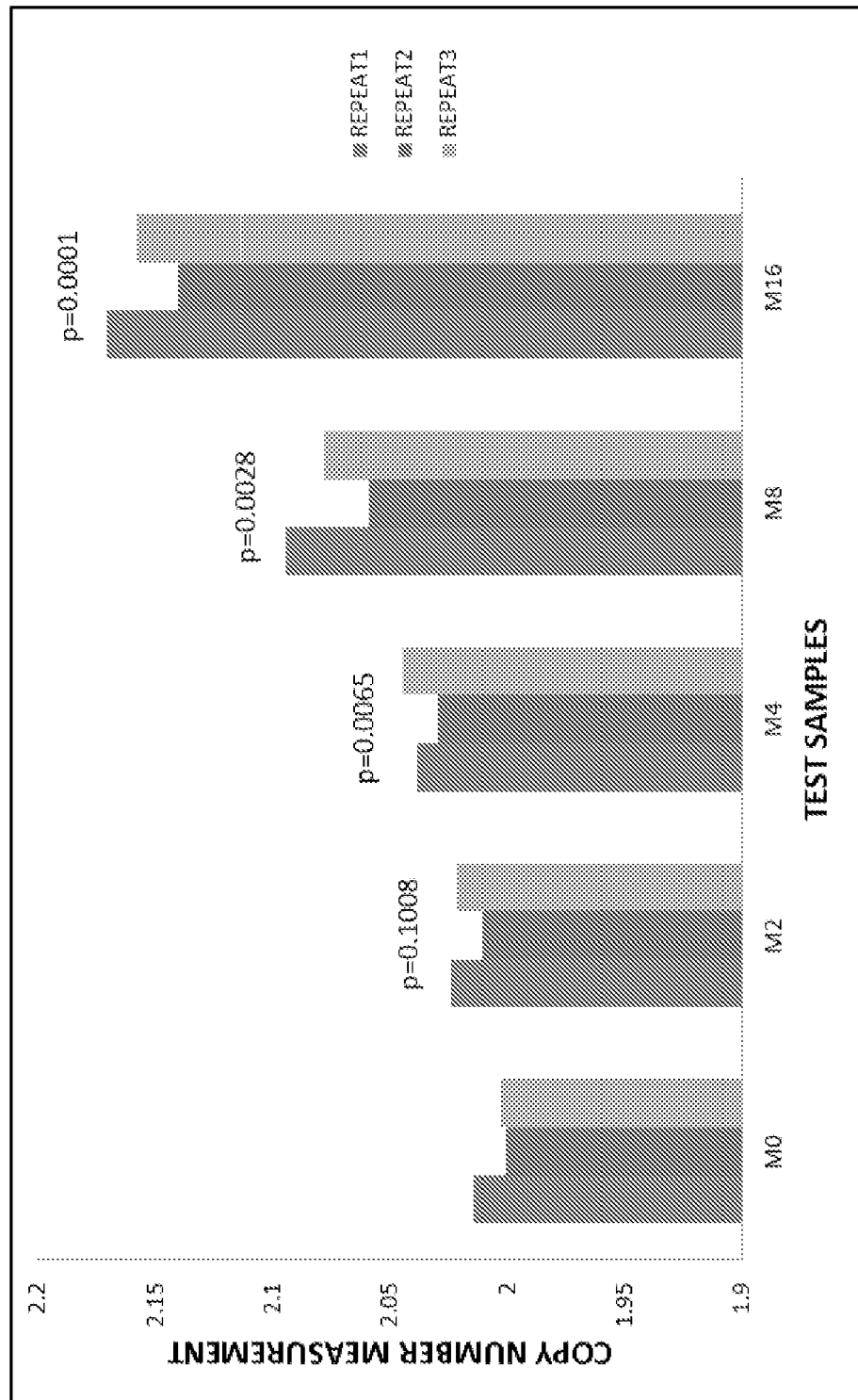


Figure 14

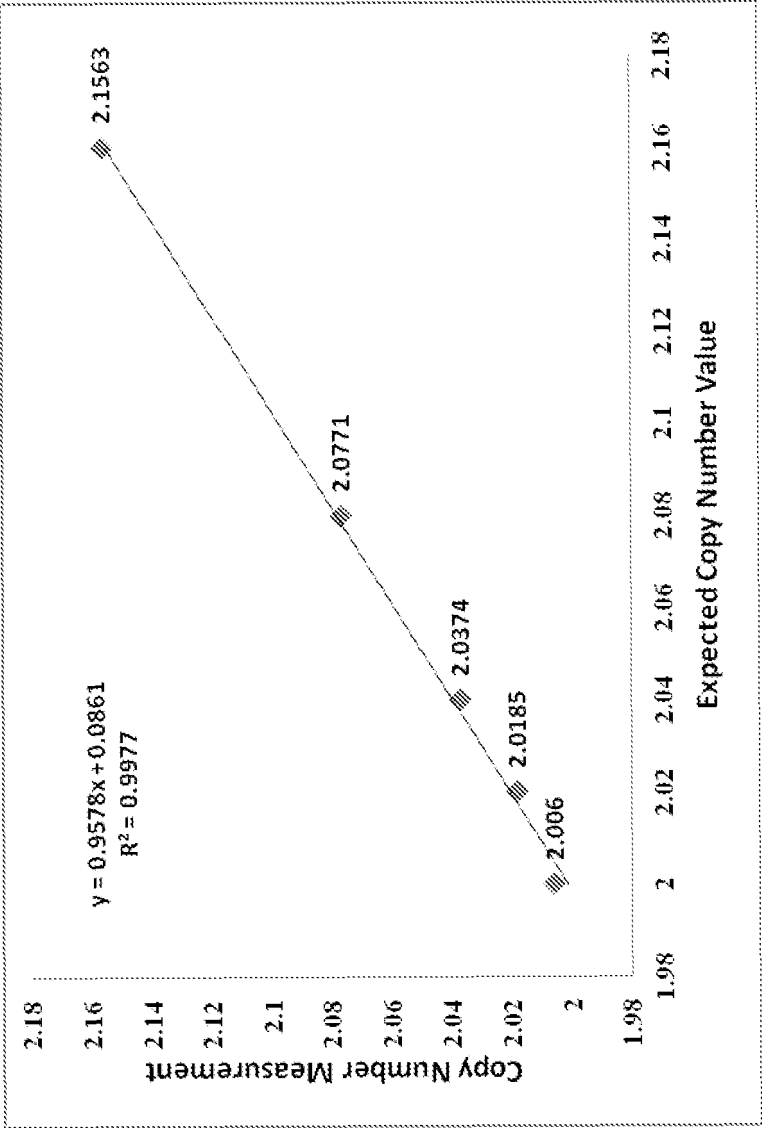


Figure 15

REFERENCES CITED IN THE DESCRIPTION

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