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(57) Abstract:

PROCESSES AND COMPOSITIONS FOR METHYLATION-BASED ENRICHMENT OF FETAL NUCLEIC ACID FROM A MATERNAL SAMPLE USEFUL FOR NON-INVASIVE PRENATAL DIAGNOSES

RELATED PATENT APPLICATION

This patent application claims the benefit of U.S. Provisional Patent Application No. 61/192,264 filed on September 16, 2008, entitled PROCESSES AND COMPOSITIONS FOR METHYLATION-BASED ENRICHMENT OF FETAL NUCLEIC ACID FROM A MATERNAL SAMPLE USEFUL FOR NON INVASIVE PRENATAL DIAGNOSES, naming Mathias Ehrich as an inventor, and having Attorney Docket No. SEQ-6022-PV. The entire content of the foregoing patent application is incorporated herein by reference, including all text, tables and drawings.

FIELD

Provided in certain embodiments are biomarkers. In some embodiments, biomarkers provided are useful for noninvasive detection of fetal genetic traits. Certain fetal genetic traits include but are not limited to presence or absence of fetal nucleic acid.

BACKGROUND

Non-invasive prenatal testing is becoming a field of rapidly growing interest. Early detection of pregnancy-related conditions, including complications during pregnancy and genetic defects of the fetus is of crucial importance, as it allows early medical intervention necessary for the safety of both the mother and the fetus. Prenatal diagnosis has been conducted using cells isolated from the fetus through procedures such as chorionic villus sampling (CVS) or amniocentesis. However, these conventional methods are invasive and present an appreciable risk to both the mother and the fetus. The National Health Service currently cites a miscarriage rate of between 1 and 2 per cent following the invasive amniocentesis and chorionic villus sampling (CVS) tests.

An alternative to these invasive approaches has been developed for prenatal screening, e.g., to detecting fetal abnormalities, following the discovery that circulating cell-free fetal nucleic acid can be detected in maternal plasma and serum (Lo et al., Lancet 350:485-487, 1997; and U.S. Patent 6,258,540). Circulating cell free fetal nucleic acid (cffNA) has several advantages making it more applicable for non-invasive prenatal testing. For example, cell free nucleic acid is present at higher levels than fetal cells and at concentrations sufficient for genetic analysis. Also, cffNA is cleared from the maternal bloodstream within hours after delivery, preventing contamination from previous pregnancies.

Examples of prenatal tests performed by detecting fetal DNA in maternal plasma or serum include fetal rhesus D (RhD) genotyping (Lo et al., N. Engl. J. Med. 339:1734-1738, 1998), fetal sex determination (Costa et al., N. Engl. J. Med. 346:1502, 2002), and diagnosis of several fetal disorders (Amicucci et al., Clin. Chem. 46:301-302, 2000; Saito et al., Lancet 356:1170, 2000; and Chiu et al., Lancet 360:998-1000, 2002). In addition, quantitative abnormalities of fetal DNA in maternal plasma/serum have been reported in preeclampsia (Lo et al., Clin. Chem. 45:184-188, 1999 and Zhong et al., Am. J. Obstet. Gynecol. 184:414-419, 2001), fetal trisomy 21 (Lo et al., Clin. Chem. 45:1747-1751, 1999 and Zhong et al., Prenat. Diagn. 20:795-798, 2000) and hyperemesis gravidarum (Sekizawa et al., Clin. Chem. 47:2164-2165, 2001).

SUMMARY

The technology provides *inter alia* human epigenetic biomarkers that are useful for the noninvasive detection of fetal genetic traits, including, but not limited to, the presence or absence of fetal nucleic acid, the absolute or relative amount of fetal nucleic acid, fetal sex, and fetal chromosomal abnormalities such as aneuploidy. The human epigenetic biomarkers of the technology represent genomic DNA that display differential CpG methylation patterns between the fetus and mother. The compositions and processes of the technology allow for the detection and quantification of fetal nucleic acid in a maternal sample based on the methylation status of the nucleic acid in said sample. More specifically, the amount of fetal nucleic acid from a maternal sample can be determined relative to the total amount of nucleic acid present, thereby providing the percentage of fetal nucleic acid in the sample. Further, the amount of fetal nucleic acid can be determined in a sequence-specific (or locus-specific) manner and with sufficient sensitivity to allow for accurate chromosomal dosage analysis (for example, to detect the presence or absence of a fetal aneuploidy).

In an aspect of the technology, a method is provided for enriching fetal nucleic acids from a maternal biological sample, based on differential methylation between fetal and maternal nucleic acid comprising the steps of: (a) binding a target nucleic acid, from a sample, and a control nucleic acid, from the sample, to a methylation-specific binding protein; and (b) eluting the bound nucleic acid based on methylation status, wherein differentially methylated nucleic acids elute at least partly into separate fractions. In an embodiment, the nucleic acid sequence includes one or more of the polynucleotide sequences of SEQ ID NOs: 1-89. SEQ ID NOs: 1-89 are provided in Table 4. The technology includes the sequences of SEQ ID NOs: 1-89, and variations thereto. In another embodiment, a control nucleic acid is not included in step (a).

In a related embodiment, a method is provided for enriching fetal nucleic acid from a maternal sample, which comprises the following steps: (a) obtaining a biological sample from a woman; (b) separating fetal and maternal nucleic acid based on the methylation status of a CpG-containing genomic sequence in the sample, wherein the genomic sequence from the fetus and the genomic sequence from the woman are differentially methylated, thereby distinguishing the genomic sequence from the woman and the genomic sequence from the fetus in the sample. In another embodiment, the genomic sequence is at least 15 nucleotides in length, comprising at least one cytosine, further wherein the region has (1) a genomic locus selected from Table 1; and (2) a DNA sequence of no more than 10 kb upstream and/or downstream from the locus. In embodiments, obtaining a biological sample from a woman does not limit the scope of the technology. This obtaining can refer to actually collecting a sample from a woman (e.g., a blood draw) or to receiving a sample from elsewhere (e.g., from a clinic or hospital) and performing steps of a method.

In another related embodiment, a method is provided for enriching fetal nucleic acid from a maternal sample, which comprises the following steps: (a) obtaining a biological sample from the woman; (b) digesting or removing maternal nucleic acid based on the methylation status of a CpG-containing genomic sequence in the sample, wherein the genomic sequence from the fetus and the genomic sequence from the woman are differentially methylated, thereby enriching for the genomic sequence from the fetus in the sample. Maternal nucleic acid may be digested using one or more methylation sensitive restriction enzymes that selectively digest or cleave maternal nucleic acid based on its methylation status. In another embodiment, the genomic sequence is at least 15 nucleotides in length, comprising at least one cytosine, further wherein the region consists of (1) a genomic locus selected from Table 1; and (2) a DNA sequence of no more than 10 kb upstream and/or downstream from the locus.

In another aspect of the technology, a method is provided for preparing nucleic acid having a nucleotide sequence of a fetal nucleic acid, which comprises the following steps: (a) providing a sample from a pregnant female; (b) separating fetal nucleic acid from maternal nucleic acid from the sample of the pregnant female according to a different methylation state between the fetal nucleic acid and the maternal nucleic acid counterpart, wherein the nucleotide sequence of the fetal nucleic acid comprises one or more CpG sites from one or more of the polynucleotide sequences of SEQ ID NOs: 1-89 within a polynucleotide sequence from a gene or locus that contains one of the polynucleotide sequences of SEQ ID NOs: 1-89; and (c) preparing nucleic acid comprising a nucleotide sequence of the fetal nucleic acid by an amplification process in which fetal nucleic acid separated in part (b) is utilized as a template. In another embodiment, a method is provided for preparing nucleic acid having a nucleotide sequence of a fetal nucleic acid, which comprises the following steps: (a) providing a sample from a pregnant female; (b) digesting or removing maternal nucleic acid from the sample of the pregnant female according to a different methylation state between the fetal nucleic acid and the maternal nucleic acid counterpart, wherein the nucleotide sequence of the fetal nucleic acid comprises one or more CpG sites from one or more of the polynucleotide sequences of SEQ ID NOs: 1-89 within a polynucleotide sequence from a gene that contains one of the polynucleotide sequences of SEQ ID NOs: 1-89; and (c) preparing nucleic acid comprising a nucleotide sequence of the fetal nucleic acid. The preparing process of step (c) may be a hybridization process, a capture process, or an amplification process in which fetal nucleic acid separated in part (b) is utilized as a template. Also, in the above embodiment wherein maternal nucleic acid is digested, the maternal nucleic acid may be digested using one or more methylation sensitive restriction enzymes that selectively digest or cleave maternal nucleic acid based on its methylation status. In either embodiment, the polynucleotide sequences of SEQ ID NOs: 1-89 may be within a polynucleotide sequence from a CpG island that contains one of the polynucleotide sequences of SEQ ID NOs: 1-89. The polynucleotide sequences of SEQ ID NOs: 1-89 are further characterized in Tables 1-3 herein, including the identification of CpG islands that overlap with the polynucleotide sequences provided in SEQ ID NOs: 1-89. In another embodiment, the nucleic acid prepared by part (c) is in solution. In yet another embodiment, the method further comprises quantifying the fetal nucleic acid from the amplification process of step (c).

In another aspect of the technology, a method is provided for enriching fetal nucleic acid from a sample from a pregnant female with respect to maternal nucleic acid, which comprises the following steps: (a) providing a sample from a pregnant female; and (b) separating or capturing fetal nucleic acid from maternal nucleic acid from the sample of the pregnant female according to a different methylation state between the fetal nucleic acid and the maternal nucleic acid, wherein the nucleotide sequence of the fetal nucleic acid comprises one or more CpG sites from one or more of the polynucleotide sequences of SEQ ID NOs: 1-89 within a polynucleotide sequence from a gene that contains one of the polynucleotide sequences of SEQ

ID NOs: 1-89. In another embodiment, the polynucleotide sequences of SEQ ID NOs: 1-89 may be within a polynucleotide sequence from a CpG island that contains one of the polynucleotide sequences of SEQ ID NOs: 1-89. The polynucleotide sequences of SEQ ID NOs: 1-89 are characterized in Table 1 herein. In another embodiment, the nucleic acid separated by part (b) is in solution. In yet another embodiment, the method further comprises amplifying and/or quantifying the fetal nucleic acid from the separation process of step (b).

In another aspect of the technology, a composition is provided comprising an isolated nucleic acid from a fetus of a pregnant female, wherein the nucleotide sequence of the nucleic acid comprises one or more of the polynucleotide sequences of SEQ ID NOs: 1-89. In one embodiment, the nucleotide sequence consists essentially of a nucleotide sequence of a gene, or portion thereof. In another embodiment, the nucleotide sequence consists essentially of a nucleotide sequence of a CpG island, or portion thereof. The polynucleotide sequences of SEQ ID NOs: 1-89 are further characterized in Table 1. In another embodiment, the nucleic acid is in solution. In another embodiment, the nucleic acid from the fetus is enriched relative to maternal nucleic acid. In another embodiment, the composition further comprises an agent that binds to methylated nucleotides. For example, the agent may be a methyl-CpG binding protein (MBD) or fragment thereof.

In another aspect of the technology, a composition is provided comprising an isolated nucleic acid from a fetus of a pregnant female, wherein the nucleotide sequence of the nucleic acid comprises one or more CpG sites from one or more of the polynucleotide sequences of SEQ ID NOs: 1-89 within a polynucleotide sequence from a gene, or portion thereof, that contains one of the polynucleotide sequences of SEQ ID NOs: 1-89. In another embodiment, the nucleotide sequence of the nucleic acid comprises one or more CpG sites from one or more of the polynucleotide sequences of SEQ ID NOs: 1-89 within a polynucleotide sequence from a CpG island, or portion thereof, that contains one of the polynucleotide sequences of SEQ ID NOs: 1-89. The polynucleotide sequences of SEQ ID NOs: 1-89 are further characterized in Table 1. In another embodiment, the nucleic acid is in solution. In another embodiment, the nucleic acid from the fetus is enriched relative to maternal nucleic acid. Hyper- and hypomethylated nucleic acid sequences of the technology are identified in Table 1. In another embodiment, the composition further comprises an agent that binds to methylated nucleotides. For example, the agent may be a methyl-CpG binding protein (MBD) or fragment thereof.

In some embodiments, a nucleotide sequence of the technology includes three or more of the CpG sites. In another embodiment, the nucleotide sequence includes five or more of the CpG sites. In another embodiment, the nucleotide sequence is from a gene region that comprises a PRC2 domain (see Table 3). In another embodiment, the nucleotide sequence is from a gene region involved with development. For example, SOX14 - which is an epigenetic marker of the present technology (See Table 1) - is a member of the SOX (SRY-related HMG-box) family of transcription factors involved in the regulation of embryonic development and in the determination of cell fate.

In some embodiments, the genomic sequence from the woman is methylated and the genomic sequence from the fetus is unmethylated. In other embodiments, the genomic sequence from the woman is unmethylated and the genomic sequence from the fetus is methylated. In another embodiment, the genomic sequence from the fetus is hypermethylated relative to the genomic sequence from the mother. Fetal genomic sequences found to be hypermethylated relative to maternal genomic sequence are provided in SEQ ID NOs: 1-59. Alternatively, the genomic sequence from the fetus is hypomethylated relative to the genomic sequence from the mother. Fetal genomic sequences found to be hypomethylated relative to maternal genomic sequence are provided in SEQ ID NOs: 60-85. Methylation sensitive restriction enzymes of the technology may be sensitive to hypo- or hyper- methylated nucleic acid.

In another embodiment, the fetal nucleic acid is extracellular nucleic acid. Generally the extracellular fetal nucleic acid is about 500, 400, 300, 250, 200 or 150 (or any number there between) nucleotide bases or less. In another embodiment, the digested maternal nucleic acid is less than about 90, 100, 110, 120, 130, 140 or 150 base pairs. In a related embodiment, the fetal nucleic acid is selectively amplified, captured or separated from or relative to the digested maternal nucleic acid based on size. For example, PCR primers may be designed to amplify nucleic acid greater than about 75, 80, 85, 90, 95, 100, 105, 110, 115 or 120 (or any number there between) base pairs thereby amplifying fetal nucleic acid and not digested maternal nucleic acid. In another embodiment, the nucleic acid is subjected to fragmentation prior to certain methodss of the technology. Examples of methods of fragmenting nucleic acid, include but are not limited to sonication and restriction enzyme digestion. In some embodiments the fetal nucleic acid is derived from the placenta. In other embodiments the fetal nucleic acid is apoptotic.

In some embodiments, the present technology provides a method in which the sample is a member selected from the following: maternal whole blood, maternal plasma or serum, amniotic fluid, a chorionic villus sample, biopsy material from a pre-implantation embryo, fetal nucleated cells or fetal cellular remnants isolated from maternal blood, maternal urine, maternal saliva, washings of the female reproductive tract and a sample obtained by celocentesis or lung lavage. In certain embodiments, the biological sample is maternal blood. In some embodiments, the biological sample is a chorionic villus sample. In certain embodiments, the maternal sample is enriched for fetal nucleic acid prior to certain methodss of the present technology. Examples of fetal enrichment methods are provided in PCT Publication Nos. WO/2007140417A2, WO2009/032781A2 and US Publication No. 20050164241.

In some embodiments, nucleated and anucleated cell populations are removed from the sample prior to practicing certain methodss of the technology (e.g., substantially all nucleated and anucleated cell populations are removed). In some embodiments, the sample is collected, stored or transported in a manner known to the person of ordinary skill in the art to minimize degradation or the quality of fetal nucleic acid present in the sample.

The sample can be from any animal, including but not limited, human, non-human, mammal, reptile, cattle, cat, dog, goat, swine, pig, monkey, ape, gorilla, bull, cow, bear, horse, sheep, poultry, mouse, rat, fish, dolphin, whale, and shark, or any animal or organism that may have a detectable pregnancy-associated disorder or chromosomal abnormality.

In some embodiments, the sample is treated with a reagent that differentially modifies methylated and unmethylated DNA. For example, the reagent may comprise bisulfite; or the reagent may comprise one or more enzymes that preferentially cleave methylated DNA; or the reagent may comprise one or more enzymes that preferentially cleave unmethylated DNA. Examples of methylation sensitive restriction enzymes include, but are not limited to, HhaI and HpaII.

In one embodiment, the fetal nucleic acid is separated from the maternal nucleic acid by an agent that specifically binds to methylated nucleotides in the fetal nucleic acid. In another embodiment, the fetal nucleic acid is separated or removed from the maternal nucleic acid by an agent that specifically binds to methylated nucleotides in the maternal nucleic acid counterpart. In an embodiment, the agent that binds to methylated nucleotides is a methyl-CpG binding protein (MBD) or fragment thereof.

In another aspect of the technology, a method is provided for determining the amount or copy number of fetal DNA in a maternal sample that comprises differentially methylated maternal and fetal DNA. The method is performed by a) distinguishing between the maternal and fetal DNA based on differential methylation status; and b) quantifying the fetal DNA of step a). In a specific embodiment, the method comprises a) digesting the maternal DNA in a maternal sample using one or more methylation sensitive restriction enzymes thereby enriching the fetal DNA; and b) determining the amount of fetal DNA from step a). The amount of fetal DNA can be used *inter alia* to confirm the presence or absence of fetal nucleic acid, determine fetal sex, diagnose fetal disease or be used in conjunction with other fetal diagnostic methods to improve sensitivity or specificity. In one embodiment, the method for determining the amount of fetal DNA does not require the use of a polymorphic sequence. For example, an allelic ratio is not used to quantify the fetal DNA in step b). In another embodiment, the method for determining the amount of fetal DNA does not require the treatment of DNA with bisulfite to convert cytosine residues to uracil. Bisulfite is known to degrade DNA, thereby, further reducing the already limited fetal nucleic acid present in maternal samples. In one embodiment, determining the amount of fetal DNA in step b) is done by introducing one or more competitors at known concentrations. In another embodiment, determining the amount of fetal DNA in step b) is done by RT-PCR, primer extension, sequencing or counting. In a related embodiment, the amount of nucleic acid is determined using BEAMing technology as described in US Patent Publication No. US20070065823. In another embodiment, the restriction efficiency is determined and the efficiency rate is used to further determine the amount of fetal DNA. Exemplary differentially methylated nucleic acids are provided in SEQ ID NOs: 1-89.

In another aspect of the technology, a method is provided for determining the concentration of fetal DNA in a maternal sample, wherein the maternal sample comprises differentially methylated maternal and fetal DNA, comprising a) determining the total amount of DNA present in the maternal sample; b) selectively digesting the maternal DNA in a maternal sample using one or more methylation sensitive restriction enzymes thereby enriching the fetal DNA; c) determining the amount of fetal DNA from step b); and d) comparing the amount of fetal DNA from step c) to the total amount of DNA from step a), thereby determining the concentration of fetal DNA in the maternal sample. The concentration of fetal DNA can be used *inter alia* in conjunction with other fetal diagnostic methods to improve sensitivity or specificity. In one embodiment, the method for determining the amount of fetal DNA does not require the use of a polymorphic sequence. For example, an allelic ratio is not used to quantify the fetal DNA in step b). In another embodiment, the method for determining the amount of fetal DNA does not require the treatment of DNA with bisulfite to convert cytosine residues to uracil. In one embodiment, determining the amount of fetal DNA in step b) is done by introducing one or more competitors at known concentrations. In another embodiment, determining the amount of fetal DNA in step b) is done by RT-PCR, sequencing or counting. In another embodiment, the restriction efficiency is determined and used to further determine the amount of total DNA and fetal DNA. Exemplary differentially methylated nucleic acids are provided in SEQ ID NOs: 1-89.

In another aspect of the technology, a method is provided for determining the presence or absence of a fetal aneuploidy using fetal DNA from a maternal sample, wherein the maternal sample comprises differentially methylated maternal and fetal DNA, comprising a) selectively digesting the maternal DNA in a maternal sample using one or more methylation sensitive restriction enzymes thereby enriching the fetal DNA; b) determining the amount of fetal DNA from a target chromosome; c) determining the amount of fetal DNA from a reference chromosome; and d) comparing the amount of fetal DNA from step b) to step c), wherein a biologically or statistically significant difference between the amount of target and reference fetal DNA is indicative of the presence of a fetal aneuploidy. In one embodiment, the method for determining the amount of fetal DNA does not require the use of a polymorphic sequence. For example, an allelic ratio is not used to quantify the fetal DNA in step b). In another embodiment, the method for determining the amount of fetal DNA does not require the treatment of DNA with bisulfite to convert cytosine residues to uracil. In one embodiment, determining the amount of fetal DNA in steps b) and c) is done by introducing one or more competitors at known concentrations. In another embodiment, determining the amount of fetal DNA in steps b) and c) is done by RT-PCR, sequencing or counting. In another embodiment, the amount of fetal DNA from a target chromosome determined in step b) is compared to a standard control, for example, the amount of fetal DNA from a target chromosome from euploid pregnancies. In another embodiment, the restriction efficiency is determined and used to further determine the amount of fetal DNA from a target chromosome and from a reference chromosome. Exemplary differentially methylated nucleic acids are provided in SEQ ID NOs: 1-89.

In another aspect of the technology, a method is provided for detecting the presence or absence of a chromosomal abnormality by analyzing the amount or copy number of target nucleic acid and control nucleic acid from a sample of differentially methylated nucleic acids comprising the steps of: (a) enriching a target nucleic acid, from a sample, and a control nucleic acid, from the sample, based on its methylation state; (b) performing a copy number analysis of the enriched target nucleic acid in at least one of the fractions; (c) performing a copy number analysis of the enriched control nucleic acid in at least one of the fractions; (d) comparing the copy number from step (b) with the copy number from step (c); and (e) determining if a chromosomal abnormality exists based on the comparison in step (d), wherein the target nucleic acid and control nucleic acid have the same or substantially the same methylation status. In a related embodiment, a method is provided for detecting the presence or absence of a chromosomal abnormality by analyzing the amount or copy number of target nucleic acid and control nucleic acid from a sample of differentially methylated nucleic acids comprising the steps of: (a) binding a target nucleic acid, from a sample, and a control nucleic acid, from the sample, to a binding agent; (b) eluting the bound nucleic acid based on methylation status, wherein differentially methylated nucleic acids elute at least partly into separate fractions; (c) performing a copy number analysis of the eluted target nucleic acid in at least one of the fractions; (d) performing a copy number analysis of the eluted control nucleic acid in at least one of the fractions; (e) comparing the copy number from step (c) with the copy number from step (d); and (f) determining if a chromosomal abnormality exists based on the comparison in step (e), wherein the target nucleic acid and control nucleic acid have the same or substantially the same methylation status. Differentially methylated nucleic acids are provided in SEQ ID NOs: 1-89.

In another aspect of the technology, a method is provided for detecting the presence or absence of a chromosomal abnormality by analyzing the allelic ratio of target nucleic acid and control nucleic acid from a sample of differentially methylated nucleic acids comprising the steps of: (a) binding a target nucleic acid, from a sample, and a control nucleic acid, from the sample, to a binding agent; (b) eluting the bound nucleic acid based on methylation status, wherein differentially methylated nucleic acids elute at least partly into separate fractions; (c) performing an allelic ratio analysis of the eluted target nucleic acid in at least one of the fractions; (d) performing an allelic ratio analysis of the eluted control nucleic acid in at least one of the fractions; (e) comparing the allelic ratio from step c with the allelic ratio from step d; and (f) determining if a chromosomal abnormality exists based on the comparison in step (e), wherein the target nucleic acid and control nucleic acid have the same or substantially the same methylation status. Differentially methylated nucleic acids are provided in SEQ ID NOs: 1-89, and SNPs within the differentially methylated nucleic acids are provided in Table 2. The methods may also be useful for detecting a pregnancy-associated disorder.

In another aspect of the technology, the amount of maternal nucleic acid is determined using the methylation-based methods of the technology. For example, fetal nucleic acid can be separated (for example, digested using a methylation-sensitive enzyme) from the maternal nucleic acid in a sample, and the maternal nucleic acid can be quantified using the methods of the technology. Once the amount of maternal nucleic acid is determined, that amount can subtracted from the total amount of nucleic acid in a sample to determine the amount of fetal nucleic acid. The amount of fetal nucleic acid can be used to detect fetal traits, including fetal aneuploidy, as described herein.

For aspects and embodiments of the technology described herein, the methods may also be useful for detecting a pregnancy-associated disorder. In some embodiments, the sample comprises fetal nucleic acid, or fetal nucleic acid and maternal nucleic acid. In the case when the sample comprises fetal and maternal nucleic acid, the fetal nucleic acid and the maternal nucleic acid may have a different methylation status. Nucleic acid species with a different methylation status can be differentiated by any method known in the art. In an embodiment, the fetal nucleic acid is enriched by the selective digestion of maternal nucleic acid by a methylation sensitive restriction enzyme. In another embodiment, the fetal nucleic acid is enriched by the selective digestion of maternal nucleic acid using two or more methylation sensitive restriction enzymes in the same assay. In an embodiment, the target nucleic acid and control nucleic acid are both from the fetus. In another embodiment, the average size of the fetal nucleic acid is about 100 bases to about 500 bases in length. In another embodiment the chromosomal abnormality is an aneuploidy, such as trisomy 21. In some embodiments, the target nucleic acid is at least a portion of a chromosome which may be abnormal and the control nucleic acid is at least a portion of a chromosome which is very rarely abnormal. For example, when the target nucleic acid is from chromosome 21, the control nucleic acid is from a chromosome other than chromosome 21 – preferably another autosome. In another embodiment, the binding agent is a methylation-specific binding protein such as MBD-Fc. Also, the enriched or eluted nucleic acid is amplified and/or quantified by any method known in the art. In an embodiment, the fetal DNA is quantified using a method that does not require the use of a polymorphic sequence. For example, an allelic ratio is not used to quantify the fetal DNA. In another embodiment, the method for quantifying the amount of fetal DNA does not require the treatment of DNA with bisulfite to convert cytosine residues to uracil.

In some embodiments, the methods of the technology include the additional step of determining the amount of one or more Y-chromosome-specific sequences in a sample. In a related embodiment, the amount of fetal nucleic acid in a sample as determined by using the methylation-based methods of the technology is compared to the amount of Y-chromosome nucleic acid present.

Methods for differentiating nucleic acid based on methylation status include, but are not limited to, methylation sensitive capture, for example using, MBD2-Fc fragment; bisulfite conversion methods, for example, MSP (methylation-sensitive PCR), COBRA, methylation-sensitive single nucleotide primer extension (Ms-SNuPE) or Sequenom MassCLEAVE™ technology; and the use of methylation sensitive restriction enzymes. Except where explicitly stated, any method for differentiating nucleic acid based on methylation status can be used with the compositions and methods of the technology.

In some embodiments, methods of the technology may further comprise an amplification step. The amplification step can be performed by PCR, such as methylation-specific PCR. In another embodiment, the amplification reaction is performed on single molecules, for example, by digital PCR, which is further described in US Patent Nos 6,143,496 and 6,440,706, both of which are hereby incorporated by reference. In other embodiments, the method does not require amplification. For example, the amount of enriched fetal DNA may be determined by counting the fetal DNA (or sequence tags attached thereto) with a flow cytometer or by sequencing means that do not require amplification. In another embodiment, the amount of fetal DNA is determined by an amplification reaction that generates amplicons larger than the digested maternal nucleic acid, thereby further enriching the fetal nucleic acid.

For embodiments that require sequence analysis, any one of the following sequencing technologies may be used: a primer extension method (e.g., iPLEX®; Sequenom, Inc.), direct DNA sequencing, restriction fragment length polymorphism (RFLP analysis), allele specific oligonucleotide (ASO) analysis, methylation-specific PCR (MSPCR), pyrosequencing analysis, acycloprime analysis, Reverse dot blot, GeneChip microarrays, Dynamic allele-specific hybridization (DASH), Peptide nucleic acid (PNA) and locked nucleic acids (LNA) probes, TaqMan, Molecular Beacons, Intercalating dye, FRET primers, fluorescence tagged dNTP/ddNTPs, AlphaScreen, SNPstream, genetic bit analysis (GBA), Multiplex minisequencing, SNaPshot, GOOD assay, Microarray miniseq, arrayed primer extension (APEX), Microarray primer extension, Tag arrays, Coded microspheres, Template-directed incorporation (TDI), fluorescence polarization, Colorimetric oligonucleotide ligation assay (OLA), Sequence-coded OLA, Microarray ligation, Ligase chain reaction, Padlock probes, Invader™ assay, hybridization using at least one probe, hybridization using at least one fluorescently labeled probe, electrophoresis, cloning and sequencing, for example as performed on the 454 platform (Roche) (Margulies, M. et al. 2005 Nature 437, 376-380), Illumina Genome Analyzer (or Solexa platform) or SOLiD System (Applied Biosystems) or the Helicos True Single Molecule DNA sequencing technology (Harris T D et al. 2008 Science, 320, 106-109), the single molecule, real-time (SMRT.TM.) technology of Pacific Biosciences, or nanopore-based sequencing (Soni GV and Meller A. 2007 Clin Chem 53: 1996-2001) and combinations thereof. Nanopore-based methods may include sequencing nucleic acid using a nanopore, or counting nucleic acid molecules using a nanopore, for example, based on size wherein sequence information is not determined.

The absolute copy number of one or more nucleic acids can be determined, for example, using mass spectrometry, a system that uses a competitive PCR approach for absolute copy number measurements. See for example, Ding C, Cantor CR (2003) A high-throughput gene expression analysis technique using competitive PCR and matrix-assisted laser desorption ionization time-of-flight MS. Proc Natl Acad Sci U S A 100:3059-3064, and US Patent Application No. 10/655762, which published as US Patent Publication No. 20040081993, both of which are hereby incorporated by reference.

In some embodiments, the amount of the genomic sequence is compared with a standard control, wherein an increase or decrease from the standard control indicates the presence or progression of a pregnancy-associated disorder. For example, the amount of fetal nucleic acid may be compared to the total amount of DNA present in the sample. Or when detecting the presence or absence of fetal aneuploidy, the amount of fetal nucleic acid from target chromosome may be compared to the amount of fetal nucleic acid from a reference chromosome. Preferably the reference chromosome is another autosome that has a low rate of aneuploidy. The ratio of target fetal nucleic acid to reference fetal nucleic acid may be compared to the same ratio from a normal, euploid pregnancy. For example, a control ratio may be determined from a DNA sample obtained from a female carrying a healthy fetus who does not have a chromosomal abnormality. Preferably, one uses a panel of control samples. Where certain chromosome anomalies are known, one can also have standards that are indicative of a specific disease or condition. Thus, for example, to screen for three different chromosomal aneuploidies in a maternal plasma of a pregnant female, one preferably uses a panel of control DNAs that have been isolated from mothers who are known to carry a fetus with, for example, chromosome 13, 18, or 21 trisomy, and a mother who is pregnant with a fetus who does not have a chromosomal abnormality.

In some embodiments, the present technology provides a method in which the alleles from the target nucleic acid and control nucleic acid are differentiated by sequence variation. The sequence variation may be a single nucleotide polymorphism (SNP) or an insertion/deletion polymorphism. In an embodiment, the fetal nucleic acid should comprise at least one high frequency heterozygous polymorphism (e.g., about 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 25% or more frequency rate), which allows the determination of the allelic-ratio of the nucleic acid in order to assess the presence or absence of the chromosomal abnormality. A list of exemplary SNPs is provided in Table 2, however, this does not represent a complete list of polymorphic alleles that can be used as part of the technology. Any SNP meeting the following criteria may also be considered: (a) the SNP has a heterozygosity frequency greater than about 2% (preferably across a range of different populations), (b) the

SNP is a heterozygous locus; and (c)(i) the SNP is within nucleic acid sequence described herein, or (c)(iii) the SNP is within about 5 to about 2000 base pairs of a SNP described herein (e.g., within about 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1250, 1500, 1750 or 2000 base pairs of a SNP described herein).

In other embodiments, the sequence variation is a short tandem repeat (STR) polymorphism. In some embodiments, the sequence variation falls in a restriction site, whereby one allele is susceptible to digestion by a restriction enzyme and the one or more other alleles are not. In some embodiments, the sequence variation is a methylation site.

In some embodiments, performing an allelic ratio analysis comprises determining the ratio of alleles of the target nucleic acid and control nucleic acid from the fetus of a pregnant woman by obtaining an nucleic acid-containing biological sample from the pregnant woman, wherein the biological sample contains fetal nucleic acid, partially or wholly separating the fetal nucleic acid from the maternal nucleic acid based on differential methylation, discriminating the alleles from the target nucleic acid and the control nucleic acid, followed by determination of the ratio of the alleles, and detecting the presence or absence of a chromosomal disorder in the fetus based on the ratio of alleles, wherein a ratio above or below a normal, euploid ratio is indicative of a chromosomal disorder. In one embodiment, the target nucleic acid is from a suspected aneuploid chromosome (e.g., chromosome 21) and the control nucleic acid is from a euploid chromosome from the same fetus.

In some embodiments, the present technology is combined with other fetal markers to detect the presence or absence of multiple chromosomal abnormalities, wherein the chromosomal abnormalities are selected from the following: trisomy 21, trisomy 18 and trisomy 13, or combinations thereof. In some embodiments, the chromosomal disorder involves the X chromosome or the Y chromosome.

In some embodiments, the compositions or processes may be multiplexed in a single reaction. For example, the amount of fetal nucleic acid may be determined at multiple loci across the genome. Or when detecting the presence or absence of fetal aneuploidy, the amount of fetal nucleic acid may be determined at multiple loci on one or more target chromosomes (e.g., chromosomes 13, 18 or 21) and on one or more reference chromosomes. If an allelic ratio is being used, one or more alleles from Table 2 can be detected and discriminated simultaneously. When determining allelic ratios, multiplexing embodiments are particularly important when the genotype at a polymorphic locus is not known. In some instances, for example when the mother and child are homozygous at the polymorphic locus, the assay may not be informative. In one embodiment, greater than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 50, 100, 200, 300 or 500, and any intermediate levels, polynucleotide sequences of the technology are enriched, separated and/or examined according the methods of the technology. When detecting a chromosomal abnormality by analyzing the copy number of target nucleic acid and control nucleic acid, less than 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 polynucleotide sequences may need to be analyzed to accurately detect the presence or absence of a chromosomal abnormality. In another embodiment, the compositions or processes of the technology may be used to assay samples that have been divided into 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 50, 100 or more replicates, or into single molecule equivalents. Methods for analyzing fetal nucleic acids from a maternal sample in replicates, including single molecule analyses, are provided in US Application No, 11/364,294, which published as US Patent Publication No. US 2007-0207466 A1, which is hereby incorporated by reference.

In a further embodiment, the present technology provides a method wherein a comparison step shows an increased risk of a fetus having a chromosomal disorder if the ratio of the alleles or absolute copy number of the target nucleic acid is higher or lower by 1 standard deviation from the standard control sequence. In some embodiments, the comparison step shows an increased risk of a fetus having a chromosomal disorder if the ratio of the alleles or absolute copy number of the target nucleic acid is higher or lower by 2 standard deviation from the standard control sequence. In some other embodiments, the comparison step shows an increased risk of a fetus having a chromosomal disorder if the ratio of the alleles or absolute copy number of the target nucleic acid is higher or lower by 3 standard deviation from the standard control sequence. In some embodiments, the comparison step shows an increased risk of a fetus having a chromosomal disorder if the ratio of the alleles or absolute copy number of the target nucleic acid is higher or lower than a statistically significant standard deviation from the control. In one embodiment, the standard control is a maternal reference, and in another embodiment the standard control is a fetal reference chromosome (e.g., non-trisomic autosome).

In some embodiments, the methods of the technology may be combined with other methods for diagnosing a chromosomal abnormality. For example, a noninvasive diagnostic method may require confirmation of the presence or absence of fetal nucleic acid, such as a sex test for a female fetus or to confirm an RhD negative female fetus in an RhD negative mother. In another embodiment, the compositions and methods of the technology may be used to determine the percentage of fetal nucleic acid in a maternal sample in order to enable another diagnostic method that requires the percentage of fetal nucleic acid be known. For example, does a sample meet certain threshold concentration requirements? When determining an allelic ratio to diagnose a fetal aneuploidy from a maternal sample, the amount or concentration of fetal nucleic acid may be required to make a diagnose with a given sensitivity and specificity. In other embodiments, the compositions and methods of the technology for detecting a chromosomal abnormality can be combined with other known methods thereby improving the overall sensitivity and specificity of the detection method. For example, mathematical models have suggested that a combined first-trimester screening program utilizing maternal age (MA), nuchal translucency (NT) thickness, serum-free beta-hCG, and serum PAPP-A will detect more than 80% of fetuses with Down's syndrome for a 5% invasive testing rate (Wald and Hackshaw, Prenat Diagn 17(9):921-9 (1997)). However, the combination of commonly used aneuploidy detection methods combined with the non-invasive free fetal nucleic acid-based methods described herein may offer improved accuracy with a lower false positive rate. Examples of combined diagnostic methods are provided in PCT Publication Number WO2008157264A2 (assigned to the Applicant), which is hereby incorporated by reference. In some embodiments, the methods of the technology may be combined with cell-based methods, wherein fetal cells are procured invasively or non-invasively.

In certain embodiments, an increased risk for a chromosomal abnormality is based on the outcome or result(s) produced from the compositions or methods provided herein. An example of an outcome is a deviation from the euploid absolute copy number or allelic ratio, which indicates the presence of chromosomal aneuploidy. This increase or decrease in the absolute copy number or ratio from the standard control indicates an increased risk of having a fetus with a chromosomal abnormality (e.g., trisomy 21). Information pertaining to a method described herein, such as an outcome, result, or risk of trisomy or aneuploidy, for example, may be transfixed, renditioned, recorded and/or displayed in any suitable medium. For example, an outcome may be transfixed in a medium to save, store, share, communicate or otherwise analyze the outcome. A medium can be tangible (e.g., paper) or intangible (e.g., electronic medium), and examples of media include, but are not limited to, computer media, databases, charts, patient charts, records, patient records, graphs and tables, and any other medium of expression. The information sometimes is stored and/or renditioned in computer readable form and sometimes is stored and organized in a database. In certain embodiments, the information may be transferred from one location to another using a physical medium (e.g., paper) or a computer readable medium (e.g., optical and/or magnetic storage or transmission medium, floppy disk, hard disk, random access memory, computer processing unit, facsimile signal, satellite signal, transmission over an internet or transmission over the world-wide web).

A CpG island may be used as the CpG-containing genomic sequence in some cases, whereas in other cases the CpG-containing genomic sequence may not be a CpG island. In some embodiments, the present technology provides a kit for performing the methods of the technology. One component of the kit is a methylation-sensitive binding agent.

In a further embodiment, the present invention provides a method for determining the amount of fetal nucleic acid in a sample comprising:

a) contacting nucleic acid present in a sample from a pregnant female, which nucleic acid comprises fetal nucleic acid and maternal nucleic acid, the combination of the fetal nucleic acid and the maternal nucleic acid comprising total nucleic acid in the sample, with a reagent that digests nucleic acid at non-methylated sites, thereby enriching methylated nucleic acid; and

b) determining the amount of fetal nucleic acid in (a) by analyzing the amount of fetal nucleic acid at a plurality of loci selected from SEQ ID NOs:1-59.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present disclosure as it existed before the priority date of each claim of this application.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1: Shows the design of the recombinant MBD-Fc protein used to separate differentially methylated DNA.

FIGURE 2: Shows the methyl-CpG-binding, antibody-like protein has a high affinity and high avidity to its "antigen", which is preferably DNA that is methylated at CpG di-nucleotides.

FIGURE 3: Shows the methyl binding domain of MBD-FC binds DNA molecules regardless of their methylation status. The strength of this protein/DNA interaction is defined by the level of DNA methylation. After binding genomic DNA, eluate solutions of increasing salt concentrations can be used to fractionate non-methylated and methylated DNA allowing for a controlled separation.

FIGURE 4: Shows the experiment used to identify differentially methylated DNA from a fetus and mother using the recombinant MBD-Fc protein and a microarray.

FIGURE 5: Shows typical results generated by Sequenom® EpiTYPER™ method, which was used to validate the results generated from the experiment illustrated in Figure 4.

FIGURE 6: Shows the correlation between the log ratios derived from microarray analysis (x axis) and methylation differences obtained by EpiTYPER analysis (y axis). Each data point represents the average for

one region across all measured samples. The microarray analysis is comparative in nature because the highly methylated fraction of the maternal DNA is hybridized together with the highly methylated fraction of placenta DNA. Positive values indicate higher methylation of the placenta samples. In mass spectrometry each samples is measured individually. We first calculated difference in methylation by subtracting the maternal methylation values from the placenta methylation value. To compare the results with the microarray data we calculated the average of the differences for all maternal / placenta DNA pairs.

FIGURE 8: Shown is the correlation between the number of gDNA molecules that were expected and the number of molecules measured by competitive PCR in combination with mass spectrometry analysis. In this experiment we used DNA derived from whole blood (black plus signs) and commercially available fully methylated DNA(red crosses) in a 90 to 10 ratio. We used the MBD-FC fusion protein to separate the non-methylated and the methylated fraction of DNA. Each fraction was subject to competitive PCR analysis with mass spectrometry readout. The method has been described earlier for the analysis of copy number variations and is commercially available for gene expression analysis. The approach allows absolute quantification of DNA molecules with the help of a synthetic oligonucleotides of know concentration. In this experiment we targeted the MGMT locus, which was not methylated in the whole blood sample used here. Using an input of 300 total gDNA copies we expect to see 270 copies of non-methylated DNA and 30 copies of methylated DNA. The measured copy numbers are largely in agreement with the expected values. The data point at 600 copies of input DNA indicates a bias in the reaction and shows that this initial proof of concept experiment needs to be followed up with more development work, before the assay can be used. However, this initial data indicates the feasibility of the approach for capturing and quantifying of a few copies of methylated DNA in the presence of an excess of unmethylated DNA species.

FIGURE 9A-9C: Shown are bar graph plots of the methylation differences obtained from the microarray analysis (dark bars) and the mass spectrometry analysis (light grey bars) with respect to their genomic location. For each of the 85 region that were identified to be differentially methylated by microarray an individual plot is provided. The x axis for each plot shows the chromosomal position of the region. The y axis depicts the log ration (in case of the microarrays) and the methylation differences (in case of the mass spectrometry results). For the microarrays each hybridization probe in the area is shown as a single black (or dark grey) bar. For the mass spectrometry results each CpG site, is shown as a light grey bar. Bars showing values greater than zero indicate higher DNA methylation in the placenta samples compared to the maternal DNA. For some genes the differences are small (i.e. RB1 or DSCR6) but still statistically significant. Those regions would be less suitable for a fetal DNA enrichment strategy.

FIGURE 10: Shows one embodiment of the Fetal Quantifier Method. Maternal nucleic acid is selectively digested and the remaining fetal nucleic acid is quantified using a competitor of known concentration. In this schema, the analyte is separated and quantified by a mass spectromter.

FIGURE 11: Shows one embodiment of the Methylation-Based Fetal Diagnostic Method. Maternal nucleic acid is selectively digested and the remaining fetal nucleic acid is quantified for three different chromosomes (13, 18 and 21). Parts 2 and 3 of the Figure illustrate the size distribution of the nucleic acid in the sample before and after digestion. The amplification reactions can be size-specific (e.g., greater than

100 base pair amplicons) such that they favor the longer, non-digested fetal nucleic acid over the digested maternal nucleic acid, thereby further enriching the fetal nucleic acid. The spectra at the bottom of the Figure show an increased amount of chromosome 21 fetal nucleic acid indicative of trisomy 21.

FIGURE 12: Shows the total number of amplifiable genomic copies from four different DNA samples isolated from the blood of non-pregnant women. Each sample was diluted to contain approximately 2500, 1250, 625 or 313 copies per reaction. Each measurement was obtained by taking the mean DNA/competitor ratio obtained from two total copy number assays (ALB and RNAseP in Table X). As Figure 12 shows, the total copy number is accurate and stable across the different samples, thus validating the usefulness of the competitor-based approach.

FIGURES 13A and B: A model system was created that contained a constant number of maternal non-methylated DNA with varying amounts of male placental methylated DNA spiked-in. The samples were spiked with male placental amounts ranging from approximately 0 to 25% relative to the maternal non-methylated DNA. The fraction of placental DNA was calculated using the ratios obtained from the methylation assays (Figure 13A) and the Y-chromosome marker (Figure 13B) as compared to the total copy number assay. The methylation and Y-chromosome markers are provided in Table X.

FIGURES 14 A and B: Show the results of the total copy number assay from plasma samples. In Figure 14A, the copy number for each sample is shown. Two samples (no 25 and 26) have a significantly higher total copy number than all the other samples. A mean of approximately 1300 amplifiable copies/ml plasma was obtained (range 766-2055). Figure 14B shows a box-and-whisker plot of the given values, summarizing the results.

FIGURES 15A and B: The amount (or copy numbers) of fetal nucleic acid from 33 different plasma samples taken from pregnant women with male fetuses are plotted. The copy numbers obtained were calculated using the methylation markers and the Y-chromosome-specific markers using the assays provided in Table X. As can be seen in Figure 15B, the box-and-whisker plot of the given values indicated minimal difference between the two different measurements, thus validating the accuracy and stability of the method.

FIGURE 16: Shows a paired correlation between the results obtained using the methylation markers versus the Y-chromosome marker from Figure 15A.

FIGURE 17: Shows the digestion efficiency of the restriction enzymes using the ratio of digestion for the control versus the competitor and comparing this value to the mean total copy number assays. Apart from sample 26 all reactions indicate the efficiency to be above about 99%.

FIGURE 18: Provides a specific method for calculating fetal DNA fraction (or concentration) in a sample using the Y-chromosome-specific markers for male pregnancies and the mean of the methylated fraction for all pregnancies (regardless of fetal sex).

FIGURE 19: Provides a specific method for calculating fetal DNA fraction (or concentration) in a sample without the Y-chromosome-specific markers. Instead, only the Assays for Methylation Quantification were used to determine the concentration of fetal DNA.

FIGURE 20: Shows a power calculation t-test for a simulated trisomy 21 diagnosis using the methods of the technology. The Figure shows the relationship between the coefficient of variation (CV) on the x-axis and the power to discriminate the assay populations using a simple t-test (y-axis). The data indicates that in 99% of all cases, one can discriminate the two population (euploid vs. aneuploid) on a significance level of 0.001 provided a CV of 5% or less.

DEFINITIONS

The term "pregnancy-associated disorder," as used in this application, refers to any condition or disease that may affect a pregnant woman, the fetus, or both the woman and the fetus. Such a condition or disease may manifest its symptoms during a limited time period, e.g., during pregnancy or delivery, or may last the entire life span of the fetus following its birth. Some examples of a pregnancy-associated disorder include ectopic pregnancy, preeclampsia, preterm labor, RhD incompatibility, fetal chromosomal abnormalities such as trisomy 21, and genetically inherited fetal disorders such as cystic fibrosis, beta-thalassemia or other monogenic disorders. The ability to enrich fetal nucleic from a maternal sample may prove particularly useful for the noninvasive prenatal diagnosis of autosomal recessive diseases such as the case when a mother and father share an identical disease causing mutation, an occurrence previously perceived as a challenge for maternal plasma-based non-trisomy prenatal diagnosis.

The term "chromosomal abnormality" or "aneuploidy" as used herein refers to a deviation between the structure of the subject chromosome and a normal homologous chromosome. The term "normal" refers to the predominate karyotype or banding pattern found in healthy individuals of a particular species, for example, a euploid genome (in humans, 46XX or 46XY). A chromosomal abnormality can be numerical or structural, and includes but is not limited to aneuploidy, polyploidy, inversion, a trisomy, a monosomy, duplication, deletion, deletion of a part of a chromosome, addition, addition of a part of chromosome, insertion, a fragment of a chromosome, a region of a chromosome, chromosomal rearrangement, and translocation. Chromosomal abnormality may also refer to a state of chromosomal abnormality where a portion of one or more chromosomes is not an exact multiple of the usual haploid number due to, for example, chromosome translocation. Chromosomal translocation (e.g. translocation between chromosome 21 and 14 where some of the 14th chromosome is replaced by extra 21st chromosome) may cause partial trisomy 21. A chromosomal abnormality can be correlated with presence of a pathological condition or with a predisposition to develop a pathological condition. A chromosomal abnormality may be detected by quantitative analysis of nucleic acid.

The terms "nucleic acid" and "nucleic acid molecule" may be used interchangeably throughout the disclosure. The terms refer to nucleic acids of any composition from, such as DNA (e.g., complementary DNA (cDNA), genomic DNA (gDNA) and the like), RNA (e.g., message RNA (mRNA), short inhibitory RNA (siRNA), ribosomal RNA (rRNA), tRNA, microRNA, RNA highly expressed by the fetus or placenta, and the like), and/or DNA or RNA analogs (e.g., containing base analogs, sugar analogs and/or a non-native backbone and the like), RNA/DNA hybrids and polyamide nucleic acids (PNAs), all of which can be in single- or double-stranded form, and unless otherwise limited, can encompass known analogs of natural nucleotides that can function in a similar manner as naturally occurring nucleotides. For example, the nucleic acids provided in SEQ ID Nos: 1-89 (see Table 4) can be in any form useful for conducting processes herein (e.g., linear, circular, supercoiled, single-stranded, double-stranded and the like) or may include variations (e.g., insertions, deletions or substitutions) that do not alter their utility as part of the present technology. A nucleic acid may be, or may be from, a plasmid, phage, autonomously replicating sequence (ARS), centromere, artificial chromosome, chromosome, or other nucleic acid able to replicate or be replicated in vitro or in a host cell, a cell, a cell nucleus or cytoplasm of a cell in certain embodiments. A template nucleic acid in some embodiments can be from a single chromosome (e.g., a nucleic acid sample may be from one chromosome of a sample obtained from a diploid organism). Unless specifically limited, the term encompasses nucleic acids containing known analogs of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions), alleles, orthologs, single nucleotide polymorphisms (SNPs), and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer et al., Nucleic Acid Res. 19:5081 (1991); Ohtsuka et al., J. Biol. Chem. 260:2605-2608 (1985); and Rossolini et al., Mol. Cell. Probes 8:91-98 (1994)). The term nucleic acid is used interchangeably with locus, gene, cDNA, and mRNA encoded by a gene. The term also may include, as equivalents, derivatives, variants and analogs of RNA or DNA synthesized from nucleotide analogs, single-stranded ("sense" or "antisense", "plus" strand or "minus" strand, "forward" reading frame or "reverse" reading frame) and double-stranded polynucleotides. Deoxyribonucleotides include deoxyadenosine, deoxycytidine, deoxyguanosine and deoxythymidine. For RNA, the base cytosine is replaced with uracil. A template nucleic acid may be prepared using a nucleic acid obtained from a subject as a template.

A "nucleic acid comprising one or more CpG sites" or a "CpG-containing genomic sequence" as used herein refers to a segment of DNA sequence at a defined location in the genome of an individual such as a human fetus or a pregnant woman. Typically, a "CpG-containing genomic sequence" is at least 15 nucleotides in length and contains at least one cytosine. Preferably, it can be at least 30, 50, 80, 100, 150, 200, 250, or 300 nucleotides in length and contains at least 2, 5, 10, 15, 20, 25, or 30 cytosines. For anyone "CpG-containing genomic sequence" at a given location, e.g., within a region centering around a given genetic locus (see Table 1), nucleotide sequence variations may exist from individual to individual and from allele to allele even for the same individual. Typically, such a region centering around a defined genetic locus (e.g., a CpG island) contains the locus as well as upstream and/or downstream sequences. Each of the upstream or downstream sequence (counting from the 5' or 3' boundary of the genetic locus, respectively) can be as long as 10 kb, in other cases may be as long as 5 kb, 2 kb, 1 kb, 500 bp, 200 bp, or 100 bp. Furthermore, a "CpG-containing genomic sequence" may encompass a nucleotide sequence transcribed or not transcribed for protein production, and the nucleotide sequence can be an inter-gene sequence, intra-gene sequence, protein-coding sequence, a non protein-coding sequence (such as a transcription promoter), or a combination thereof.

As used herein, a "methylated nucleotide" or a "methylated nucleotide base" refers to the presence of a methyl moiety on a nucleotide base, where the methyl moiety is not present in a recognized typical nucleotide base. For example, cytosine does not contain a methyl moiety on its pyrimidine ring, but 5-

methylcytosine contains a methyl moiety at position 5 of its pyrimidine ring. Therefore, cytosine is not a methylated nucleotide and 5-methylcytosine is a methylated nucleotide. In another example, thymine contains a methyl moiety at position 5 of its pyrimidine ring, however, for purposes herein, thymine is not considered a methylated nucleotide when present in DNA since thymine is a typical nucleotide base of DNA. Typical nucleoside bases for DNA are thymine, adenine, cytosine and guanine. Typical bases for RNA are uracil, adenine, cytosine and guanine. Correspondingly a "methylation site" is the location in the target gene nucleic acid region where methylation has, or has the possibility of occurring. For example a location containing CpG is a methylation site wherein the cytosine may or may not be methylated.

As used herein, a "CpG site" or "methylation site" is a nucleotide within a nucleic acid that is susceptible to methylation either by natural occurring events in vivo or by an event instituted to chemically methylate the nucleotide in vitro.

As used herein, a "methylated nucleic acid molecule" refers to a nucleic acid molecule that contains one or more methylated nucleotides that is/are methylated.

A "CpG island" as used herein describes a segment of DNA sequence that comprises a functionally or structurally deviated CpG density. For example, Yamada et al. (Genome Research 14:247-266, 2004) have described a set of standards for determining a CpG island: it must be at least 400 nucleotides in length, has a greater than 50% GC content, and an OCF/ECF ratio greater than 0.6. Others (Takai et al., Proc. Natl. Acad. Sci. U.S.A. 99:3740-3745, 2002) have defined a CpG island less stringently as a sequence at least 200 nucleotides in length, having a greater than 50% GC content, and an OCF/ECF ratio greater than 0.6.

The term "epigenetic state" or "epigenetic status" as used herein refers to any structural feature at a molecular level of a nucleic acid (e.g., DNA or RNA) other than the primary nucleotide sequence. For instance, the epigenetic state of a genomic DNA may include its secondary or tertiary structure determined or influenced by, e.g., its methylation pattern or its association with cellular proteins.

The term "methylation profile" "methylation state" or "methylation status," as used herein to describe the state of methylation of a genomic sequence, refers to the characteristics of a DNA segment at a particular genomic locus relevant to methylation. Such characteristics include, but are not limited to, whether any of the cytosine (C) residues within this DNA sequence are methylated, location of methylated C residue(s), percentage of methylated C at any particular stretch of residues, and allelic differences in methylation due to, e.g., difference in the origin of the alleles. The term "methylation" profile" or "methylation status" also refers to the relative or absolute concentration of methylated C or unmethylated C at any particular stretch of residues in a biological sample. For example, if the cytosine (C) residue(s) within a DNA sequence are methylated it may be referred to as "hypermethylated"; whereas if the cytosine (C) residue(s) within a DNA sequence are not methylated it may be referred to as "hypomethylated". Likewise, if the cytosine (C) residue(s) within a DNA sequence (e.g., fetal nucleic acid) are methylated as compared to another sequence from a different region or from a different individual (e.g., relative to maternal nucleic acid), that sequence is considered hypermethylated compared to the other sequence. Alternatively, if the cytosine (C) residue(s) within a DNA sequence are not methylated as compared to another sequence from a different region or from a different individual (e.g., the mother), that sequence is considered hypomethylated compared to the other sequence. These sequences are said to be "differentially methylated", and more specifically, when the methylation status differs between mother and fetus, the sequences are considered "differentially methylated maternal and fetal nucleic acid".

The term "agent that binds to methylated nucleotides" as used herein refers to a substance that is capable of binding to methylated nucleic acid. The agent may be naturally-occurring or synthetic, and may be modified or unmodified. In one embodiment, the agent allows for the separation of different nucleic acid species according to their respective methylation states. An example of an agent that binds to methylated nucleotides is described in PCT Patent Application No. PCT/EP2005/012707, which published as WO06056480A2 and is hereby incorporated by reference. The described agent is a bifunctional polypeptide comprising the DNA-binding domain of a protein belonging to the family of Methyl-CpG binding proteins (MBDs) and an Fc portion of an antibody (see Figure 1). The recombinant methyl-CpG-binding, antibody-like protein can preferably bind CpG methylated DNA in an antibody-like manner. That means, the methyl-CpG-binding, antibody-like protein has a high affinity and high avidity to its "antigen", which is preferably DNA that is methylated at CpG dinucleotides. The agent may also be a multivalent MBD (see Figure 2).

The term "polymorphism" as used herein refers to a sequence variation within different alleles of the same genomic sequence. A sequence that contains a polymorphism is considered "polymorphic sequence". Detection of one or more polymorphisms allows differentiation of different alleles of a single genomic sequence or between two or more individuals. As used herein, the term "polymorphic marker" or "polymorphic sequence" refers to segments of genomic DNA that exhibit heritable variation in a DNA sequence between individuals. Such markers include, but are not limited to, single nucleotide polymorphisms (SNPs), restriction fragment length polymorphisms (RFLPs), short tandem repeats, such as di-, tri- or tetra-nucleotide repeats (STRs), and the like. Polymorphic markers according to the present technology can be used to specifically differentiate between a maternal and paternal allele in the enriched fetal nucleic acid sample.

The terms "single nucleotide polymorphism" or "SNP" as used herein refer to the polynucleotide sequence variation present at a single nucleotide residue within different alleles of the same genomic sequence. This variation may occur within the coding region or non-coding region (i.e., in the promoter or intronic region) of a genomic sequence, if the genomic sequence is transcribed during protein production. Detection of one or more SNP allows differentiation of different alleles of a single genomic sequence or between two or more individuals.

The term "allele" as used herein is one of several alternate forms of a gene or non-coding regions of DNA that occupy the same position on a chromosome. The term allele can be used to describe DNA from any organism including but not limited to bacteria, viruses, fungi, protozoa, molds, yeasts, plants, humans, non-humans, animals, and archeabacteria.

The terms "ratio of the alleles" or "allelic ratio" as used herein refer to the ratio of the population of one allele and the population of the other allele in a sample. In some trisomic cases, it is possible that a fetus may be tri-allelic for a particular locus. In such cases, the term "ratio of the alleles" refers to the ratio of the population of any one allele against one of the other alleles, or any one allele against the other two alleles.

The term "non-polymorphism-based quantitative method" as used herein refers to a method for determining the amount of an analyte (e.g., total nucleic acid, Y-chromosome nucleic acid, or fetal nucleic acid) that does not require the use of a polymorphic marker or sequence. Although a polymorphism may be present in the sequence, said polymorphism is not required to quantify the sequence. Examples of non-polymorphism-based quantitative methods include, but are not limited to, RT-PCR, digital PCR, array-based methods, sequencing methods, nanopore-based methods, nucleic acid-bound bead-based counting methods and competitor-based methods wherein one or more competitors are introduced at a known concentration(s) to determine the amount of one or more analytes. In some embodiments, some of the above exemplary methods (for example, sequencing) may need to be actively modified or designed such that one or more polymorphisms are not interrogated.

The terms "absolute amount" or "copy number" as used herein refers to the amount or quantity of an analyte (e.g., total nucleic acid or fetal nucleic acid). The present technology provides compositions and processes for determining the absolute amount of fetal nucleic acid in a mixed maternal sample. Absolute amount or copy number represents the number of molecules available for detection, and may be expressed as the genomic equivalents per unit. The term "concentration" refers to the amount or proportion of a substance in a mixture or solution (e.g., the amount of fetal nucleic acid in a maternal sample that comprises a mixture of maternal and fetal nucleic acid). The concentration may be expressed as a percentage, which is used to express how large/small one quantity is, relative to another quantity as a fraction of 100. Platforms for determining the quantity or amount of an analyte (e.g., target nucleic acid) include, but are not limited to, mass spectrometery, digital PCR, sequencing by synthesis platforms (e.g., pyrosequencing), fluorescence spectroscopy and flow cytometry.

The term "sample" as used herein refers to a specimen containing nucleic acid. Examples of samples include, but are not limited to, tissue, bodily fluid (for example, blood, serum, plasma, saliva, urine, tears, peritoneal fluid, ascitic fluid, vaginal secretion, breast fluid, breast milk, lymph fluid, cerebrospinal fluid or mucosa secretion), umbilical cord blood, chorionic villi, amniotic fluid, an embryo, a two-celled embryo, a four-celled embryo, an eight-celled embryo, a 16-celled embryo, a 32-celled embryo, a 64-celled embryo, a 128-celled embryo, a 256-celled embryo, a 512-celled embryo, a 1024-celled embryo, embryonic tissues, lymph fluid, cerebrospinal fluid, mucosa secretion, or other body exudate, fecal matter, an individual cell or extract of the such sources that contain the nucleic acid of the same, and subcellular structures such as mitochondria, using protocols well established within the art.

Fetal DNA can be obtained from sources including but not limited to maternal blood, maternal serum, maternal plasma, fetal cells, umbilical cord blood, chorionic villi, amniotic fluid, urine, saliva, lung lavage, cells or tissues.

The term "blood" as used herein refers to a blood sample or preparation from a pregnant woman or a woman being tested for possible pregnancy. The term encompasses whole blood or any fractions of blood, such as serum and plasma as conventionally defined.

The term "bisulfite" as used herein encompasses any suitable types of bisulfites, such as sodium bisulfite, that are capable of chemically converting a cytosine (C) to a uracil (U) without chemically modifying a methylated cytosine and therefore can be used to differentially modify a DNA sequence based on the methylation status of the DNA.

As used herein, a reagent that "differentially modifies" methylated or non-methylated DNA encompasses any reagent that modifies methylated and/or unmethylated DNA in a process through which distinguishable products result from methylated and non-methylated DNA, thereby allowing the identification of the DNA methylation status. Such processes may include, but are not limited to, chemical reactions (such as a C.fwdarw.U conversion by bisulfite) and enzymatic treatment (such as cleavage by a methylation-dependent endonuclease). Thus, an enzyme that preferentially cleaves or digests methylated DNA is one capable of cleaving or digesting a DNA molecule at a much higher efficiency when the DNA is methylated, whereas an enzyme that preferentially cleaves or digests unmethylated DNA exhibits a significantly higher efficiency when the DNA is not methylated.

The terms "non-bisulfite-based method" and "non-bisulfite-based quantitative method" as used herein refer to any method for quantifying methylated or non-methylated nucleic acid that does not require the use of bisulfite. The terms also refer to methods for preparing a nucleic acid to be quantified that do not require bisulfite treatment. Examples of non-bisulfite-based methods include, but are not limited to, methods for digesting nucleic acid using one or more methylation sensitive enzymes and methods for separating nucleic acid using agents that bind nucleic acid based on methylation status.

The terms "methyl-sensitive enzymes" and "methylation sensitive restriction enzymes" are DNA restriction endonucleases that are dependent on the methylation state of their DNA recognition site for activity. For example, there are methyl-sensitive enzymes that cleave or digest at their DNA recognition sequence only if it is not methylated. Thus, an unmethylated DNA sample will be cut into smaller fragments than a methylated DNA sample. Similarly, a hypermethylated DNA sample will not be cleaved. In contrast, there are methyl-sensitive enzymes that cleave at their DNA recognition sequence only if it is methylated. As used herein, the terms "cleave", "cut" and "digest" are used interchangeably.

The term "target nucleic acid" as used herein refers to a nucleic acid examined using the methods disclosed herein to determine if the nucleic acid is part of a pregnancy-related disorder or chromosomal abnormality. For example, a target nucleic acid from chromosome 21 could be examined using the methods of the technology to detect Down's Syndrome.

The term "control nucleic acid" as used herein refers to a nucleic acid used as a reference nucleic acid according to the methods disclosed herein to determine if the nucleic acid is part of a chromosomal abnormality. For example, a control nucleic acid from a chromosome other than chromosome 21 (herein referred to as a "reference chromosome") could be as a reference sequence to detect Down's Syndrome. In some embodiments, the control sequence has a known or predetermined quantity.

The term “sequence-specific” or “locus-specific method” as used herein refers to a method that interrogates (for example, quantifies) nucleic acid at a specific location (or locus) in the genome based on the sequence composition. Sequence-specific or locus-specific methods allow for the quantification of specific regions or chromosomes.

The term "gene" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region (leader and trailer) involved in the transcription/translation of the gene product and the regulation of the transcription/translation, as well as intervening sequences (introns) between individual coding segments (exons).

In this application, the terms "polypeptide," "peptide," and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymers. As used herein, the terms encompass amino acid chains of any length, including full-length proteins (i.e., antigens), wherein the amino acid residues are linked by covalent peptide bonds.

The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, e.g., hydroxyproline, .gamma.-carboxyglutamate, and O-phosphoserine.

Amino acids may be referred to herein by either the commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

"Primers" as used herein refer to oligonucleotides that can be used in an amplification method, such as a polymerase chain reaction (PCR), to amplify a nucleotide sequence based on the polynucleotide sequence corresponding to a particular genomic sequence, e.g., one located within the CpG island CGI137, PDE9A, or CGI009 on chromosome 21, in various methylation status. At least one of the PCR primers for amplification of a polynucleotide sequence is sequence-specific for the sequence.

The term "template" refers to any nucleic acid molecule that can be used for amplification in the technology. RNA or DNA that is not naturally double stranded can be made into double stranded DNA so as to be used as template DNA. Any double stranded DNA or preparation containing multiple, different double stranded DNA molecules can be used as template DNA to amplify a locus or loci of interest contained in the template DNA.

The term “amplification reaction” as used herein refers to a process for copying nucleic acid one or more times. In embodiments, the method of amplification includes but is not limited to polymerase chain reaction, self-sustained sequence reaction, ligase chain reaction, rapid amplification of cDNA ends, polymerase chain reaction and ligase chain reaction, Q-beta phage amplification, strand displacement amplification, or splice overlap extension polymerase chain reaction. In some embodiments, a single molecule of nucleic acid is amplified, for example, by digital PCR.

The term "sensitivity" as used herein refers to the number of true positives divided by the number of true positives plus the number of false negatives, where sensitivity (sens) may be within the range of $0 \leq \text{sens} \leq 1$. Ideally, method embodiments herein have the number of false negatives equaling zero or close to equaling zero, so that no subject is wrongly identified as not having at least one chromosome abnormality or other genetic disorder when they indeed have at least one chromosome abnormality or other genetic disorder. Conversely, an assessment often is made of the ability of a prediction algorithm to classify negatives correctly, a complementary measurement to sensitivity. The term "specificity" as used herein refers to the number of true negatives divided by the number of true negatives plus the number of false positives, where sensitivity (spec) may be within the range of $0 \leq \text{spec} \leq 1$. Ideally, methods embodiments herein have the number of false positives equaling zero or close to equaling zero, so that no subject wrongly identified as having at least one chromosome abnormality other genetic disorder when they do not have the chromosome abnormality other genetic disorder being assessed. Hence, a method that has sensitivity and specificity equaling one, or 100%, sometimes is selected.

One or more prediction algorithms may be used to determine significance or give meaning to the detection data collected under variable conditions that may be weighed independently of or dependently on each other. The term "variable" as used herein refers to a factor, quantity, or function of an algorithm that has a value or set of values. For example, a variable may be the design of a set of amplified nucleic acid species, the number of sets of amplified nucleic acid species, percent fetal genetic contribution tested, percent maternal genetic contribution tested, type of chromosome abnormality assayed, type of genetic disorder assayed, type of sex-linked abnormalities assayed, the age of the mother and the like. The term "independent" as used herein refers to not being influenced or not being controlled by another. The term "dependent" as used herein refers to being influenced or controlled by another. For example, a particular chromosome and a trisomy event occurring for that particular chromosome that results in a viable being are variables that are dependent upon each other.

One of skill in the art may use any type of method or prediction algorithm to give significance to the data of the present technology within an acceptable sensitivity and/or specificity. For example, prediction algorithms such as Chi-squared test, z-test, t-test, ANOVA (analysis of variance), regression analysis, neural nets, fuzzy logic, Hidden Markov Models, multiple model state estimation, and the like may be used. One or more methods or prediction algorithms may be determined to give significance to the data having different independent and/or dependent variables of the present technology. And one or more methods or prediction algorithms may be determined not to give significance to the data having different independent and/or dependent variables of the present technology. One may design or change parameters of the different variables of methods described herein based on results of one or more prediction algorithms (e.g., number of sets analyzed, types of nucleotide species in each set). For example, applying the Chi-squared test to detection data may suggest that specific ranges of maternal age are correlated to a higher likelihood of having an offspring with a specific chromosome abnormality, hence the variable of maternal age may be weighed differently verses being weighed the same as other variables.

In certain embodiments, several algorithms may be chosen to be tested. These algorithms can be trained with raw data. For each new raw data sample, the trained algorithms will assign a classification to that sample (i.e. trisomy or normal). Based on the classifications of the new raw data samples, the trained algorithms' performance may be assessed based on sensitivity and specificity. Finally, an algorithm with the highest sensitivity and/or specificity or combination thereof may be identified.

DETAILED DESCRIPTION

Introduction

The presence of fetal nucleic acid in maternal plasma was first reported in 1997 and offers the possibility for non-invasive prenatal diagnosis simply through the analysis of a maternal blood sample (Lo et al., Lancet 350:485-487, 1997). To date, numerous potential clinical applications have been developed. In particular, quantitative abnormalities of fetal nucleic acid, for example DNA, concentrations in maternal plasma have been found to be associated with a number of pregnancy-associated disorders, including preeclampsia, preterm labor, antepartum hemorrhage, invasive placentation, fetal Down syndrome, and other fetal chromosomal aneuploidies. Hence, fetal nucleic acid analysis in maternal plasma represents a powerful mechanism for the monitoring of fetomaternal well-being.

However, fetal DNA co-exists with background maternal DNA in maternal plasma. Hence, most reported applications have relied on the detection of Y-chromosome sequences as these are most conveniently distinguishable from maternal DNA. Such an approach limits the applicability of the existing assays to only 50% of all pregnancies, namely those with male fetuses. Thus, there is much need for the development of sex-independent compositions and methods for enriching and analyzing fetal nucleic acid from a maternal sample. Also, methods that rely on polymorphic markers to quantify fetal nucleic acid may be susceptible to varying heterozygosity rates across different ethnicities thereby limiting their applicability (e.g., by increasing the number of markers that are needed).

It was previously demonstrated that fetal and maternal DNA can be distinguished by their differences in methylation status (U.S. Patent No. 6,927,028, which is hereby incorporated by reference). Methylation is an epigenetic phenomenon, which refers to processes that alter a phenotype without involving changes in the DNA sequence. By exploiting the difference in the DNA methylation status between mother and fetus, one can successfully detect and analyze fetal nucleic acid in a background of maternal nucleic acid.

The present inventors provides novel genomic polynucleotides that are differentially methylated between the fetal DNA from the fetus (e.g., from the placenta) and the maternal DNA from the mother, for example from peripheral blood cells. This discovery thus provides a new approach for distinguishing fetal and maternal genomic DNA and new methods for accurately quantifying fetal nucleic which may be used for non-invasive prenatal diagnosis.

Methodology

Practicing the technology utilizes routine techniques in the field of molecular biology. Basic texts disclosing the general methods of use in the technology include Sambrook and Russell, Molecular Cloning, A Laboratory Manual (3rd ed. 2001); Kriegler, Gene Transfer and Expression: A Laboratory Manual (1990); and Current Protocols in Molecular Biology (Ausubel et al., eds., 1994)).

For nucleic acids, sizes are given in either kilobases (kb) or base pairs (bp). These are estimates derived from agarose or acrylamide gel electrophoresis, from sequenced nucleic acids, or from published DNA sequences. For proteins, sizes are given in kilodaltons (kDa) or amino acid residue numbers. Protein sizes are estimated from gel electrophoresis, from sequenced proteins, from derived amino acid sequences, or from published protein sequences.

Oligonucleotides that are not commercially available can be chemically synthesized, e.g., according to the solid phase phosphoramidite triester method first described by Beaucage & Caruthers, Tetrahedron Lett. 22: 1859-1862 (1981), using an automated synthesizer, as described in Van Devanter et. al., Nucleic Acids Res. 12: 6159-6168 (1984). Purification of oligonucleotides is performed using any art-recognized strategy, e.g., native acrylamide gel electrophoresis or anion-exchange high performance liquid chromatography (HPLC) as described in Pearson & Reanier, J. Chrom. 255: 137-149 (1983).

Acquisition of Blood Samples and Extraction of DNA

The present technology relates to separating, enriching and analyzing fetal DNA found in maternal blood as a non-invasive means to detect the presence and/or to monitor the progress of a pregnancy-associated condition or disorder. Thus, the first steps of practicing the technology are to obtain a blood sample from a pregnant woman and extract DNA from the sample.

A. Acquisition of Blood Samples

A blood sample is obtained from a pregnant woman at a gestational age suitable for testing using a method of the present technology. The suitable gestational age may vary depending on the disorder tested, as discussed below. Collection of blood from a woman is performed in accordance with the standard protocol hospitals or clinics generally follow. An appropriate amount of peripheral blood, e.g., typically between 5-50 ml, is collected and may be stored according to standard procedure prior to further preparation. Blood samples may be collected, stored or transported in a manner known to the person of ordinary skill in the art to minimize degradation or the quality of nucleic acid present in the sample.

B. Preparation of Blood Samples

The analysis of fetal DNA found in maternal blood according to the present technology may be performed using, e.g., the whole blood, serum, or plasma. The methods for preparing serum or plasma from maternal blood are well known among those of skill in the art. For example, a pregnant woman's blood can be placed in a tube containing EDTA or a specialized commercial product such as Vacutainer SST (Becton Dickinson, Franklin Lakes, N.J.) to prevent blood clotting, and plasma can then be obtained from whole blood through centrifugation. On the other hand, serum may be obtained with or without centrifugation-following blood clotting. If centrifugation is used then it is typically, though not exclusively, conducted at an appropriate speed, e.g., 1,500-3,000 times g. Plasma or serum may be subjected to additional centrifugation steps before being transferred to a fresh tube for DNA extraction.

In addition to the acellular portion of the whole blood, DNA may also be recovered from the cellular fraction, enriched in the buffy coat portion, which can be obtained following centrifugation of a whole blood sample from the woman and removal of the plasma.

C. Extraction of DNA

There are numerous known methods for extracting DNA from a biological sample including blood. The general methods of DNA preparation (e.g., described by Sambrook and Russell, Molecular Cloning: A Laboratory Manual 3d ed., 2001) can be followed; various commercially available reagents or kits, such as Qiagen's QIAamp Circulating Nucleic Acid Kit, QiaAmp DNA Mini Kit or QiaAmp DNA Blood Mini Kit (Qiagen, Hilden, Germany), GenomicPrep™ Blood DNA Isolation Kit (Promega, Madison, Wis.), and GFX™ Genomic Blood DNA Purification Kit (Amersham, Piscataway, N.J.), may also be used to obtain DNA from a blood sample from a pregnant woman. Combinations of more than one of these methods may also be used.

In some embodiments, the sample may first be enriched or relatively enriched for fetal nucleic acid by one or more methods. For example, the discrimination of fetal and maternal DNA can be performed using the compositions and processes of the present technology alone or in combination with other discriminating factors. Examples of these factors include, but are not limited to, single nucleotide differences between chromosome X and Y, chromosome Y-specific sequences, polymorphisms located elsewhere in the genome, size differences between fetal and maternal DNA and differences in methylation pattern between maternal and fetal tissues.

Other methods for enriching a sample for a particular species of nucleic acid are described in PCT Patent Application Number PCT/US07/69991, filed May 30, 2007, PCT Patent Application Number PCT/US2007/071232, filed June 15, 2007, US Provisional Application Numbers 60/968,876 and 60/968,878 (assigned to the Applicant), (PCT Patent Application Number PCT/EP05/012707, filed November 28, 2005) which are all hereby incorporated by reference. In certain embodiments, maternal nucleic acid is selectively removed (either partially, substantially, almost completely or completely) from the sample.

Methylation Specific Separation of Nucleic Acid

The methods provided herein offer an alternative approach for the enrichment of fetal DNA based on the methylation-specific separation of differentially methylated DNA. It has recently been discovered that many genes involved in developmental regulation are controlled through epigenetics in embryonic stem cells.

Consequently, multiple genes can be expected to show differential DNA methylation between nucleic acid of fetal origin and maternal origin. Once these regions are identified, a technique to capture methylated DNA can be used to specifically enrich fetal DNA. For identification of differentially methylated regions, a novel approach was used to capture methylated DNA. This approach uses a protein, in which the methyl binding domain of MBD2 is fused to the Fc fragment of an antibody (MBD-FC) (Gebhard C, Schwarzfischer L, Pham TH, Schilling E, Klug M, Andreesen R, Rehli M (2006) Genomewide profiling of CpG methylation identifies novel targets of aberrant hypermethylation in myeloid leukemia. Cancer Res 66:6118-6128). This fusion protein has several advantages over conventional methylation specific antibodies. The MBD-FC has a higher affinity to methylated DNA and it binds double stranded DNA. Most importantly the two proteins differ in the way they bind DNA. Methylation specific antibodies bind DNA stochastically, which means that only a binary answer can be obtained. The methyl binding domain of MBD-FC on the other hand binds DNA molecules regardless of their methylation status. The strength of this protein - DNA interaction is defined by the level of DNA methylation. After binding genomic DNA, eluate solutions of increasing salt concentrations can be used to fractionate non-methylated and methylated DNA allowing for a more controlled separation (Gebhard C, Schwarzfischer L, Pham TH, Andreesen R, Mackensen A, Rehli M (2006) Rapid and sensitive detection of CpG-methylation using methyl-binding (MB)-PCR. Nucleic Acids Res 34:e82). Consequently this method, called Methyl-CpG immunoprecipitation (MCIP), cannot only enrich, but also fractionate genomic DNA according to methylation level, which is particularly helpful when the unmethylated DNA fraction should be investigated as well.

Methylation Sensitive Restriction Enzyme Digestion

The technology also provides compositions and processes for determining the amount of fetal nucleic acid from a maternal sample. The technology allows for the enrichment of fetal nucleic acid regions in a maternal sample by selectively digesting nucleic acid from said maternal sample with an enzyme that selectively and completely or substantially digests the maternal nucleic acid to enrich the sample for at least one fetal nucleic acid region. Preferably, the digestion efficiency is greater than about 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%. Following enrichment, the amount of fetal nucleic acid can be determined by quantitative methods that do not require polymorphic sequences or bisulfite treatment, thereby, offering a solution that works equally well for female fetuses and across different ethnicities and preserves the low copy number fetal nucleic acid present in the sample.

For example, there are methyl-sensitive enzymes that preferentially or substantially cleave or digest at their DNA recognition sequence if it is non-methylated. Thus, an unmethylated DNA sample will be cut into smaller fragments than a methylated DNA sample. Similarly, a hypermethylated DNA sample will not be cleaved. In contrast, there are methyl-sensitive enzymes that cleave at their DNA recognition sequence only if it is methylated.

Methyl-sensitive enzymes that digest unmethylated DNA suitable for use in methods of the technology include, but are not limited to, HpaII, HhaI, MaeII, BstUI and AciI. An enzyme that can be used is HpaII that cuts only the unmethylated sequence CCGG. Another enzyme that can be used is HhaI that cuts only the unmethylated sequence GCGC. Both enzymes are available from New England BioLabs®, Inc. Combinations of two or more methyl-sensitive enzymes that digest only unmethylated DNA can also be used. Suitable enzymes that digest only methylated DNA include, but are not limited to, DpnI, which cuts at a recognition sequence GATC, and McrBC, which belongs to the family of AAA.sup.+ proteins and cuts DNA containing modified cytosines and cuts at recognition site 5' . . . Pu.sup.mC(N.sub.40-3000) Pu.sup.mC . . . 3' (New England BioLabs, Inc., Beverly, Mass.).

Cleavage methods and procedures for selected restriction enzymes for cutting DNA at specific sites are well known to the skilled artisan. For example, many suppliers of restriction enzymes provide information on conditions and types of DNA sequences cut by specific restriction enzymes, including New England BioLabs, Pro-Mega Biochems, Boehringer-Mannheim, and the like. Sambrook et al. (See Sambrook et al., Molecular Biology: A laboratory Approach, Cold Spring Harbor, N.Y. 1989) provide a general description of methods for using restriction enzymes and other enzymes. In methods of the present technology enzymes often are used under conditions that will enable cleavage of the maternal DNA with about 95%-100% efficiency, preferably with about 98%-100% efficiency.

Other Methods for Methylation Analysis

Various methylation analysis procedures are known in the art, and can be used in conjunction with the present technology. These assays allow for determination of the methylation state of one or a plurality of CpG islands within a DNA sequence. In addition, the methods maybe used to quantify methylated nucleic acid. Such assays involve, among other techniques, DNA sequencing of bisulfite-treated DNA, PCR (for sequence-specific amplification), Southern blot analysis, and use of methylation-sensitive restriction enzymes.

Genomic sequencing is a technique that has been simplified for analysis of DNA methylation patterns and 5-methylcytosine distribution by using bisulfite treatment (Frommer et al., Proc. Natl. Acad. Sci. USA 89:1827-1831, 1992). Additionally, restriction enzyme digestion of PCR products amplified from bisulfite-converted DNA may be used, e.g., the method described by Sadri & Hornsby (Nucl. Acids Res. 24:5058-5059, 1996), or COBRA (Combined Bisulfite Restriction Analysis) (Xiong & Laird, Nucleic Acids Res. 25:2532-2534, 1997).

COBRA analysis is a quantitative methylation assay useful for determining DNA methylation levels at specific gene loci in small amounts of genomic DNA (Xiong & Laird, Nucleic Acids Res. 25:2532-2534, 1997). Briefly, restriction enzyme digestion is used to reveal methylation-dependent sequence differences in PCR products of sodium bisulfite-treated DNA. Methylation-dependent sequence differences are first introduced into the genomic DNA by standard bisulfite treatment according to the procedure described by Frommer et al. (Proc. Natl. Acad. Sci. USA 89:1827-1831, 1992). PCR amplification of the bisulfite converted DNA is then performed using primers specific for the interested CpG islands, followed by restriction endonuclease digestion, gel electrophoresis, and detection using specific, labeled hybridization probes. Methylation levels in the original DNA sample are represented by the relative amounts of digested and undigested PCR product in a linearly quantitative fashion across a wide spectrum of DNA methylation levels. In addition, this technique can be reliably applied to DNA obtained from microdissected paraffin-embedded tissue samples. Typical reagents (e.g., as might be found in a typical COBRA-based kit) for

COBRA analysis may include, but are not limited to: PCR primers for specific gene (or methylation-altered DNA sequence or CpG island); restriction enzyme and appropriate buffer; gene-hybridization oligo; control hybridization oligo; kinase labeling kit for oligo probe; and radioactive nucleotides. Additionally, bisulfite conversion reagents may include: DNA denaturation buffer; sulfonation buffer; DNA recovery reagents or kits (e.g., precipitation, ultrafiltration, affinity column); desulfonation buffer; and DNA recovery components.

The MethyLight™ assay is a high-throughput quantitative methylation assay that utilizes fluorescence-based real-time PCR (TaqMan.RTM.) technology that requires no further manipulations after the PCR step (Eads et al., Cancer Res. 59:2302-2306, 1999). Briefly, the MethyLight.TM. process begins with a mixed sample of genomic DNA that is converted, in a sodium bisulfite reaction, to a mixed pool of methylation-dependent sequence differences according to standard procedures (the bisulfite process converts unmethylated cytosine residues to uracil). Fluorescence-based PCR is then performed either in an "unbiased" (with primers that do not overlap known CpG methylation sites) PCR reaction, or in a "biased" (with PCR primers that overlap known CpG dinucleotides) reaction. Sequence discrimination can occur either at the level of the amplification process or at the level of the fluorescence detection process, or both.

The MethyLight assay may be used as a quantitative test for methylation patterns in the genomic DNA sample, wherein sequence discrimination occurs at the level of probe hybridization. In this quantitative version, the PCR reaction provides for unbiased amplification in the presence of a fluorescent probe that overlaps a particular putative methylation site. An unbiased control for the amount of input DNA is provided by a reaction in which neither the primers, nor the probe overlie any CpG dinucleotides. Alternatively, a qualitative test for genomic methylation is achieved by probing of the biased PCR pool with either control oligonucleotides that do not "cover" known methylation sites (a fluorescence-based version of the "MSP" technique), or with oligonucleotides covering potential methylation sites.

The MethyLight process can by used with a "TaqMan" probe in the amplification process. For example, double-stranded genomic DNA is treated with sodium bisulfite and subjected to one of two sets of PCR reactions using TaqMan.RTM. probes; e.g., with either biased primers and TaqMan.RTM. probe, or unbiased primers and TaqMan.RTM. probe. The TaqMan.RTM. probe is dual-labeled with fluorescent "reporter" and "quencher" molecules, and is designed to be specific for a relatively high GC content region so that it melts out at about 10.degree. C. higher temperature in the PCR cycle than the forward or reverse primers. This allows the TaqMan.RTM. probe to remain fully hybridized during the PCR annealing/extension step. As the Taq polymerase enzymatically synthesizes a new strand during PCR, it will eventually reach the annealed TaqMan.RTM. probe. The Taq polymerase 5' to 3' endonuclease activity will then displace the TaqMan.RTM. probe by digesting it to release the fluorescent reporter molecule for quantitative detection of its now unquenched signal using a real-time fluorescent detection system.

Typical reagents (e.g., as might be found in a typical MethyLight.TM.-based kit) for MethyLight.TM. analysis may include, but are not limited to: PCR primers for specific gene (or methylation-altered DNA sequence or CpG island); TaqMan.RTM. probes; optimized PCR buffers and deoxynucleotides; and Taq polymerase.

The Ms-SNuPE technique is a quantitative method for assessing methylation differences at specific CpG sites based on bisulfite treatment of DNA, followed by single-nucleotide primer extension (Gonzalgo & Jones, Nucleic Acids Res. 25:2529-2531, 1997).

Briefly, genomic DNA is reacted with sodium bisulfite to convert unmethylated cytosine to uracil while leaving 5-methylcytosine unchanged. Amplification of the desired target sequence is then performed using PCR primers specific for bisulfite-converted DNA, and the resulting product is isolated and used as a template for methylation analysis at the CpG site(s) of interest.

Small amounts of DNA can be analyzed (e.g., microdissected pathology sections), and it avoids utilization of restriction enzymes for determining the methylation status at CpG sites.

Typical reagents (e.g., as might be found in a typical Ms-SNuPE-based kit) for Ms-SNuPE analysis may include, but are not limited to: PCR primers for specific gene (or methylation-altered DNA sequence or CpG island); optimized PCR buffers and deoxynucleotides; gel extraction kit; positive control primers; Ms-SNuPE primers for specific gene; reaction buffer (for the Ms-SNuPE reaction); and radioactive nucleotides. Additionally, bisulfite conversion reagents may include: DNA denaturation buffer; sulfonation buffer; DNA recovery regents or kit (e.g., precipitation, ultrafiltration, affinity column); desulfonation buffer; and DNA recovery components.

MSP (methylation-specific PCR) allows for assessing the methylation status of virtually any group of CpG sites within a CpG island, independent of the use of methylation-sensitive restriction enzymes (Herman et al. Proc. Nat. Acad. Sci. USA 93:9821-9826, 1996; U.S. Pat. No. 5,786,146). Briefly, DNA is modified by sodium bisulfite converting unmethylated, but not methylated cytosines to uracil, and subsequently amplified with primers specific for methylated versus umnethylated DNA. MSP requires only small quantities of DNA, is sensitive to 0.1% methylated alleles of a given CpG island locus, and can be performed on DNA extracted from paraffin-embedded samples. Typical reagents (e.g., as might be found in a typical MSP-based kit) for MSP analysis may include, but are not limited to: methylated and unmethylated PCR primers for specific gene (or methylation-altered DNA sequence or CpG island), optimized PCR buffers and deoxynucleotides, and specific probes.

The MCA technique is a method that can be used to screen for altered methylation patterns in genomic DNA, and to isolate specific sequences associated with these changes (Toyota et al., Cancer Res. 59:2307-12, 1999). Briefly, restriction enzymes with different sensitivities to cytosine methylation in their recognition sites are used to digest genomic DNAs from primary tumors, cell lines, and normal tissues prior to arbitrarily primed PCR amplification. Fragments that show differential methylation are cloned and sequenced after resolving the PCR products on high-resolution polyacrylamide gels. The cloned fragments are then used as probes for Southern analysis to confirm differential methylation of these regions. Typical reagents (e.g., as might be found in a typical MCA-based kit) for MCA analysis may include, but are not limited to: PCR primers for arbitrary priming Genomic DNA; PCR buffers and nucleotides, restriction enzymes and appropriate buffers; gene-hybridization oligos or probes; control hybridization oligos or probes.

Another method for analyzing methylation sites is a primer extension assay, including an optimized PCR amplification reaction that produces amplified targets for subsequent primer extension genotyping analysis using mass spectrometry. The assay can also be done in multiplex. This method (particularly as it relates to genotyping single nucleotide polymorphisms) is described in detail in PCT publication WO05012578A1 and US publication US20050079521A1. For methylation analysis, the assay can be adopted to detect bisulfite introduced methylation dependent C to T sequence changes. These methods are particularly useful for performing multiplexed amplification reactions and multiplexed primer extension reactions (e.g., multiplexed homogeneous primer mass extension (hME) assays) in a single well to further increase the throughput and reduce the cost per reaction for primer extension reactions.

Four additional methods for DNA methylation analysis include restriction landmark genomic scanning (RLGS, Costello et al., 2000), methylation-sensitive-representational difference analysis (MS-RDA), methylation-specific AP-PCR (MS-AP-PCR) and methyl-CpG binding domain column/segregation of partly melted molecules (MBD/SPM).

Additional methylation analysis methods that may be used in conjunction with the present technology are described in the following papers: Laird, P.W. Nature Reviews Cancer 3, 253-266 (2003); Biotechniques; Uhlmann, K. et al. Electrophoresis 23:4072-4079 (2002) - PyroMeth; Colella et al. Biotechniques. 2003 Jul;35(1):146-50; Dupont JM, Tost J, Jammes H, and Gut IG. Anal Biochem, Oct 2004; 333(1): 119-27; and Tooke N and Pettersson M. IVDT. Nov 2004; 41.

Polynucleotide Sequence Amplification and Determination

Following separation of nucleic acid in a methylation-differential manner, the nucleic acid may be subjected to sequence-based analysis. Furthermore, once it is determined that one particular genomic sequence of fetal origin is hypermethylated or hypomethylated compared to the maternal counterpart, the amount of this fetal genomic sequence can be determined. Subsequently, this amount can be compared to a standard control value and serve as an indication for the potential of certain pregnancy-associated disorder.

A. Amplification of Nucleotide Sequences

In many instances, it is desirable to amplify a nucleic acid sequence of the technology using any of several nucleic acid amplification procedures which are well known in the art (listed above and described in greater detail below). Specifically, nucleic acid amplification is the enzymatic synthesis of nucleic acid amplicons (copies) which contain a sequence that is complementary to a nucleic acid sequence being amplified. Nucleic acid amplification is especially beneficial when the amount of target sequence present in a sample is very low. By amplifying the target sequences and detecting the amplicon synthesized, the sensitivity of an assay can be vastly improved, since fewer target sequences are needed at the beginning of the assay to better ensure detection of nucleic acid in the sample belonging to the organism or virus of interest.

A variety of polynucleotide amplification methods are well established and frequently used in research. For instance, the general methods of polymerase chain reaction (PCR) for polynucleotide sequence amplification are well known in the art and are thus not described in detail herein. For a review of PCR methods, protocols, and principles in designing primers, see, e.g., Innis, et al., PCR Protocols: A Guide to Methods and Applications, Academic Press, Inc. N.Y., 1990. PCR reagents and protocols are also available from commercial vendors, such as Roche Molecular Systems.

PCR is most usually carried out as an automated process with a thermostable enzyme. In this process, the temperature of the reaction mixture is cycled through a denaturing region, a primer annealing region, and an extension reaction region automatically. Machines specifically adapted for this purpose are commercially available.

Although PCR amplification of a polynucleotide sequence is typically used in practicing the present technology, one of skill in the art will recognize that the amplification of a genomic sequence found in a maternal blood sample may be accomplished by any known method, such as ligase chain reaction (LCR), transcription-mediated amplification, and self-sustained sequence replication or nucleic acid sequence-based amplification (NASBA), each of which provides sufficient amplification. More recently developed branched-DNA technology may also be used to qualitatively demonstrate the presence of a particular genomic sequence of the technology, which represents a particular methylation pattern, or to quantitatively determine the amount of this particular genomic sequence in the maternal blood. For a review of branched-DNA signal amplification for direct quantitation of nucleic acid sequences in clinical samples, see Nolte, Adv. Clin. Chem. 33:201-235, 1998.

The compositions and processes of the technology are also particularly useful when practiced with digital PCR. Digital PCR was first developed by Kalinina and colleagues (Kalinina et al., “Nanoliter scale PCR with TaqMan detection.” Nucleic Acids Research. 25; 1999-2004, (1997)) and further developed by Vogelstein and Kinzler (Digital PCR. Proc Natl Acad Sci U S A. 96; 9236-41, (1999)). The application of digital PCR for use with fetal diagnostics was first described by Cantor et al. (PCT Patent Publication No. WO05023091A2) and subsequently described by Quake et al. (US Patent Publication No. US 20070202525), which are both hereby incorporated by reference. Digital PCR takes advantage of nucleic acid (DNA, cDNA or RNA) amplification on a single molecule level, and offers a highly sensitive method for quantifying low copy number nucleic acid. Fluidigm® Corporation offers systems for the digital analysis of nucleic acids.

B. Determination of Polynucleotide Sequences

Techniques for polynucleotide sequence determination are also well established and widely practiced in the relevant research field. For instance, the basic principles and general techniques for polynucleotide sequencing are described in various research reports and treatises on molecular biology and recombinant genetics, such as Wallace et al., supra; Sambrook and Russell, supra, and Ausubel et al., supra. DNA sequencing methods routinely practiced in research laboratories, either manual or automated, can be used for practicing the present technology. Additional means suitable for detecting changes in a polynucleotide sequence for practicing the methods of the present technology include but are not limited to mass spectrometry, primer extension, polynucleotide hybridization, real-time PCR, and electrophoresis.

Use of a primer extension reaction also can be applied in methods of the technology. A primer extension reaction operates, for example, by discriminating the SNP alleles by the incorporation of deoxynucleotides and/or dideoxynucleotides to a primer extension primer which hybridizes to a region adjacent to the SNP site. The primer is extended with a polymerase. The primer extended SNP can be detected physically by mass spectrometry or by a tagging moiety such as biotin. As the SNP site is only extended by a complementary deoxynucleotide or dideoxynucleotide that is either tagged by a specific label or generates a primer extension product with a specific mass, the SNP alleles can be discriminated and quantified.

Reverse transcribed and amplified nucleic acids may be modified nucleic acids. Modified nucleic acids can include nucleotide analogs, and in certain embodiments include a detectable label and/or a capture agent. Examples of detectable labels include without limitation fluorophores, radioisotopes, colormetric agents, light emitting agents, chemiluminescent agents, light scattering agents, enzymes and the like. Examples of capture agents include without limitation an agent from a binding pair selected from antibody/antigen, antibody/antibody, antibody/antibody fragment, antibody/antibody receptor, antibody/protein A or protein G, hapten/anti-hapten, biotin/avidin, biotin/streptavidin, folic acid/folate binding protein, vitamin B12/intrinsic factor, chemical reactive group/complementary chemical reactive group (e.g., sulfhydryl/maleimide, sulfhydryl/haloacetyl derivative, amine/isotriocyanate, amine/succinimidyl ester, and amine/sulfonyl halides) pairs, and the like. Modified nucleic acids having a capture agent can be immobilized to a solid support in certain embodiments

Mass spectrometry is a particularly effective method for the detection of a polynucleotide of the technology, for example a PCR amplicon, a primer extension product or a detector probe that is cleaved from a target nucleic acid. The presence of the polynucleotide sequence is verified by comparing the mass of the detected signal with the expected mass of the polynucleotide of interest. The relative signal strength, e.g., mass peak on a spectra, for a particular polynucleotide sequence indicates the relative population of a specific allele, thus enabling calculation of the allele ratio directly from the data. For a review of genotyping methods using Sequenom® standard iPLEX™ assay and MassARRAY® technology, see Jurinke, C., Oeth, P., van den Boom, D., "MALDI-TOF mass spectrometry: a versatile tool for high-performance DNA analysis." Mol. Biotechnol. 26, 147-164 (2004); and Oeth, P. et al., "iPLEX™ Assay: Increased Plexing Efficiency and Flexibility for MassARRAY® System through single base primer extension with mass-modified Terminators." SEQUENOM Application Note (2005), both of which are hereby incorporated by reference. For a review of detecting and quantifying target nucleic using cleavable detector probes that are cleaved during the amplification process and detected by mass spectrometry, see US Patent Application Number 11/950,395, which was filed December 4, 2007, and is hereby incorporated by reference.

Sequencing technologies are improving in terms of throughput and cost. Sequencing technologies, such as that achievable on the 454 platform (Roche) (Margulies, M. et al. 2005 Nature 437, 376-380), Illumina Genome Analyzer (or Solexa platform) or SOLiD System (Applied Biosystems) or the Helicos True Single Molecule DNA sequencing technology (Harris T D et al. 2008 Science, 320, 106-109), the single molecule, real-time (SMRT.TM.) technology of Pacific Biosciences, and nanopore sequencing (Soni GV and Meller A. 2007 Clin Chem 53: 1996-2001), allow the sequencing of many nucleic acid molecules isolated from a specimen at high orders of multiplexing in a parallel fashion (Dear Brief Funct Genomic Proteomic 2003; 1: 397-416).

Each of these platforms allow sequencing of clonally expanded or non-amplified single molecules of nucleic acid fragments. Certain platforms involve, for example, (i) sequencing by ligation of dye-modified probes (including cyclic ligation and cleavage), (ii) pyrosequencing, and (iii) single-molecule sequencing. Nucleotide sequence species, amplification nucleic acid species and detectable products generated there from can be considered a "study nucleic acid" for purposes of analyzing a nucleotide sequence by such sequence analysis platforms.

Sequencing by ligation is a nucleic acid sequencing method that relies on the sensitivity of DNA ligase to base-pairing mismatch. DNA ligase joins together ends of DNA that are correctly base paired. Combining the ability of DNA ligase to join together only correctly base paired DNA ends, with mixed pools of fluorescently labeled oligonucleotides or primers, enables sequence determination by fluorescence detection. Longer sequence reads may be obtained by including primers containing cleavable linkages that can be cleaved after label identification. Cleavage at the linker removes the label and regenerates the 5' phosphate on the end of the ligated primer, preparing the primer for another round of ligation. In some embodiments primers may be labeled with more than one fluorescent label (e.g., 1 fluorescent label, 2, 3, or 4 fluorescent labels).

An example of a system that can be used by a person of ordinary skill based on sequencing by ligation generally involves the following steps. Clonal bead populations can be prepared in emulsion microreactors containing study nucleic acid ("template"), amplification reaction components, beads and primers. After amplification, templates are denatured and bead enrichment is performed to separate beads with extended templates from undesired beads (e.g., beads with no extended templates). The template on the selected beads undergoes a 3' modification to allow covalent bonding to the slide, and modified beads can be deposited onto a glass slide. Deposition chambers offer the ability to segment a slide into one, four or eight chambers during the bead loading process. For sequence analysis, primers hybridize to the adapter sequence. A set of four color dye-labeled probes competes for ligation to the sequencing primer. Specificity of probe ligation is achieved by interrogating every 4th and 5th base during the ligation series. Five to seven rounds of ligation, detection and cleavage record the color at every 5th position with the number of rounds determined by the type of library used. Following each round of ligation, a new complimentary primer offset by one base in the 5' direction is laid down for another series of ligations. Primer reset and ligation rounds (5–7 ligation cycles per round) are repeated sequentially five times to generate 25–35 base pairs of sequence for a single tag. With mate-paired sequencing, this process is repeated for a second tag. Such a system can be used to exponentially amplify amplification products generated by a process described herein, e.g., by ligating a heterologous nucleic acid to the first amplification product generated by a process described herein and performing emulsion amplification using the same or a different solid support originally used to generate the first amplification product. Such a system also may be used to analyze amplification products directly generated by a process described herein by bypassing an exponential amplification process and directly sorting the solid supports described herein on the glass slide.

Pyrosequencing is a nucleic acid sequencing method based on sequencing by synthesis, which relies on detection of a pyrophosphate released on nucleotide incorporation. Generally, sequencing by synthesis involves synthesizing, one nucleotide at a time, a DNA strand complimentary to the strand whose sequence is being sought. Study nucleic acids may be immobilized to a solid support, hybridized with a sequencing primer, incubated with DNA polymerase, ATP sulfurylase, luciferase, apyrase, adenosine 5' phosphsulfate and luciferin. Nucleotide solutions are sequentially added and removed. Correct incorporation of a nucleotide releases a pyrophosphate, which interacts with ATP sulfurylase and produces ATP in the presence of adenosine 5' phosphsulfate, fueling the luciferin reaction, which produces a chemiluminescent signal allowing sequence determination.

An example of a system that can be used by a person of ordinary skill based on pyrosequencing generally involves the following steps: ligating an adaptor nucleic acid to a study nucleic acid and hybridizing the study nucleic acid to a bead; amplifying a nucleotide sequence in the study nucleic acid in an emulsion; sorting beads using a picoliter multiwell solid support; and sequencing amplified nucleotide sequences by pyrosequencing methodology (e.g., Nakano et al., "Single-molecule PCR using water-in-oil emulsion;" Journal of Biotechnology 102: 117-124 (2003)). Such a system can be used to exponentially amplify amplification products generated by a process described herein, e.g., by ligating a heterologous nucleic acid to the first amplification product generated by a process described herein.

Certain single-molecule sequencing embodiments are based on the principal of sequencing by synthesis, and utilize single-pair Fluorescence Resonance Energy Transfer (single pair FRET) as a mechanism by which photons are emitted as a result of successful nucleotide incorporation. The emitted photons often are detected using intensified or high sensitivity cooled charge-couple-devices in conjunction with total internal reflection microscopy (TIRM). Photons are only emitted when the introduced reaction solution contains the correct nucleotide for incorporation into the growing nucleic acid chain that is synthesized as a result of the sequencing process. In FRET based single-molecule sequencing, energy is transferred between two fluorescent dyes, sometimes polymethine cyanine dyes Cy3 and Cy5, through long-range dipole interactions. The donor is excited at its specific excitation wavelength and the excited state energy is transferred, non-radiatively to the acceptor dye, which in turn becomes excited. The acceptor dye eventually returns to the ground state by radiative emission of a photon. The two dyes used in the energy transfer process represent the "single pair", in single pair FRET. Cy3 often is used as the donor fluorophore and often is incorporated as the first labeled nucleotide. Cy5 often is used as the acceptor fluorophore and is used as the nucleotide label for successive nucleotide additions after incorporation of a first Cy3 labeled nucleotide. The fluorophores generally are within 10 nanometers of each for energy transfer to occur successfully.

An example of a system that can be used based on single-molecule sequencing generally involves hybridizing a primer to a study nucleic acid to generate a complex; associating the complex with a solid phase; iteratively extending the primer by a nucleotide tagged with a fluorescent molecule; and capturing an image of fluorescence resonance energy transfer signals after each iteration (e.g., U.S. Patent No. 7,169,314; Braslavsky et al., PNAS 100(7): 3960-3964 (2003)). Such a system can be used to directly sequence amplification products generated by processes described herein. In some embodiments the released linear amplification product can be hybridized to a primer that contains sequences complementary to immobilized capture sequences present on a solid support, a bead or glass slide for example. Hybridization of the primer--released linear amplification product complexes with the immobilized capture sequences, immobilizes released linear amplification products to solid supports for single pair FRET based sequencing by synthesis. The primer often is fluorescent, so that an initial reference image of the surface of the slide with immobilized nucleic acids can be generated. The initial reference image is useful for determining locations at which true nucleotide incorporation is occurring. Fluorescence signals detected in array locations not initially identified in the "primer only" reference image are discarded as non-specific fluorescence. Following immobilization of the primer--released linear amplification product complexes, the bound nucleic acids often are sequenced in parallel by the iterative steps of, a) polymerase extension in the presence of one fluorescently labeled nucleotide, b) detection of fluorescence using appropriate microscopy, TIRM for example, c) removal of fluorescent nucleotide, and d) return to step a with a different fluorescently labeled nucleotide.

In some embodiments, nucleotide sequencing may be by solid phase single nucleotide sequencing methods and processes. Solid phase single nucleotide sequencing methods involve contacting sample nucleic acid and solid support under conditions in which a single molecule of sample nucleic acid hybridizes to a single molecule of a solid support. Such conditions can include providing the solid support molecules and a single molecule of sample nucleic acid in a "microreactor." Such conditions also can include providing a mixture in which the sample nucleic acid molecule can hybridize to solid phase nucleic acid on the solid support. Single nucleotide sequencing methods useful in the embodiments described herein are described in United States Provisional Patent Application Serial Number 61/021,871 filed January 17, 2008.

In certain embodiments, nanopore sequencing detection methods include (a) contacting a nucleic acid for sequencing ("base nucleic acid," e.g., linked probe molecule) with sequence-specific detectors, under conditions in which the detectors specifically hybridize to substantially complementary subsequences of the base nucleic acid; (b) detecting signals from the detectors and (c) determining the sequence of the base nucleic acid according to the signals detected. In certain embodiments, the detectors hybridized to the base nucleic acid are disassociated from the base nucleic acid (e.g., sequentially dissociated) when the detectors interfere with a nanopore structure as the base nucleic acid passes through a pore, and the detectors disassociated from the base sequence are detected. In some embodiments, a detector disassociated from a base nucleic acid emits a detectable signal, and the detector hybridized to the base nucleic acid emits a different detectable signal or no detectable signal. In certain embodiments, nucleotides in a nucleic acid (e.g., linked probe molecule) are substituted with specific nucleotide sequences corresponding to specific nucleotides ("nucleotide representatives"), thereby giving rise to an expanded nucleic acid (e.g., U.S. Patent No. 6,723,513), and the detectors hybridize to the nucleotide representatives in the expanded nucleic acid, which serves as a base nucleic acid. In such embodiments, nucleotide representatives may be arranged in a binary or higher order arrangement (e.g., Soni and Meller, Clinical Chemistry 53(11): 1996-2001 (2007)). In some embodiments, a nucleic acid is not expanded, does not give rise to an expanded nucleic acid, and directly serves a base nucleic acid (e.g., a linked probe molecule serves as a non-expanded base nucleic acid), and detectors are directly contacted with the base nucleic acid. For example, a first detector may hybridize to a first subsequence and a second detector may hybridize to a second subsequence, where the first detector and second detector each have detectable labels that can be distinguished from one another, and where the signals from the first detector and second detector can be distinguished from one another when the detectors are disassociated from the base nucleic acid. In certain embodiments, detectors include a region that hybridizes to the base nucleic acid (e.g., two regions), which can be about 3 to about 100 nucleotides in length (e.g., about 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 50, 55, 60, 65, 70, 75, 80, 85, 90, or 95 nucleotides in length). A detector also may include one or more regions of nucleotides that do not hybridize to the base nucleic acid. In some embodiments, a detector is a molecular beacon. A detector often comprises one or more detectable labels independently selected from those described herein. Each detectable label can be detected by any convenient detection process capable of detecting a signal generated by each label (e.g., magnetic, electric, chemical, optical and the like). For example, a CD camera can be used to detect signals from one or more distinguishable quantum dots linked to a detector.

In certain sequence analysis embodiments, reads may be used to construct a larger nucleotide sequence, which can be facilitated by identifying overlapping sequences in different reads and by using identification sequences in the reads. Such sequence analysis methods and software for constructing larger sequences from reads are known to the person of ordinary skill (e.g., Venter et al., Science 291: 1304-1351 (2001)). Specific reads, partial nucleotide sequence constructs, and full nucleotide sequence constructs may be compared between nucleotide sequences within a sample nucleic acid (i.e., internal comparison) or may be compared with a reference sequence (i.e., reference comparison) in certain sequence analysis embodiments. Internal comparisons sometimes are performed in situations where a sample nucleic acid is prepared from multiple samples or from a single sample source that contains sequence variations. Reference comparisons sometimes are performed when a reference nucleotide sequence is known and an objective is to determine whether a sample nucleic acid contains a nucleotide sequence that is substantially similar or the same, or different, than a reference nucleotide sequence. Sequence analysis is facilitated by sequence analysis apparatus and components known to the person of ordinary skill in the art.

Methods provided herein allow for high-throughput detection of nucleic acid species in a plurality of nucleic acids (e.g., nucleotide sequence species, amplified nucleic acid species and detectable products generated from the foregoing). Multiplexing refers to the simultaneous detection of more than one nucleic acid species. General methods for performing multiplexed reactions in conjunction with mass spectrometry, are known (see, e.g., U.S. Pat. Nos. 6,043,031, 5,547,835 and International PCT application No. WO 97/37041). Multiplexing provides an advantage that a plurality of nucleic acid species (e.g., some having different sequence variations) can be identified in as few as a single mass spectrum, as compared to having to perform a separate mass spectrometry analysis for each individual target nucleic acid species. Methods provided herein lend themselves to high-throughput, highly-automated processes for analyzing sequence variations with high speed and accuracy, in some embodiments. In some embodiments, methods herein may be multiplexed at high levels in a single reaction.

In certain embodiments, the number of nucleic acid species multiplexed include, without limitation, about 1 to about 500 (e.g., about 1-3, 3-5, 5-7, 7-9, 9-11, 11-13, 13-15, 15-17, 17-19, 19-21, 21-23, 23-25, 25-27, 27-29, 29-31, 31-33, 33-35, 35-37, 37-39, 39-41, 41-43, 43-45, 45-47, 47-49, 49-51, 51-53, 53-55, 55-57, 57-59, 59-61, 61-63, 63-65, 65-67, 67-69, 69-71, 71-73, 73-75, 75-77, 77-79, 79-81, 81-83, 83-85, 85-87, 87-89, 89-91, 91-93, 93-95, 95-97, 97-101, 101-103, 103-105, 105-107, 107-109, 109-111, 111-113, 113-115, 115-117, 117-119, 121-123, 123-125, 125-127, 127-129, 129-131, 131-133, 133-135, 135-137, 137-139, 139-141, 141-143, 143-145, 145-147, 147-149, 149-151, 151-153, 153-155, 155-157, 157-159, 159-161, 161-163, 163-165, 165-167, 167-169, 169-171, 171-173, 173-175, 175-177, 177-179, 179-181, 181-183, 183-185, 185-187, 187-189, 189-191, 191-193, 193-195, 195-197, 197-199, 199-201, 201-203, 203-205, 205-207, 207-209, 209-211, 211-213, 213-215, 215-217, 217-219, 219-221, 221-223, 223-225, 225-227, 227-229, 229-231, 231-233, 233-235, 235-237, 237-239, 239-241, 241-243, 243-245, 245-247, 247-249, 249-251, 251-253, 253-255, 255-257, 257-259, 259-261, 261-263, 263-265, 265-267, 267-269, 269-271, 271-273, 273-275, 275-277, 277-279, 279-281, 281-283, 283-285, 285-287, 287-289, 289-291, 291-293, 293-295, 295-297, 297-299, 299-301, 301- 303, 303- 305, 305- 307, 307- 309, 309- 311, 311- 313, 313- 315, 315- 317, 317- 319, 319-321, 321-323, 323-325, 325-327, 327-329, 329-331, 331-333, 333- 335, 335-337, 337-339, 339-341, 341-343, 343-345, 345-347, 347-349, 349-351, 351-353, 353-355, 355-357, 357-359, 359-361, 361-363, 363-365, 365-367, 367-369, 369-371, 371-373, 373-375, 375-377, 377-379, 379-381, 381-383, 383-385, 385-387, 387-389, 389-391, 391-393, 393-395, 395-397, 397-401, 401- 403, 403- 405, 405- 407, 407- 409, 409- 411, 411- 413, 413- 415, 415- 417, 417- 419, 419-421, 421-423, 423-425, 425-427, 427-429, 429-431, 431-433, 433- 435, 435-437, 437-439, 439-441, 441-443, 443-445, 445-447, 447-449, 449-451, 451-453, 453-455, 455-457, 457-459, 459-461, 461-463, 463-465, 465-467, 467-469, 469-471, 471-473, 473-475, 475-477, 477-479, 479-481, 481-483, 483-485, 485-487, 487-489, 489-491, 491-493, 493-495, 495-497, 497-501).

Design methods for achieving resolved mass spectra with multiplexed assays can include primer and oligonucleotide design methods and reaction design methods. See, for example, the multiplex schemes provided in Tables X and Y. For primer and oligonucleotide design in multiplexed assays, the same general guidelines for primer design applies for uniplexed reactions, such as avoiding false priming and primer dimers, only more primers are involved for multiplex reactions. For mass spectrometry applications, analyte peaks in the mass spectra for one assay are sufficiently resolved from a product of any assay with which that assay is multiplexed, including pausing peaks and any other by-product peaks. Also, analyte peaks optimally fall within a user-specified mass window, for example, within a range of 5,000-8,500 Da. In some embodiments multiplex analysis may be adapted to mass spectrometric detection of chromosome abnormalities, for example. In certain embodiments multiplex analysis may be adapted to various single nucleotide or nanopore based sequencing methods described herein. Commercially produced micro-reaction chambers or devices or arrays or chips may be used to facilitate multiplex analysis, and are commercially available.

Detection of Fetal Aneuploidy

For the detection of fetal aneuploidies, some methods rely on measuring the ratio between maternally and paternally inherited alleles. However, the ability to quantify chromosomal changes is impaired by the maternal contribution of cell free nucleic acids, which makes it necessary to deplete the sample from maternal DNA prior to measurement. Promising approaches take advantage of the different size distribution of fetal and maternal DNA or measure RNA that is exclusively expressed by the fetus (see for example, US Patent Application No. 11/384128, which published as US20060252071 and is hereby incorporated by reference). Assuming fetal DNA makes up only about 5% of all cell free DNA in the maternal plasma, there is a decrease of the ratio difference from 1.6% to only about 1.2% between a trisomy sample and a healthy control. Consequently, reliable detection of allele ratio changes requires enriching the fetal fraction of cell free DNA, for example, using the compositions and methods of the present technology.

Some methods rely on measuring the ratio of maternal to paternally inherited alleles to detect fetal chromosomal aneuploidies from maternal plasma. A diploid set yields a 1:1 ratio while trisomies can be detected as a 2:1 ratio. Detection of this difference is impaired by statistical sampling due to the low abundance of fetal DNA, presence of excess maternal DNA in the plasma sample and variability of the measurement technique. The latter is addressed by using methods with high measurement precision, like digital PCR or mass spectrometry. Enriching the fetal fraction of cell free DNA in a sample is currently achieved by either depleting maternal DNA through size exclusion or focusing on fetal-specific nucleic acids, like fetal-expressed RNA. Another differentiating feature of fetal DNA is its DNA methylation pattern. Thus, provided herein are novel compositions and methods for accurately quantifying fetal nucleic acid based on differential methylation between a fetus and mother. The methods rely on sensitive absolute copy number analysis to quantify the fetal nucleic acid portion of a maternal sample, thereby allowing for the prenatal detection of fetal traits. The methods of the technology have identified approximately 3000 CpG rich regions in the genome that are differentially methylated between maternal and fetal DNA. The selected regions showed highly conserved differential methylation across all measured samples. In addition the set of regions is enriched for genes important in developmental regulation, indicating that epigenetic regulation of these areas is a biologically relevant and consistent process (see Table 3). Enrichment of fetal DNA can now be achieved by using our MBD-FC protein to capture cell free DNA (e.g., substantially all cell free DNA) and then elute the highly methylated DNA fraction with high salt concentrations. Using the low salt eluate fractions, the MBD-FC is equally capable of enriching non-methylated fetal DNA.

The present technology provides 63 confirmed genomic regions on chromosomes 13, 18 and 21 with low maternal and high fetal methylation levels. After capturing these regions, SNPs can be used to determine the aforementioned allele ratios. When high frequency SNPs are used around 10 markers have to be measured to achieve a high confidence of finding at least one SNP where the parents have opposite homozygote genotypes and the child has a heterozygote genotype.

In another embodiment, a method for chromosomal abnormality detection is provided that utilizes absolute copy number quantification. A diploid chromosome set will show the same number of copies for differentially methylated regions across all chromosomes, but, for example, a trisomy 21 sample would show 1.5 times more copies for differentially methylated regions on chromosome 21. Normalization of the genomic DNA amounts for a diploid chromosome set can be achieved by using unaltered autosomes as reference (also provided herein – see Table 1). Comparable to other approaches, a single marker is less likely to be sufficient for detection of this difference, because the overall copy numbers are low. Typically there are approximately 100 to 200 copies of fetal DNA from 1 ml of maternal plasma at 10 to 12 weeks of gestation. However, the methods of the present technology offer a redundancy of detectable markers that enables highly reliable discrimination of diploid versus aneuploid chromosome sets.

Data Processing and Identifying Presence or Absence of a Chromosome Abnormality

The term "detection" of a chromosome abnormality as used herein refers to identification of an imbalance of chromosomes by processing data arising from detecting sets of amplified nucleic acid species, nucleotide sequence species, or a detectable product generated from the foregoing (collectively "detectable product"). Any suitable detection device and method can be used to distinguish one or more sets of detectable products, as addressed herein. An outcome pertaining to the presence or absence of a chromosome abnormality can be expressed in any suitable form, including, without limitation, probability (e.g., odds ratio, p-value), likelihood, percentage, value over a threshold, or risk factor, associated with the presence of a chromosome abnormality for a subject or sample. An outcome may be provided with one or more of sensitivity, specificity, standard deviation, coefficient of variation (CV) and/or confidence level, or combinations of the foregoing, in certain embodiments.

Detection of a chromosome abnormality based on one or more sets of detectable products may be identified based on one or more calculated variables, including, but not limited to, sensitivity, specificity, standard deviation, coefficient of variation (CV), a threshold, confidence level, score, probability and/or a combination thereof. In some embodiments, (i) the number of sets selected for a diagnostic method, and/or (ii) the particular nucleotide sequence species of each set selected for a diagnostic method, is determined in part or in full according to one or more of such calculated variables.

In certain embodiments, one or more of sensitivity, specificity and/or confidence level are expressed as a percentage. In some embodiments, the percentage, independently for each variable, is greater than about 90% (e.g., about 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99%, or greater than 99% (e.g., about 99.5%, or greater, about 99.9% or greater, about 99.95% or greater, about 99.99% or greater)). Coefficient of variation (CV) in some embodiments is expressed as a percentage, and sometimes the percentage is about 10% or less (e.g., about 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1%, or less than 1% (e.g., about 0.5% or less, about 0.1% or less, about 0.05% or less, about 0.01% or less)). A probability (e.g., that a particular outcome determined by an algorithm is not due to chance) in certain embodiments is expressed as a p-value, and sometimes the p-value is about 0.05 or less (e.g., about 0.05, 0.04, 0.03, 0.02 or 0.01, or less than 0.01 (e.g., about 0.001 or less, about 0.0001 or less, about 0.00001 or less, about 0.000001 or less)).

For example, scoring or a score may refer to calculating the probability that a particular chromosome abnormality is actually present or absent in a subject/sample. The value of a score may be used to determine for example the variation, difference, or ratio of amplified nucleic detectable product that may correspond to the actual chromosome abnormality. For example, calculating a positive score from detectable products can lead to an identification of a chromosome abnormality, which is particularly relevant to analysis of single samples.

In certain embodiments, simulated (or simulation) data can aid data processing for example by training an algorithm or testing an algorithm. Simulated data may for instance involve hypothetical various samples of different concentrations of fetal and maternal nucleic acid in serum, plasma and the like. Simulated data may be based on what might be expected from a real population or may be skewed to test an algorithm and/or to assign a correct classification based on a simulated data set. Simulated data also is referred to herein as "virtual" data. Fetal/maternal contributions within a sample can be simulated as a table or array of numbers (for example, as a list of peaks corresponding to the mass signals of cleavage products of a reference biomolecule or amplified nucleic acid sequence), as a mass spectrum, as a pattern of bands on a gel, or as a representation of any technique that measures mass distribution. Simulations can be performed in most instances by a computer program. One possible step in using a simulated data set is to evaluate the confidence of the identified results, i.e. how well the selected positives/negatives match the sample and whether there are additional variations. A common approach is to calculate the probability value (p-value) which estimates the probability of a random sample having better score than the selected one. As p-value calculations can be prohibitive in certain circumstances, an empirical model may be assessed, in which it is assumed that at least one sample matches a reference sample (with or without resolved variations). Alternatively other distributions such as Poisson distribution can be used to describe the probability distribution.

In certain embodiments, an algorithm can assign a confidence value to the true positives, true negatives, false positives and false negatives calculated. The assignment of a likelihood of the occurrence of a chromosome abnormality can also be based on a certain probability model.

Simulated data often is generated in an *in silico* process. As used herein, the term "in silico" refers to research and experiments performed using a computer. In silico methods include, but are not limited to, molecular modeling studies, karyotyping, genetic calculations, biomolecular docking experiments, and virtual representations of molecular structures and/or processes, such as molecular interactions.

As used herein, a "data processing routine" refers to a process, that can be embodied in software, that determines the biological significance of acquired data (i.e., the ultimate results of an assay). For example, a data processing routine can determine the amount of each nucleotide sequence species based upon the data collected. A data processing routine also may control an instrument and/or a data collection routine based upon results determined. A data processing routine and a data collection routine often are integrated and provide feedback to operate data acquisition by the instrument, and hence provide assay-based judging methods provided herein.

As used herein, software refers to computer readable program instructions that, when executed by a computer, perform computer operations. Typically, software is provided on a program product containing program instructions recorded on a computer readable medium, including, but not limited to, magnetic media including floppy disks, hard disks, and magnetic tape; and optical media including CD-ROM discs, DVD discs, magneto-optical discs, and other such media on which the program instructions can be recorded.

Different methods of predicting abnormality or normality can produce different types of results. For any given prediction, there are four possible types of outcomes: true positive, true negative, false positive, or false negative. The term "true positive" as used herein refers to a subject correctly diagnosed as having a chromosome abnormality. The term "false positive" as used herein refers to a subject wrongly identified as having a chromosome abnormality. The term "true negative" as used herein refers to a subject correctly identified as not having a chromosome abnormality. The term "false negative" as used herein refers to a subject wrongly identified as not having a chromosome abnormality. Two measures of performance for any given method can be calculated based on the ratios of these occurrences: (i) a sensitivity value, the fraction of predicted positives that are correctly identified as being positives (e.g., the fraction of nucleotide sequence sets correctly identified by level comparison detection/determination as indicative of chromosome abnormality, relative to all nucleotide sequence sets identified as such, correctly or incorrectly), thereby reflecting the accuracy of the results in detecting the chromosome abnormality; and (ii) a specificity value, the fraction of predicted negatives correctly identified as being negative (the fraction of nucleotide sequence sets correctly identified by level comparison detection/determination as indicative of chromosomal normality, relative to all nucleotide sequence sets identified as such, correctly or incorrectly), thereby reflecting accuracy of the results in detecting the chromosome abnormality.

EXAMPLES

The following examples are provided by way of illustration only and not by way of limitation. Those of skill in the art will readily recognize a variety of non-critical parameters that could be changed or modified to yield essentially the same or similar results.

In Example 1 below, the Applicants used a new fusion protein that captures methylated DNA in combination with CpG Island array to identify genomic regions that are differentially methylated between fetal placenta tissue and maternal blood. A stringent statistical approach was used to only select regions which show little variation between the samples, and hence suggest an underlying biological mechanism. Eighty-five differentially methylated genomic regions predominantly located on chromosomes 13, 18 and 21 were validated. For this validation, a quantitative mass spectrometry based approach was used that interrogated 261 PCR amplicons covering these 85 regions. The results are in very good concordance (95% confirmation), proving the feasibility of the approach.

Next, the Applicants provide an innovative approach for aneuploidy testing, which relies on the measurement of absolute copy numbers rather than allele ratios.

Example 1

In the below Example, ten paired maternal and placental DNA samples were used to identify differentially methylated regions. These results were validated using a mass spectrometry-based quantitative methylation assay. First, genomic DNA from maternal buffy coat and corresponding placental tissue was first extracted. Next the MBD-FC was used to capture the methylated fraction of each DNA sample. See Figures 1-3. The two tissue fractions were labeled with different fluorescent dyes and hybridized to an Agilent® CpG Island microarray. See Figure 4. This was done to identify differentially methylated regions that could be utilized for prenatal diagnoses. Therefore, two criteria were employed to select genomic regions as potential enrichment markers: the observed methylation difference had to be present in all tested sample pairs, and the region had to be more than 200 bp in length.

DNA preparation and fragmentation

Genomic DNA (gDNA) from maternal buffy coat and placental tissue was prepared using the QIAamp DNA Mini Kit™ and QIAamp DNA Blood Mini Kit™, respectively, from Qiagen® (Hilden, Germany). For MCIp, gDNA was quantified using the NanoDrop ND 1000™ spectrophotometer (Thermo Fisher®, Waltham, MA,USA). Ultrasonication of 2.5 µg DNA in 500 µl TE buffer to a mean fragment size of 300-500 bp was carried out with the Branson Digital Sonifier 450™ (Danbury, CT, USA) using the following settings: amplitude 20%, sonication time 110 seconds, pulse on/pulse off time 1.4/0.6 seconds. Fragment range was monitored using gel electrophoresis.

Methyl-CpG Immunoprecipitation

Per sample, 56 µg purified MBD-Fc protein and 150 µl of Protein A Sepharose 4 Fast Flow beads (Amersham Biosciences®, Piscataway, NJ, USA) were rotated in 15 ml TBS overnight at 4°C. Then, theMBD-Fc beads (150 µl/assay) were transferred and dispersed in to 2 ml Ultrafree-CL centrifugal filter devices (Millipore®, Billerica, MA, USA) and spin-washed three times with Buffer A (20 mM Tris-HCl, pH8.0, 2 mM MgCl2, 0.5 mM EDTA 300 mM NaCl, 0.1% NP-40). Sonicated DNA (2 µg) was added to the washed MBD-Fc beads in 2 ml Buffer A and rotated for 3 hours at 4°C. Beads were centrifuged to recover unbound DNA fragments (300 mM fraction) and subsequently washed twice with 600 µl of buffers containing increasing NaCl concentrations (400, 500, 550, 600, and 1000 mM). The flow through of each wash step was collected in separate tubes and desalted using a MinElute PCR Purification Kit™ (Qiagen®). In parallel, 200 ng sonicated input DNA was processed as a control using the MinElute PCR Purification Kit™ (Qiagen®).

Microarray handling and analysis

To generate fluorescently labeled DNA for microarray hybridization, the 600 mM and 1M NaCl fractions (enriched methylated DNA) for each sample were combined and labeled with either Alexa Fluor 555–aha–dCTP (maternal) or Alexa Fluor 647–aha–dCTP (placental) using the BioPrime Total Genomic Labeling System™ (Invitrogen®, Carlsbad, CA, USA). The labeling reaction was carried out according to the manufacturer's manual. The differently labeled genomic DNA fragments of matched maternal/placental pairs were combined to a final volume of 80 µl, supplemented with 50 µg Cot-1 DNA (Invitrogen®), 52 µl of Agilent 10X blocking reagent (Agilent Technologies®, Santa Clara, CA, USA), 78 µl of deionized formamide, and 260 µl Agilent 2X hybridization buffer. The samples were heated to 95°C for 3 min, mixed, and subsequently incubated at 37°C for 30 min. Hybridization on Agilent CpG Island Microarray Kit™ was then carried out at 67°C for 40 hours using an Agilent SureHyb™ chamber and an Agilent hybridization oven. Slides were washed in Wash I (6X SSPE, 0.005% N-lauroylsarcosine) at room temperature for 5 min and in Wash II (0.06X SSPE) at 37°C for an additional 5 min. Next, the slides were submerged in acetonitrile and Agilent Ozone Protection Solution™, respectively, for 30 seconds. Images were scanned immediately and analyzed using an Agilent DNA Microarray Scanner™. Microarray images were processed using Feature Extraction Software v9.5 and the standard CGH protocol.

Bisulfite Treatment

Genomic DNA sodium bisulfite conversion was performed using EZ-96 DNA Methylation Kit™ (ZymoResearch, Orange County, CA). The manufacturer's protocol was followed using 1ug of genomic DNA and the alternative conversion protocol (a two temperature DNA denaturation).

Quantitative Methylation Analysis

Sequenom's MassARRAY® System was used to perform quantitative methylation analysis. This system utilizes matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry in combination with RNA base specific cleavage (Sequenom® MassCLEAVE™). A detectable pattern is then analyzed for methylation status. PCR primers were designed using Sequenom® *EpiDESIGNER™* (www.epidesigner.com). A total of 261 amplicons, covering 85 target regions, were used for validation (median amplification length = 367 bp, min = 108, max = 500; median number of CpG's per amplicon =23, min = 4, max = 65). For each reverse primer, an additional T7 promoter tag for *in-vivo* transcription was added, as well as a 10mer tag on the forward primer to adjust for melting temperature differences. The MassCLEAVE(tm) biochemistry was performed as previously described (Ehrich M, et al. (2005) Quantitative high-throughput analysis of DNA methylation patterns by base specific cleavage and mass spectrometry. *Proc Natl Acad Sci U S A* 102:15785-15790). Mass spectra were acquired using a MassARRAY™ Compact MALDI-TOF (Sequenom®, San Diego) and methylation ratios were generated by the EpiTYPER™ software v1.0 (Sequenom®, San Diego).

Statistical analysis

All statistical calculations were performed using the R statistical software package (www.r-project.org). First, the array probes were grouped based on their genomic location. Subsequent probes that were less than 1000 bp apart were grouped together. To identify differentially methylated regions, a control sample was used as reference. In the control sample, the methylated fraction of a blood derived control DNA was hybridized against itself. Ideally this sample should show log ratios of the two color channels around 0. However because of the variability in hybridization behavior, the probes show a mean log ratio of 0.02 and a standard deviation of 0.18. Next the log ratios observed in our samples were compared to the control sample. A two way, paired t-test was used to test the NULL hypothesis that the groups are identical. Groups that contained less than 4 probes were excluded from the analysis. For groups including four or five probes, all probes were used in a paired t-test. For Groups with six or more probes, a sliding window test consisting of five probes at a time was used, whereby the window was moved by one probe increments. Each test sample was compared to the control sample and the p-values were recorded. Genomic regions were selected as being differentially methylated if eight out of ten samples showed a p value < 0.01, or if six out of ten samples showed a p value < 0.001. The genomic regions were classified as being not differentially

methylated when the group showed less than eight samples with a p value < 0.01 and less than six samples with a p value < 0.001. Samples that didn't fall in either category were excluded from the analysis. For a subset of genomic regions that have been identified as differentially methylated, the results were confirmed using quantitative methylation analysis.
The Go analysis was performed using the online GOstat tool (http://gostat.wehi.edu.au/cgibin/-goStat.pl). P values were calculated using Fisher's exact test.

Microarray-based marker discovery results

To identify differentially methylated regions a standard sample was used, in which the methylated DNA fraction of monocytes was hybridized against itself. This standard provided a reference for the variability of fluorescent measurements in a genomic region. Differentially methylated regions were then identified by comparing the log ratios of each of the ten placental/maternal samples against this standard. Because the goal of this study was to identify markers that allow the reliable separation of maternal and fetal DNA, the target selection was limited to genes that showed a stable, consistent methylation difference over a contiguous stretch of genomic DNA. This focused the analysis on genomic regions where multiple probes indicated differential methylation. The selection was also limited to target regions where all samples showed differential methylation, excluding those with strong inter-individual differences. Two of the samples showed generally lower log ratios in the microarray analysis. Because a paired test was used for target selection, this did not negatively impact the results.
Based on these selection criteria, 3043 genomic regions were identified that were differentially methylated between maternal and fetal DNA. 21778 regions did not show a methylation difference. No inter-chromosomal bias in the distribution of differentially methylated regions was observed. The differentially methylated regions were located next to or within 2159 known genes. The majority of differentially methylated regions are located in the promoter area (18%) and inside the coding region (68%), while only few regions are located downstream of the gene (7%) or at the transition from promoter to coding region (7%). Regions that showed no differential methylation showed a similar distribution for promoter (13%) and downstream (5%) locations, but the fraction of regions located in the transition of promoter to coding region was higher (39%) and the fraction inside the coding region was lower (43%).

It has been shown in embryonic stem cells (ES) that genes targeted by the polycomb repressive complex2 (PRC2) are enriched for genes regulating development (Lee TI, et al. (2006) Control of developmental regulators by Polycomb in human embryonic stem cells. *Cell* 125:301-313). It has also been shown that differentially methylated genes are enriched for genes targeted by PRC2 in many cancer types (Ehrich M, et al. (2008) Cytosine methylation profiling of cancer cell lines. *Proc Natl Acad Sci U S A* 105:4844-48). The set of genes identified as differentially methylated in this study is also enriched for genes targeted by PRC2 (p-value < 0.001, odds ratio = 3.6, 95% CI for odds ratio= 3.1 – 4.2). A GO analysis of the set of differentially methylated genes reveals that this set is significantly enriched for functions important during development. Six out of the ten most enriched functions include developmental or morphogenic processes [anatomical structure morphogenesis (GO:0009653, p value =0), developmental process (GO:0032502, p value = 0), multicellular organismal development (GO:0007275, p value = 0), developmental of an organ (GO:0048513, p value = 0), system development (GO:0048731, p value = 0) and development of an anatomical structure (GO:0048856, p value = 0)].

Validation using Sequenom® EpiTYPER™

To validate the microarray findings, 63 regions from chromosomes 13, 18 and 21 and an additional 26 regions from other autosomes were selected for confirmation by a different technology. Sequenom EpiTYPER™ technology was used to quantitatively measure DNA methylation in maternal and placental samples. For an explanation of the EpiTYPER™ methods, see Ehrich M, Nelson MR, Stanssens P, Zabeau M, Liloglou T, Xinarianos G, Cantor CR, Field JK, van den Boom D (2005) Quantitative high-throughput analysis of DNA methylation patterns by base specific cleavage and mass spectrometry. Proc Natl Acad Sci U S A 102:15785-15790). For each individual CpG site in a target region the average methylation value across all maternal DNA samples and across all placenta samples was calculated. The difference between average maternal and placenta methylation was then compared to the microarray results. The results from the two technologies were in good concordance (see Figure7). For 85 target regions the quantitative results confirm the microarray results (95% confirmation rate). For 4 target regions, all located on chromosome 18, the results could not be confirmed. The reason for this discrepancy is currently unclear.

In contrast to microarrays, which focus on identification of methylation differences, the quantitative measurement of DNA methylation allowed analysis of absolute methylation values. In the validation set of 85 confirmed differentially methylated regions, a subset of 26 regions is more methylated in the maternal DNA sample and 59 regions are more methylated in the placental sample (see Table 1). Interestingly, genes that are hypomethylated in the placental samples tend to show larger methylation differences than genes that are hypermethylated in the placental sample (median methylation difference for hypomethylated genes = 39%, for hypermethylated genes = 20%).

Example 2

Example 2 describes a non-invasive approach for detecting the amount of fetal nucleic acid present in a maternal sample (herein referred to as the "Fetal Quantifier Method"), which may be used to detect or confirm fetal traits (e.g., fetal sex of RhD compatibility), or diagnose chromosomal abnormalities such as Trisomy 21 (both of which are herein referred to as the "Methylation-Based Fetal Diagnostic Method"). Figure 10 shows one embodiment of the Fetal Quantifier Method, and Figure 11 shows one embodiment of the Methylation-Based Fetal Diagnostic Method. Both processes use fetal DNA obtained from a maternal sample. The sample comprises maternal and fetal nucleic acid that is differentially methylated. For example, the sample may be maternal plasma or serum. Fetal DNA comprises approximately 2-30% of the total DNA in maternal plasma. The actual amount of fetal contribution to the total nucleic acid present in a sample varies from pregnancy to pregnancy and can change based on a number of factors, including, but not limited to, gestational age, the mother's health and the fetus' health.

As described herein, the technical challenge posed by analysis of fetal DNA in maternal plasma lies in the need to be able to discriminate the fetal DNA from the co-existing background maternal DNA. The methods of the present technology exploit such differences, for example, the differential methylation that is observed between fetal and maternal DNA, as a means to enrich for the relatively small percentage of fetal DNA present in a sample from the mother. The non-invasive nature of the approach provides a major advantage over conventional methods of prenatal diagnosis such as, amniocentesis, chronic villus sampling and cordocentesis, which are associated with a small but finite risk of fetal loss. Also, because the method is not dependent on fetal cells being in any particular cell phase, the method provides a rapid detection means to determine the presence and also the nature of the chromosomal abnormality. Further, the approach is sex-independent (i.e., does not require the presence of a Y-chromosome) and polymorphic-independent (i.e., an allelic ratio is not determined). Thus, the compositions and methods of the technology represent improved universal, noninvasive approaches for accurately determining the amount of fetal nucleic acid present in a maternal sample.

Assay design and advantages

There is a need for accurate detection and quantification of fetal DNA isolated noninvasively from a maternal sample. The present technology takes advantage of the presence of circulating, cell free fetal nucleic acid (ccfDNA) in maternal plasma or serum. In order to be commercially and clinically practical, the methods of the technology should only consume a small portion of the limited available fetal DNA. For example, less than 50%, 40%, 30%, 25%, 20%, 15%, 10%, 5% or less of the sample. Further, the approach should preferably be developed in a multiplex assay format in which one or more (preferably all) of the following assays are included:

- Assays for the detection of total amount of genomic equivalents present in the sample, i.e., assays recognizing both maternal and fetal DNA species;
- Assays for the detection of fetal DNA isolated from a male pregnancy, i.e., sequences specific for chromosome Y;
- Assays specific for regions identified as differentially methylated between the fetus and mother; or
- Assays specific for regions known to be hypomethylated in all tissues to be investigated, which can serve as a control for restriction efficiency.

Other features of the assay may include one or more of the following:

- For each assay, a target-specific, competitor oligonucleotide that is identical, or substantially identical, to the target sequence apart from a distinguishable feature of the competitor, such as a difference in one or more nucleotides relative to the target sequence. This oligonucleotide when added into the PCR reaction will be co-amplified with the target and a ratio obtained between these two PCR amplicons will indicate the number of target specific DNA sequences (e.g., fetal DNA from a specific locus) present in the maternal sample.

- The amplicon lengths should preferably be of similar length in order not to skew the amplification towards the shorter fragments. However, as long as the amplification efficiency is about equal, different lengths may be used.
- Differentially methylated targets can be selected from Table 1 or from any other targets known to be differentially methylated between mother and fetus. These targets can be hypomethylated in DNA isolated from non-pregnant women and hypermethylated in samples obtained from fetal samples. These assays will serve as controls for the restriction efficiency.
- The results obtained from the different assays can be used to quantify one or more of the following:
    - Total number of amplifiable genomes present in the sample (total amount of genomic equivalents);
    - The fetal fraction of the amplifiable genomes (fetal concentration or percentage); or
    - Differences in copy number between fetally-derived DNA sequences (for example, between fetal chromosome 21 and a reference chromosome such as chromosome 3).

Examples of assays used in the test

Below is an outline of the reaction steps used to perform a method of the technology, for example, as provided in Figure 10. This outline is not intended to limit the scope of the technology. Rather it provides one embodiment of the technology using the Sequenom® MassARRAY® technology.

1) DNA isolation from plasma samples.
2) Digestion of the DNA targets using methylation sensitive restriction enzymes (for example, HhaI and HpaII).

   For each reaction the available DNA was mixed with water to a final volume of 25 ul.

   10 ul of a reaction mix consisting of 10 units HhaI, 10 units HpaII and a reaction buffer were added. The sample was incubated at an optimal temperature for the restriction enzymes. HhaI and HpaII digest non-methylated DNA (and will not digest hemi- or completely methylated DNA). Following digestion, the enzymes were denatured using a heating step.
3) Genomic Amplification- PCR was performed in a total volume of 50 ul by adding PCR reagents (Buffer, dNTPs, primers and polymerase). Exemplary PCR and extend primers are provided below. In addition, synthetic competitor oligonucleotide was added at known concentrations.
4) Replicates (optional) - Following PCR the 50 ul reaction was split into 5 ul parallel reactions (replicates) in order to minimize variation introduced during the post PCR steps of the test. Post PCR steps include SAP, primer extension (MassEXTEND® technology), resin treatment, dispensing of spectrochip and MassARRAY.

5) Quantification of the Amplifiable Genomes – Sequenom MassARRAY® technology was used to determine the amount of amplification product for each assay. Following PCR, a single base extension assay was used to interrogate the amplified regions (including the competitor oligonucleotides introduced in step 3). Specific extend primers designed to hybridize directly adjacent to the site of interest were introduced. See extend primers provided below. These DNA oligonucleotides are referred to as iPLEX® MassEXTEND® primers. In the extension reaction, the iPLEX primers were hybridized to the complementary DNA templates and extended with a DNA polymerase. Special termination mixtures that contain different combinations of deoxy- and dideoxynucleotide triphosphates along with enzyme and buffer, directed limited extension of the iPLEX primers. Primer extension occurs until a complementary dideoxynucleotide is incorporated.

   The extension reaction generated primer products of varying length, each with a unique molecular weight. As a result, the primer extension products can be simultaneously separated and detected using Matrix Assisted Laser Desorption/Ionization, Time-Of-Flight (MALDI-TOF) mass spectrometry on the MassARRAY® Analyzer Compact. Following this separation and detection, SEQUENOM's proprietary software automatically analyzes the data.

6) Calculating the amount and concentration of fetal nucleic acid – Methods for calculating the total amount of genomic equivalents present in the sample, the amount (and concentration) of fetal nucleic acid isolated from a male pregnancy, and the amount (and concentration) of fetal nucleic based on differentially methylated targets are provided below and in Figures 18 and 19.

The above protocol can be used to perform one or more of the assays described below. In addition to the sequences provided immediately below, a multiplex scheme that interrogates multiple is provided in Table X below.

1) *Assay for the quantification of the total number of amplifiable genomic equivalents in the sample.*

Targets were selected in housekeeping genes not located on the chromosomes 13, 18, 21, X or Y. The targets should be in a single copy gene and not contain any recognition sites for the methylation sensitive restriction enzymes.

Underlined sequences are PCR primer sites, italic is the site for the single base extend primer and bold letter (C) is the nucleotide extended on human DNA

ApoE Chromosome 19:45409835-45409922 DNA target sequence with interrogated nucleotide C in bold. All of the chromosome positions provided in this section are from the February 2009 UCSC Genome Build.
GATTGACAGTTTCTCCTTCCCCAGACTGGCCAATCACAG*GCAGGAAGATGAAGGTT***C**TGTGGGCTGCGTTGCTGGTCACATTCCTGGC

ApoE Forward Primer: 5'-ACGTTGGATG-TTGACAGTTTCTCCTTCCCC (Primer contains a 5' 10 bp MassTag separated by a dash)

ApoE Reverse Primer: 5'-ACGTTGGATG-GAATGTGACCAGCAACGCAG (Primer contains a 5' 10 bp MassTag separated by a dash)

ApoE Extension Primer: 5'-GCAGGAAGATGAAGGTT [C/T] Primer extends C on human DNA targets and T on synthetic DNA targets

ApoE synthetic competitor oligonucleotide: 5'-
GATTGACAGTTTCTCCTTCCCCAGACTGGCCAATCACAGGCAGGAAGATGAAGGTT**T**TGTGGGCTGCGTTGCTGG
TCACATTCCTGGC (Bold T at position 57 is different from human DNA)

2) *Assay for the quantification of the total number of chromosome Y sequences in the sample.*

Targets specific for the Y-chromosome were selected, with no similar or paralog sequences elsewhere in the genome. The targets should preferably be in a single copy gene and not contain any recognition sites for the methylation sensitive restriction enzyme(s).

Underlined sequences are PCR primer sites, and italic nucleotide(s) is the site for the single-base extend primer and bold letter (C) is the nucleotide extended on human DNA.

SRY on chrY:2655628-2655717 (reverse complement)
GAGTTTTGGATAGTAAAATAAGTTTCGAACTCTGGCACC*TTTCAATTTTGTCGCACT***C**TCCTTGTTTTTGACAATGC
AATCATATGCTTC

SRY Forward Primer: 5'-ACG-TGGATAGTAAAATAAGTTTCGAACTCTG (Primer contains a 5' 3 bp MassTag separated by a dash)

SRY Reverse Primer: 5'- GAAGCATATGATTGCATTGTCAAAAAC

SRY Extension Primer: 5'-aTTTCAATTTTGTCGCACT [C/T] Primer extends C on human DNA targets and T on synthetic DNA targets. 5' Lower case "a" is a non-complementary nucleotide

SRY synthetic competitor oligonucleotide: 5'-
GAGTTTTGGATAGTAAAATAAGTTTCGAACTCTGGCACCTTTCAATTTTGTCGCACT**T**TCCTTGTTTTTGACAATGC
AATCATATGCTTC

3) *Assay for the quantification of fetal methylated DNA sequences present in the sample.*
Targets were selected in regions known to be differentially methylated between maternal and fetal DNA. Sequences were selected to contain several restriction sites for methylation sensitive enzymes. For this study the HhaI (GCGC) and HpaII (CCGG) enzymes were used.

Underlined sequences are PCR primer sites, italic is the site for the single base extend primer and bold letter (C) is the nucleotide extended on human DNA, lower case letter are recognition sites for the methylation sensitive restriction enzymes.

TBX3 on chr12:115124905-115125001

GAACTCCTCTTTGTCTCTGCGTGCccggcgcgcCC*CCCTCccggTGGGTGATAAA***C**CCACTCTGgcgccggCCATgcgcTGGGTGATTAATTTGCGA

TBX3 Forward Primer: 5'- ACGTTGGATG-TCTTTGTCTCTGCGTGCCC (Primer contains a 5' 10 bp MassTag separated by a dash)

TBX3 Reverse Primer: 5'- ACGTTGGATG-TTAATCACCCAGCGCATGGC (Primer contains a 5' 10 bp MassTag separated by a dash)

TBX3 Extension Primer: 5'- CCCCTCCCGGTGGGTGATAAA [C/T] Primer extends C on human DNA targets and T on synthetic DNA targets. 5' Lower case "a" is a non-complementary nucleotide

TBX3 synthetic competitor oligonucleotide: 5'-GAACTCCTCTTTGTCTCTGCGTGCCCGGCGCGCCCCCCTCCCGGTGGGTGATAAATCCACTCTGGCGCCGGCCATGCGCTGGGTGATTAATTTGCGA

4) Control Assay for the enzyme restriction efficiency.

Targets were selected in regions known not to be methylated in any tissue to be investigated. Sequences were selected to contain no more than one site for each restriction enzyme to be used.

Underlined sequences are PCR primer sites, italic nucleotide(s) represent the site for the single-base extend primer and bold letter (G) is the reverse nucleotide extended on human DNA, lower case letter are recognition sites for the methylation sensitive restriction enzymes.

CACNA1G chr17:48637892-48637977 (reverse complement)
CCATTGGCCGTCCGCCGTGGCAGTGCGGGCGGGAgcgcAG**G***GAGAGAACCACAGCTGGAAT*CCGATTCCCACCCCAAAACCCAGGA

HhaI Forward Primer: 5'- ACGTTGGATG-CCATTGGCCGTCCGCCGTG (Primer contains a 5' 10 bp MassTag separated by a dash)

HhaI Reverse Primer: 5'- ACGTTGGATG-TCCTGGGTTTTGGGGTGGGAA (Primer contains a 5' 10 bp MassTag separated by a dash)

HhaI Extension Primer: 5'- TTCCAGCTGTGGTTCTCTC

HhaI synthetic competitor oligonucleotide: 5'-CCATTGGCCGTCCGCCGTGGCAGTGCGGGCGGGAGCGCAG**A***GAGAGAACCACAGCTGGAAT*CCGATTCCCACCCCAAAACCCAGGA

Validation experiments

The sensitivity and accuracy of the present technology was measured using both a model system and clinical samples. In the different samples, a multiplex assay was run that contains 2 assays for total copy number quantification, 3 assays for methylation quantification, 1 assay specific for chromosome Y and 1 digestion control assay. See Tables X1 and X2. Another multiplex scheme with additional assays is provided in Tables Y1 and Y2.

TABLE X1: PCR Primers and Extend Primers

Gene ID	*	First Primer	Second Primer	Extend Primer
SOX14	M	ACGTTGGATGACATGGTCGGCCCCACGGAAT	ACGTTGGATGCTCCTTCCTAGTGTGAGAACCG	CAGGTTCCGGGGCTTGGG
Hhal_CTRL	D	ACGTTGGATGACCCATTGGCCGTCCGCCGT	ACGTTGGATGTTTTGGGGTGGGAATCGGATT	CGCAGGGAGAGAACCACAG
TBX3	M	ACGTTGGATGGAACTCCTCTTTGTCTCTGCG	ACGTTGGATGTGGCATGGCCGGCGCCAGA	CCCCTCCCGGTGGGTGATAAA
SRY	Y	ACGTTGGATGCGCAGCAACGGGACCGCTACA	ACGTTGGCATCTAGGTAGGTCTTTGTAGCCAA	AAAGCTGTAGGACAATCGGGT
ALB	T	ACGTTGCGTAGCAACCTGTTACATATTAA	ACGTTGGATCTGAGCAAAGGCAATCAACACCC	CATTTTTCTACATCCTTTGTTT
EDG6	M	ACGTTGGATGCATAGAGGCCCATGATGGTGG	ACGTTGGATGACCTTCTGCCCCTCTACTCCAA	agAAGATCACCAGGCAGAAGAGG
RNaseP	T	ACGTTGGATGGTGTGGTCAGCTCTTCCCTTCAT	ACGTTGGCCCACATGTAATGTGTTGAAAAAGCA	ACTTGGAGAACAAAGGACACCGTTA

TABLE X2: Competitor Oligonucleotide Sequence

Gene ID	*	Competitor Oligonucleotide Sequence
SOX14	M	GGTCGGCCCCACGGAATCCCGGCTCTGTGTGCGCCCAGGTTCCGGGGCTTGGGTGTTGCCGGTTCTCACACTAGGAAGGAG
Hhal_CTRL	D	CCATTGGCCGTCCGCCGTGGCAGTGCGGGCGGGAGCGCAGAGAGAGAACCACAGCTGGAATCCGATTCCCACCCCAAAA
TBX3	M	GAACTCCTCTTTGTCTCTGCGTGCCCGGCGCGCCCCCCTCCCGGTGGGTGATAAATCCACTCTGGCGCCGGCCATGC
SRY	Y	GCAGCAACGGGACCGCTACAGCCACTGGACAAAGCCGTAGGACAATCGGGTAACATTGGCTACAAAGACCTACCTAGATGC
ALB	T	GCGTAGCAACCTGTTACATATTAAAGTTTTATTATACTACATTTTTCTACATCCTTTGTTTCAGAGTGTTGATTGCCTTTGCTCAGTATCTTCAG
EDG6	M	CCTTCTGCCCCTCTACTCCAAGCGCTACACCCTCTTCTGCCTGGTGATCTTTGCCGGCGTCCTGGCCACCATCATGGGCCTCTATG
RNaseP	T	GTGTGGTCAGCTCTTCCCTTCATCACATACTTGGAGAACAAAGGACACCGTTATCCATGCTTTTTCAACACATTACATGTGGG

TABLE Y1: PCR Primers and Extend Primers

Gene ID	*	First Primer	Second Primer	Extend Primer
EDG6	M	ACGTTGGATGTTCTGCCCCTCTACTCCAAG	ACGTTGGATGCATAGAGGCCCATGATGGTG	TTCTGCCTGGTGATCTT
RNAseP	T	ACGTTGGATGTCAGCTCTTCCCTTCATCAC	ACGTTGGATGCCTACCTCCCACATGTAATGT	AACAAAGGACACCGTTA
ApoE	T	ACGTTGGATGTTGACAGTTTCTCCTTCCCC	ACGTTGGATGGAATGTGACCAGCAACGCAG	GCAGGAAGATGAAGGTT
SOX14	M	ACGTTGGATGCGGTCGGCCCCACGGAAT	ACGTTGGATGCTCCTTCCTAGTGTGAGAACCG	aAGGTTCCGGGGCTTGGG

Gene ID	*	First Primer	Second Primer	Extend Primer
SRY no2	Y	ACGTGGATAGTAAAATAAGTTTCGAACTCTG	GAAGCATATGATTGCATTGTCAAAAAC	aTTTCAATTTTGTCGCACT
SRY no1	Y	ACGTTGGATGCACAGCTCACCGCAGCAACG	ACGTTGGATGCTAGGTAGGTCTTTGTAGCCAA	AGCTGTAGGACAATCGGGT
TBX3	M	ACGTTGGATGTCTTTGTCTCTGCGTGCCC	ACGTTGGATGTTAATCACCCAGCGCATGGC	CCCTCCCGGTGGGTGATAAA
CACNA1G dig CTRL 1	D	ACGTTGGATGGACTGAGCCCCAGAACTCG	ACGTTGGATGGTGGGTTTGTGCTTTCCACG	AGGGCCGGGGTCTGCGCGTG
DAPK1 dig CTRL 2	D	ACGTTGGATGAAGCCAAGTTTCCCTCCGC	ACGTTGGATGCTTTTGCTTTCCCAGCCAGG	GAGGCACTGCCCGGACAAACC
ALB	T	ACGTTAGCGTAGCAACCTGTTACATATTAA	ACGTTGGATGCTGAGCAAAGGCAATCAACA	CATTTTTCTACATCCTTTGTTT

TABLE Y2: Competitor Oligonucleotide Sequence

Gene ID	*	Competitor
EDG6	M	CCTTCTGCCCCTCTACTCCAAGCGCTACACCCTCTTCTGCCTGGTGATCTTTGCCGGCGTCCTGGCCACCATCATGGGCCTCTATG
RNAseP	T	GTGTGGTCAGCTCTTCCCTTCATCACATACTTGGAGAACAAAGGACACCGTTATCCATGCTTTTTCAACACATTACATGTGGGAGGTAGG
ApoE	T	GATTGACAGTTTCTCCTTCCCCAGACTGGCCAATCACAGGCAGGAAGATGAAGGTTTTGTGGGCTGCGTTGCTGGTCACATTCCTGGC
SOX14	M	AAAACCAGAGATTCGCGGTCGGCCCCACGGAATCCCGGCTCTGTGTGCGCCCAGGTTCCGGGGCTTGGGTGTTGCCGGTTCTCACACTAGGAAGGAGC
SRY no2	Y	GAGTTTTGGATAGTAAAATAAGTTTCGAACTCTGGCACCTTTCAATTTTGTCGCACTTTCCTTGTTTTTGACAATGCAATCATATGCTTC
SRY no1	Y	GCAGCCAGCTCACCGCAGCAACGGGACCGCTACAGCCACTGGACAAAGCTGTAGGACAATCGGGTGACATTGGCTACAAAGACCTACCTAGATGC
TBX3	M	GAACTCCTCTTTGTCTCTGCGTGCCCGGCGCGCCCCCCTCCCGGTGGGTGATAAATCCACTCTGGCGCCGGCCATGCGCTGGGTGATTAATTTGCGA
CACNA1G dig CTRL 1	D	GTGGGTTTGTGCTTTCCACGCGTGCACACACACGCGCAGACCCCGGCCCTTGCCCCGCCTACCTCCCCGAGTTCTGGGGCTCAGTC
DAPK1 dig CTRL 2	D	GCGCCAGCTTTTGCTTTCCCAGCCAGGGCGCGGTGAGGTTTGTCCGGGCAGTGCCTCGAGCAACTGGGAAGGCCAAGGCGGAGGGAAAC
ALB	T	GCGTAGCAACCTGTTACATATTAAAGTTTTATTATACTACATTTTTCTACATCCTTTGTTTTAGGGTGTTGATTGCCTTTGCTCAGTATCTTCAGC

T=Assay for Total Amount; M=Assay for Methylation quantification; Y= Y-Chromosome Specific Assay; D=Digestion control

Model system using genomic DNA

In order to determine the sensitivity and accuracy of the method when determining the total number of amplifiable genomic copies in a sample, a subset of different DNA samples isolated from the blood of non-pregnant women was tested. Each sample was diluted to contain approximately 2500, 1250, 625 or 313 copies per reaction. The total number of amplifiable genomic copies was obtained by taking the mean DNA/competitor ratio obtained from the three total copy number assays. The results from the four different samples are shown in Figure 12.

To optimize the reaction, a model system was developed to simulate DNA samples isolated from plasma. These samples contained a constant number of maternal non-methylated DNA and were spiked with different amounts of male placental methylated DNA. The samples were spiked with amounts ranging from approximately 0 to 25% relative to the maternal non-methylated DNA. The results are shown in Figures 13A and B. The fraction of placental DNA was calculated using the ratios obtained from the methylation assays (Figure 13A), the SRY markers (Figure 13B) and the total copy number assays. The primer sequences for the methylation assays (TBX), Y-chromosome assays (SRY) and total copy number (APOE) are provided above. The model system demonstrated that the methylation-based method performed equal to the Y-chromosome method (SRY markers), thus validating the methylation-based method as a sex-independent fetal quantifier.

Plasma samples

To investigate the sensitivity and accuracy of the methods in clinical samples, 33 plasma samples obtained from women pregnant with a male fetus were investigated using the multiplex scheme from Table X. For each reaction, a quarter of the DNA obtained from a 4ml extraction was used in order to meet the important requirement that only a portion of the total sample is used.

Total copy number quantification

The results from the total copy number quantification can be seen in Figures 14A and B. In Figure 14A, the copy number for each sample is shown. Two samples (nos. 25 and 26) have a significantly higher total copy number than all the other samples. In general, a mean of approximately 1300 amplifiable copies/ml plasma was obtained (range 766-2055). Figure 14B shows a box-and-whisker plot of the given values, summarizing the results.

Correlation between results obtained from the methylation markers and the Y-chromosome marker

In Figures 15A and B, the numbers of fetal copies for each sample are plotted. As all samples were from male pregnancies. The copy numbers obtained can be calculated using either the methylation or the Y-chromosome-specific markers. As can be seen in Figure 15B, the box-and-whisker plot of the given values indicated minimal difference between the two different measurements.

The results showing the correlation between results obtained from the methylation markers and the Y-chromosome marker (SRY) is shown in Figure 16. Again, the methylation-based method performed equal to the Y-chromosome method (SRY markers), further validating the methylation-based method as a sex-independent and polymorphism-independent fetal quantifier. The multiplexed assays disclosed in Table X were used to determine the amount fetal nucleic.

Finally, the digestion efficiency was determined by using the ratio of digestion for the control versus the competitor and comparing this value to the mean total copy number assays. See Figure 17. Apart from sample 26 all reactions indicate the efficiency to be above 99%.

Data Analysis

Mass spectra analysis was done using Typer 4 (a Sequenom software product). The peak height (signal over noise) for each individual DNA analyte and competitor assay was determined and exported for further analysis.

The total number of molecules present for each amplicon was calculated by dividing the DNA specific peak by the competitor specific peak to give a ratio. (The "DNA" Peak in Figures 18 and 19 can be thought of as the analyte peak for a given assay). Since the number of competitor molecules added into the reaction is known, the total number of DNA molecules can be determined by multiplying the ratio by the number of added competitor molecules.

The fetal DNA fraction (or concentration) in each sample was calculated using the Y-chromosome-specific markers for male pregnancies and the mean of the methylated fraction for all pregnancies. In brief, for chromosome Y, the ratio was obtained by dividing the analyte (DNA) peak by the competitor peak and multiplying this ratio by the number of competitor molecules added into the reaction. This value was divided by a similar ratio obtained from the total number of amplifiable genome equivalents determination (using the Assay(s) for Total Amount). See Figure 18. Since the total amount of nucleic acid present in a sample is a sum of maternal and fetal nucleic acid, the fetal contribution can be considered to be a fraction of the larger, background maternal contribution. Therefore, translating this into the equation shown in Figure 18, the fetal fraction (k) of the total nucleic acid present in the sample is equal to the equation: $k=2xR/(1-2R)$, where R is the ratio between the Y-chromosome amount and the total amount. Since the Y-chromosome is haploid and Assays for the Total Amount are determined using diploid targets, this calculation is limited to a fetal fraction smaller than 50% of the maternal fraction.

In Figure 19, a similar calculation for the fetal concentration is shown by using the methylation specific markers (see Assays for Methylation Quantification). In contrast to Y-chromosome specific markers, these markers are from diploid targets, therefore, the limitations stated for the Y-Chromosome Specific Assay can be omitted. Thus, the fetal fraction (k) can be determined using the equation: $k=R(1-R)$, where R is the ratio between the methylation assay and the total assay.

Simulation

A first simple power calculation was performed that assumes a measurement system that uses 20 markers from chromosome 21, and 20 markers from one or more other autosomes. Starting with 100 copies of fetal DNA, a measurement standard deviation of 25 copies and the probability for a type I error to be lower than 0.001, it was found that the methods of the technology will be able to differentiate a diploid from a triploid chromosome set in 99.5% of all cases. The practical implementation of such an approach could for example be achieved using mass spectrometry, a system that uses a competitive PCR approach for absolute copy number measurements. The method can run 20 assays in a single reaction and has been shown to have a standard deviation in repeated measurements of around 3 to 5%. This method was used in combination with known methods for differentiating methylated and non-methylated nucleic acid, for example, using methyl-binding agents to separate nucleic acid or using methylation-sensitive enzymes to digest maternal nucleic acid. Figure 8 shows the effectiveness of MBD-FC protein (a methyl-binding agent) for capturing and thereby separating methylated DNA in the presence of an excess of unmethylated DNA (see Figure 8).

A second statistical power analysis was performed to assess the predictive power of an embodiment of the Methylation-Based Fetal Diagnostic Method described herein. The simulation was designed to demonstrate the likelihood of differentiating a group of trisomic chromosome 21 specific markers from a group of reference markers (for example, autosomes excluding chromosome 21). Many parameters influence the ability to discriminate the two populations of markers reliably. For the present simulation, values were chosen for each parameter that have been shown to be the most likely to occur based on experimentation. The following parameters and respective values were used:

Copy Numbers

Maternal copy numbers = 2000

Fetal copy numbers for chromosomes other than 21, X and Y = 200

Fetal copy numbers for chromosome 21 in case of euploid fetus = 200

Fetal copy numbers for chromosome 21 in case of aneuploid T21 fetus = 300

Percent fetal DNA (before methylation-based enrichment) = 10% (see above)

Methylation Frequency

Average methylation percentage in a target region for maternal DNA = 10%

Average methylation percentage in a target region for fetal DNA = 80%

Average percentage of non-methylated and non-digested maternal DNA (i.e., a function of restriction efficiency (among other things) = 5%

Number of assays targeting chromosome 21 = 10

Number of assays targeting chromosomes other than 21, X and Y = 10

The results are displayed in Figure 20. Shown is the relationship between the coefficient of variation (CV) on the x-axis and the power to discriminate the assay populations using a simple t-test (y-axis). The data indicates that in 99% of all cases, one can discriminate the two population (euploid vs. aneuploid) on a significance level of 0.001 provided a CV of 5% or less.. Based on this simulation, the method represents a powerful noninvasive diagnostic method for the prenatal detection of fetal aneuploidy that is sex-independent and will work in all ethnicities (i.e., no allelic bias).

TABLE 1

GENE NAME	CHROM	START	END	CpG ISLAND	MEAN LOG RATIO MICRO-ARRAY	MEAN MATERNAL METHY-LATION EPITYPER	MEAN PLACENTA METHY-LATION EPITYPER	METHY-LATION DIFFERENCE PLACENTA-MATERNAL	RELATIVE METHYLATION PLACENTA TO MATERNAL
chr13 group00016	chr13	19773745	19774050	chr13:19773518-19774214	0.19	0.22	0.32	0.1	HYPERMETHYLATION
chr13 group00005	chr13	19290394	19290768	:-	-0.89	0.94	0.35	-0.59	HYPOMETHYLATION
CRYL1	chr13	19887090	19887336	chr13:19887007-19887836	-0.63	0.74	0.21	-0.53	HYPOMETHYLATION
IL17D	chr13	20193675	20193897	chr13:20193611-20194438	-1.01	0.53	0.13	-0.39	HYPOMETHYLATION
CENPJ	chr13	24404023	24404359	:-	0.57	0.17	0.49	0.32	HYPERMETHYLATION
ATP8A2	chr13	25484475	25484614	chr13:25484287-25484761	0.81	0.16	0.43	0.27	HYPERMETHYLATION
GSH1	chr13	27265542	27265834	chr13:27264549-27266505	0.57	0.13	0.19	0.05	HYPERMETHYLATION
PDX1	chr13	27393789	27393979	chr13:27392001-27394099	0.55	0.06	0.2	0.14	HYPERMETHYLATION
PDX1	chr13	27400459	27401165	chr13:27400362-27400744; chr13:27401057-27401374	0.73	0.12	0.26	0.14	HYPERMETHYLATION
MAB21L1	chr13	34947737	34948062	chr13:34947570-34948159	0.66	0.11	0.17	0.06	HYPERMETHYLATION
RB1	chr13	47790983	47791646	chr13:47790636-47791858	0.18	0.45	0.48	0.03	HYPERMETHYLATION
PCDH17	chr13	57104856	57106841	chr13:57104527-57106931	0.46	0.15	0.21	0.06	HYPERMETHYLATION
KLHL1	chr13	69579933	69580146	chr13:69579733-69580220	0.79	0.09	0.28	0.2	HYPERMETHYLATION
POU4F1	chr13	78079515	78081073	chr13:78079328-78079615; chr13:78080860-78081881	0.66	0.12	0.23	0.11	HYPERMETHYLATION
GPC6	chr13	92677402	92678666	chr13:92677246-92678878	0.66	0.06	0.19	0.13	HYPERMETHYLATION

GENE NAME	CHROM	START	END	CpG ISLAND	MEAN LOG RATIO MICRO-ARRAY	MEAN MATERNAL METHY-LATION EPITYPER	MEAN PLACENTA METHY-LATION EPITYPER	METHY-LATION DIFFERENCE PLACENTA-MATERNAL	RELATIVE METHYLATION PLACENTA TO MATERNAL
SOX21	chr13	94152286	94153047	chr13:94152190-94153185	0.94	0.16	0.4	0.25	HYPERMETHYLATION
ZIC2	chr13	99439660	99440858	chr13:99439335-99440189; chr13:99440775-99441095	0.89	0.13	0.35	0.22	HYPERMETHYLATION
IRS2	chr13	109232856	109235065	chr13:109232467-109238181	-0.17	0.73	0.38	-0.35	HYPOMETHYLATION
chr13 group00350	chr13	109716455	109716604	chr13:109716325-109716726	-0.37	0.77	0.41	-0.36	HYPOMETHYLATION
chr13 group00385	chr13	111595578	111595955	chr13:111595459-111596131	0.87	0.06	0.2	0.14	HYPERMETHYLATION
chr13 group00390	chr13	111756337	111756593	chr13:111755805-111756697	0.71	0.12	0.34	0.22	HYPERMETHYLATION
chr13 group00391	chr13	111759856	111760045	chr13:111757885-111760666	0.86	0.11	0.36	0.25	HYPERMETHYLATION
chr13 group00395	chr13	111808255	111808962	chr13:111806599-111808492; chr13:111808866-111809114	0.96	0.13	0.35	0.22	HYPERMETHYLATION
chr13 group00399	chr13	112033503	112033685	chr13:112032967-112033734	0.38	0.26	0.43	0.18	HYPERMETHYLATION
MCF2L	chr13	112724910	112725742	chr13:112724782-112725121; chr13:112725628-112725837	-0.47	0.91	0.33	-0.58	HYPOMETHYLATION
F7	chr13	112799123	112799379	chr13:112798487-112799566	-0.05	0.97	0.55	-0.41	HYPOMETHYLATION
PROZ	chr13	112855566	112855745	chr13:112855289-112855866	0.29	0.15	0.3	0.16	HYPERMETHYLATION
chr18 group00039	chr18	6919797	6919981	chr18:6919450-6920088	-0.38	0.88	0.39	-0.49	HYPOMETHYLATION
CIDEA	chr18	12244327	12244696	chr18:12244147-12245089	0.23	0.14	0.23	0.1	HYPERMETHYLATION
chr18 group00091	chr18	12901467	12901643	chr18:12901024-12902704	0.16	0.15	0.43	0.29	HYPERMETHYLATION
chr18 group00094	chr18	13126819	13126986	chr18:13126596-	0.41	0.07	0.34	0.27	HYPERMETHYLATION

GENE NAME	CHROM	START	END	CpG ISLAND	MEAN LOG RATIO MICRO-ARRAY	MEAN MATERNAL METHY-LATION EPITYPER	MEAN PLACENTA METHY-LATION EPITYPER	METHY-LATION DIFFERENCE PLACENTA-MATERNAL	RELATIVE METHYLATION PLACENTA TO MATERNAL
				13127564					
C18orf1	chr18	13377536	13377654	chr18:13377385-13377686	-0.12	0.95	0.69	-0.26	HYPOMETHYLATION
KLHL14	chr18	28603978	28605183	chr18:28603688-28606300	0.83	0.07	0.19	0.12	HYPERMETHYLATION
CD33L3	chr18	41671477	41673011	chr18:41671386-41673101	-0.34	0.49	0.44	-0.05	HYPOMETHYLATION
ST8SIA3	chr18	53171265	53171309	chr18:53170705-53172603	1.02	0.09	0.25	0.16	HYPERMETHYLATION
ONECUT2	chr18	53254808	53259810	chr18:53254152-53259851	0.74	0.09	0.23	0.14	HYPERMETHYLATION
RAX	chr18	55086286	55086436	chr18:55085813-55087807	0.88	0.11	0.26	0.16	HYPERMETHYLATION
chr18 group00277	chr18	57151972	57152311	chr18:57151663-57152672	0.58	0.08	0.21	0.13	HYPERMETHYLATION
TNFRSF11A	chr18	58203013	58203282	chr18:58202849-58203367	-0.33	0.88	0.28	-0.6	HYPOMETHYLATION
NETO1	chr18	68685099	68687060	chr18:68684945-68687851	0.65	0.09	0.22	0.13	HYPERMETHYLATION
chr18 group00304	chr18	70133945	70134397	chr18:70133732-70134724	0.12	0.93	0.92	-0.01	NOT CONFIRMED
TSHZ1	chr18	71128742	71128974	chr18:71128638-71129076	0.23	0.95	0.92	-0.03	NOT CONFIRMED
ZNF236	chr18	72664454	72664736	chr18:72662797-72664893	-0.62	0.17	0.1	-0.07	HYPOMETHYLATION
MBP	chr18	72953150	72953464	chr18:72953137-72953402	0.6	0.44	0.72	0.28	HYPERMETHYLATION
chr18 group00342	chr18	74170347	74170489	chr18:74170210-74170687	-0.2	0.78	0.48	-0.3	HYPOMETHYLATION
NFATC1	chr18	75385424	75386008	chr18:75385279-75386532	0.23	0.14	0.84	0.7	HYPERMETHYLATION
CTDP1	chr18	75596358	75596579	chr18:75596009-75596899	0.07	0.97	0.96	-0.01	NOT CONFIRMED
chr18 group00430	chr18	75653272	75653621	:-	0.52	0.24	0.62	0.39	HYPERMETHYLATION
KCNG2	chr18	75760343	75760820	chr18:75759900-75760988	0.01	0.84	0.75	-0.09	NOT CONFIRMED

GENE NAME	CHROM	START	END	CpG ISLAND	MEAN LOG RATIO MICRO-ARRAY	MEAN MATERNAL METHY-LATION EPITYPER	MEAN PLACENTA METHY-LATION EPITYPER	METHY-LATION DIFFERENCE PLACENTA-MATERNAL	RELATIVE METHYLATION PLACENTA TO MATERNAL
OLIG2	chr21	33317673	33321183	chr21:33316998-33322115	0.66	0.11	0.2	0.09	HYPERMETHYLATION
OLIG2	chr21	33327593	33328334	chr21:33327447-33328408	-0.75	0.77	0.28	-0.49	HYPOMETHYLATION
RUNX1	chr21	35180938	35185436	chr21:35180822-35181342; chr21:35182320-35185557	-0.68	0.14	0.07	-0.07	HYPOMETHYLATION
SIM2	chr21	36994965	36995298	chr21:36990063-36995761	0.83	0.08	0.26	0.18	HYPERMETHYLATION
SIM2	chr21	36999025	36999410	chr21:36998632-36999555	0.87	0.06	0.24	0.18	HYPERMETHYLATION
DSCR6	chr21	37300407	37300512	chr21:37299807-37301307	0.22	0.04	0.14	0.11	HYPERMETHYLATION
DSCAM	chr21	41135559	41135706	chr21:41135380-41135816	1.03	0.06	0.29	0.23	HYPERMETHYLATION
chr21 group00165	chr21	43643421	43643786	chr21:43643322-43643874	1.14	0.16	0.81	0.65	HYPERMETHYLATION
AIRE	chr21	44529935	44530388	chr21:44529856-44530472	-0.55	0.62	0.27	-0.35	HYPOMETHYLATION
SUMO3	chr21	45061293	45061853	chr21:45061154-45063386	-0.41	0.55	0.46	-0.09	HYPOMETHYLATION
C21orf70	chr21	45202815	45202972	chr21:45202706-45203073	-0.46	0.96	0.51	-0.46	HYPOMETHYLATION
C21orf123	chr21	45671984	45672098	chr21:45671933-45672201	-0.63	0.92	0.43	-0.49	HYPOMETHYLATION
COL18A1	chr21	45754383	45754487	chr21:45753653-45754639	-0.18	0.97	0.72	-0.25	HYPOMETHYLATION
PRMT2	chr21	46911967	46912385	chr21:46911628-46912534	1.08	0.04	0.25	0.21	HYPERMETHYLATION
SIX2	chr2	45081223	45082129	chr2:45081148-45082287	1.15	0.08	0.36	0.28	HYPERMETHYLATION
SIX2	chr2	45084851	45085711	chr2:45084715-45084986; chr2:45085285-45086054	1.21	0.07	0.35	0.28	HYPERMETHYLATION

GENE NAME	CHROM	START	END	CpG ISLAND	MEAN LOG RATIO MICRO-ARRAY	MEAN MATERNAL METHY-LATION EPITYPER	MEAN PLACENTA METHY-LATION EPITYPER	METHY-LATION DIFFERENCE PLACENTA-MATERNAL	RELATIVE METHYLATION PLACENTA TO MATERNAL
SOX14	chr3	138971870	138972322	chr3:138971738-138972096; chr3:138972281-138973691	1.35	0.08	0.33	0.25	HYPERMETHYLATION
TLX3	chr5	170674439	170676431	chr5:170674208-170675356; chr5:170675783-170676712	0.91	0.11	0.35	0.24	HYPERMETHYLATION
FOXP4	chr6	41623666	41624114	chr6:41621630-41624167	1.1	0.07	0.27	0.2	HYPERMETHYLATION
FOXP4	chr6	41636384	41636779	chr6:41636244-41636878	1.32	0.04	0.33	0.29	HYPERMETHYLATION
chr7 group00267	chr7	12576755	12577246	chr7:12576690-12577359	0.94	0.08	0.26	0.17	HYPERMETHYLATION
NPY	chr7	24290224	24291508	chr7:24290083-24291605	0.93	0.09	0.3	0.21	HYPERMETHYLATION
SHH	chr7	155291537	155292091	chr7:155288453-155292175	0.98	0.19	0.52	0.33	HYPERMETHYLATION
OSR2	chr8	100029764	100030536	chr8:100029673-100030614	1.21	0.08	0.43	0.35	HYPERMETHYLATION
GLIS3	chr9	4288283	4289645	chr9:4287817-4290182	1.24	0.06	0.24	0.18	HYPERMETHYLATION
PRMT8	chr12	3472714	3473190	chr12:3470227-3473269	0.86	0.07	0.23	0.16	HYPERMETHYLATION
TBX3	chr12	113609153	113609453	chr12:113609112-113609535	1.45	0.09	0.56	0.48	HYPERMETHYLATION
chr12 group00801	chr12	118516189	118517435	chr12:118515877-118517595	1.1	0.06	0.25	0.19	HYPERMETHYLATION
PAX9	chr14	36201402	36202386	chr14:36200932-36202536	0.89	0.11	0.32	0.21	HYPERMETHYLATION
SIX1	chr14	60178801	60179346	chr14:60178707-60179539	0.95	0.1	0.33	0.22	HYPERMETHYLATION
ISL2	chr15	74420013	74421546	chr15:74419317-74422570	1.08	0.08	0.27	0.19	HYPERMETHYLATION
DLX4	chr17	45397228	45397930	chr17:45396281-45398063	1.25	0.1	0.32	0.22	HYPERMETHYLATION
CBX4	chr17	75428613	75431793	chr17:75427586-	1	0.07	0.27	0.21	HYPERMETHYLATION

GENE NAME	CHROM	START	END	CpG ISLAND	MEAN LOG RATIO MICRO-ARRAY	MEAN MATERNAL METHY-LATION EPITYPER	MEAN PLACENTA METHY-LATION EPITYPER	METHY-LATION DIFFERENCE PLACENTA-MATERNAL	RELATIVE METHYLATION PLACENTA TO MATERNAL
				75433676					
EDG6	chr19	3129836	3130874	chr19:3129741-3130986	1.35	0.04	0.87	0.83	HYPERMETHYLATION
PRRT3	chr3	9963364	9964023	chr3:9962895-9964619	-0.85	0.9	0.09	-0.81	HYPOMETHYLATION
MGC29506	chr5	138757911	138758724	chr5:138755609-138758810	-0.63	0.93	0.17	-0.76	HYPOMETHYLATION
TEAD3	chr6	35561812	35562252	chr6:35561754-35562413	-1.17	0.92	0.13	-0.8	HYPOMETHYLATION
chr12 group00022	chr12	1642456	1642708	chr12:1642195-1642774	-1.33	0.66	0.09	-0.57	HYPOMETHYLATION
CENTG1	chr12	56406249	56407788	chr12:56406176-56407818	-1.07	0.95	0.19	-0.77	HYPOMETHYLATION
CENTG1	chr12	56416146	56418794	chr12:56416095-56416628; chr12:56418745-56419001	-0.94	0.85	0.16	-0.69	HYPOMETHYLATION

Information based on the March 2006 human reference sequence (NCBI Build 36.1), which was produced by the International Human Genome Sequencing Consortium.

TABLE 2

GENE NAME	CHROM	START	END	SNPs
chr13 group00016	chr13	19773745	19774050	rs7996310; rs12870878
chr13 group00005	chr13	19290394	19290768	rs11304938
CENPJ	chr13	24404023	24404359	rs7326661
ATP8A2	chr13	25484475	25484614	rs61947088
PDX1	chr13	27400459	27401165	rs58173592; rs55836809; rs61944011
RB1	chr13	47790983	47791646	rs2804094; rs4151432; rs4151433; rs4151434; rs4151435
PCDH17	chr13	57104856	57106841	rs35287822; rs34642962; rs41292834; rs45500496; rs45571031; rs41292836; rs28374395; rs41292838
KLHL1	chr13	69579933	69580146	rs3751429
POU4F1	chr13	78079515	78081073	rs11620410; rs35794447; rs2765065
GPC6	chr13	92677402	92678666	rs35689696; rs11839555; rs55695812; rs35259892
SOX21	chr13	94152286	94153047	rs41277652; rs41277654; rs35276096; rs5805873; rs35109406
ZIC2	chr13	99439660	99440858	rs9585309; rs35501321; rs9585310; rs7991728; rs1368511
IRS2	chr13	109232856	109235065	rs61747993; rs1805097; rs9583424; rs35927012; rs1056077; rs1056078; rs34889228; rs1056080; rs1056081; rs12853546; rs4773092; rs35223808; rs35894564; rs3742210; rs34412495; rs61962699; rs45545638; rs61743905
chr13 group00395	chr13	111808255	111808962	rs930346
MCF2L	chr13	112724910	112725742	rs35661110; rs2993304; rs1320519; rs7320418; rs58416100
F7	chr13	112799123	112799379	rs2480951; rs2476320
CIDEA	chr18	12244327	12244696	rs60132277
chr18 group00091	chr18	12901467	12901643	rs34568924; rs8094284; rs8094285
C18orf1	chr18	13377536	13377654	rs9957861
KLHL14	chr18	28603978	28605183	rs61737323; rs61737324; rs12960414
CD33L3	chr18	41671477	41673011	rs62095363; rs2919643
ONECUT2	chr18	53254808	53259810	rs35685953; rs61735644; rs8084084; rs35937482; rs35427632; rs7232930; rs3786486; rs34286480; rs3786485; rs28655657; rs4940717; rs4940719; rs3786484; rs34040569;

GENE NAME	CHROM	START	END	SNPs
				rs35542747; rs33946478; rs35848049; rs7231349; rs7231354; rs34481218; rs12962172; rs3911641
RAX	chr18	55086286	55086436	rs58797899; rs45501496
chr18 group00277	chr18	57151972	57152311	rs17062547
TNFRSF11A	chr18	58203013	58203282	rs35114461
NETO1	chr18	68685099	68687060	rs4433898; rs34497518; rs35135773; rs6566677; rs57425572; rs36026929; rs34666288; rs10627137; rs35943684; rs9964226; rs4892054; rs9964397; rs4606820; rs12966677; rs8095606
chr18 group00304	chr18	70133945	70134397	rs8086706; rs8086587; rs8090367; rs999332; rs17806420; rs58811193
TSHZ1	chr18	71128742	71128974	rs61732783; rs3744910; rs1802180
chr18 group00342	chr18	74170347	74170489	rs7226678
NFATC1	chr18	75385424	75386008	rs28446281; rs56384153; rs4531815; rs3894049
chr18 group00430	chr18	75653272	75653621	rs34967079; rs35465647
KCNG2	chr18	75760343	75760820	rs3744887; rs3744886
OLIG2	chr21	33317673	33321183	rs2236618; rs11908971; rs9975039; rs6517135; rs2009130; rs1005573; rs1122807; rs10653491; rs10653077; rs35086972; rs28588289; rs7509766; rs62216114; rs35561747; rs7509885; rs11547332
OLIG2	chr21	33327593	33328334	rs7276788; rs7275842; rs7275962; rs7276232; rs16990069; rs13051692; rs56231743; rs35931056
RUNX1	chr21	35180938	35185436	rs2843956; rs55941652; rs56020428; rs56251824; rs13051109; rs13051111; rs3833348; rs7510136; rs743289; rs5843690; rs33915227; rs11402829; rs2843723; rs8128138; rs8131386; rs2843957; rs57537540; rs13048584; rs7281361; rs2843965; rs2843958
SIM2	chr21	36994965	36995298	rs2252821
SIM2	chr21	36999025	36999410	rs58347144; rs737380
DSCAM	chr21	41135559	41135706	rs35298822
AIRE	chr21	44529935	44530388	rs35110251; rs751032; rs9978641
SUMO3	chr21	45061293	45061853	rs9979741; rs235337; rs7282882
C21orf70	chr21	45202815	45202972	rs61103857; rs9979028; rs881318; rs881317
COL18A1	chr21	45754383	45754487	rs35102708; rs9980939
PRMT2	chr21	46911967	46912385	rs35481242; rs61743122; rs8131044; rs2839379
SIX2	chr2	45081223	45082129	rs62130902

GENE NAME	CHROM	START	END	SNPs
SIX2	chr2	45084851	45085711	rs35417092; rs57340219
SOX14	chr3	138971870	138972322	rs57343003
TLX3	chr5	170674439	170676431	rs11134682; rs35704956; rs2964533; rs35601828
FOXP4	chr6	41623666	41624114	rs12203107; rs1325690
FOXP4	chr6	41636384	41636779	rs56835416
chr7 group00267	chr7	12576755	12577246	rs56752985; rs17149965; rs6948573; rs2240572
NPY	chr7	24290224	24291508	rs2390965; rs2390966; rs2390967; rs2390968; rs3025123; rs16146; rs16145; rs16144; rs13235842; rs13235935; rs13235938; rs13235940; rs13235944; rs36083509; rs3025122; rs16143; rs16478; rs16142; rs16141; rs16140; rs16139; rs2229966; rs1042552; rs5571; rs5572
SHH	chr7	155291537	155292091	rs9333622; rs1233554; rs9333620; rs1233555
GLIS3	chr9	4288283	4289645	rs56728573; rs12340657; rs12350099; rs35338539; rs10974444; rs7852293
PRMT8	chr12	3472714	3473190	rs12172776
TBX3	chr12	113609153	113609453	rs60114979
chr12 group00801	chr12	118516189	118517435	rs966246; rs17407022; rs970095; rs2711748
PAX9	chr14	36201402	36202386	rs17104893; rs12883298; rs17104895; rs35510737; rs12882923; rs12883049; rs28933970; rs28933972; rs28933971; rs28933373; rs61734510
SIX1	chr14	60178801	60179346	rs761555
ISL2	chr15	74420013	74421546	rs34173230; rs11854453
DLX4	chr17	45397228	45397930	rs62059964; rs57481357; rs56888011; rs17638215; rs59056690; rs34601685; rs17551082
CBX4	chr17	75428613	75431793	rs1285243; rs35035500; rs12949177; rs3764374; rs62075212; rs62075213; rs3764373; rs3764372; rs55973291
EDG6	chr19	3129836	3130874	rs34728133; rs34573539; rs3826936; rs34914134; rs61731111; rs34205484
MGC29506	chr5	138757911	138758724	rs11748963; rs7447765; rs35262202
CENTG1	chr12	56406249	56407788	rs61935742; rs12318065; rs238519; rs238520; rs238521; rs808930; rs2640595; rs2640596; rs2640597; rs2640598; rs34772922
CENTG1	chr12	56416146	56418794	rs11830475; rs34482618; rs2650057; rs2518686; rs12829991

TABLE 3

GENE NAME	RELATIVE METHYLATION PLACENTA TO MATERNAL	PRC2 TARGET
CRYL1	HYPOMETHYLATION	TRUE
IL17D	HYPOMETHYLATION	TRUE
GSH1	HYPERMETHYLATION	TRUE
MAB21L1	HYPERMETHYLATION	TRUE
PCDH17	HYPERMETHYLATION	TRUE
KLHL1	HYPERMETHYLATION	TRUE
POU4F1	HYPERMETHYLATION	TRUE
SOX21	HYPERMETHYLATION	TRUE
ZIC2	HYPERMETHYLATION	TRUE
CIDEA	HYPERMETHYLATION	TRUE
KLHL14	HYPERMETHYLATION	TRUE
ONECUT2	HYPERMETHYLATION	TRUE
RAX	HYPERMETHYLATION	TRUE
TNFRSF11A	HYPOMETHYLATION	TRUE
OLIG2	HYPERMETHYLATION	TRUE
OLIG2	HYPOMETHYLATION	TRUE
SIM2	HYPERMETHYLATION	TRUE
SIM2	HYPERMETHYLATION	TRUE
SIX2	HYPERMETHYLATION	TRUE
SIX2	HYPERMETHYLATION	TRUE
SOX14	HYPERMETHYLATION	TRUE
TLX3	HYPERMETHYLATION	TRUE
SHH	HYPERMETHYLATION	TRUE
OSR2	HYPERMETHYLATION	TRUE
TBX3	HYPERMETHYLATION	TRUE
PAX9	HYPERMETHYLATION	TRUE
SIX1	HYPERMETHYLATION	TRUE
ISL2	HYPERMETHYLATION	TRUE
DLX4	HYPERMETHYLATION	TRUE
CBX4	HYPERMETHYLATION	TRUE
CENTG1	HYPOMETHYLATION	TRUE
CENTG1	HYPOMETHYLATION	TRUE

TABLE 4

SEQ ID NO	GENE NAME	SEQUENCE
1	chr13 group-00016	CAGCAGGCGCGCTCCCGGCGAATCTGCCTGAATCGCCGTGAATGCGGTGGGGTGCAGGGCAGGGGCTGGTTTTCTCAGCCGGTCTTGGCTTTTCTCTTTCTCTCCTGCTCCACCAGCAGCCCCTCCGCGGGTCCCATGGGCTCCGCGCTCAGAACAGCCCGGAACCAGGCGCCGCTCGCCGCTCGCTGGGGGCCACCCGCCTCTCCCCGGAACAGCCTCCCGCGGGCCTCTTGGCCTCGCACTGGCGCCCTCACCCACACATCGTCCCTTTATCCGCTCAGACGCTGCAAAGGGCCTTCTGTCTC
2	CENPJ	GCTTTGGATTTATCCTCATTGGCTAAATCCCTCCTGAAACATGAAACTGAAACAAAGCCCTGAACCCCCTCAGGCTGAAAAGACAAACCCCGCCTGAG

SEQ ID NO	GENE NAME	SEQUENCE
		GCCGGGTCCCGCTCCCCACCTGGAGGGACCCAATTCTGGGCGCCTTCTGGCGACGGTCCCTGCTAGGGACGCTGCGCTCTCCGAGTGCGAGTTTTCGCCAAACTGATAAAGCACGCAGAACCGCAATCCCCAAACTAACACTGAACCCGGACCCGCGATCCCCAAACTGACAAGGGACCCGGAACAGCGACCCCCAAACCGACACGGGACTCGGGAACCGCTATCTCCAAAGGGCAGC
3	ATP8A2	TTTCCACAACAGGGAGCCAGCATTGAGGCGCCCAGATGGCATCTGCTGGAAATCACGGGCCGCTGGTGAAGCACCACGCCTTACCCGACGTGGGGAGGTGATCCCCCACCTCATCCCACCCCCTTCTGTCTGTCTCCTT
4	GSH1	GCTGGACAAGGAGCGCTCACTGTAGCTCTGCTGTGGATTGTGTTGGGGCGAAGAGATGGGTAAGAGGTCAAAGTCGTAGGATTCTGGCGACCGCCTACCAAGGGATTGGGTCCACAGCACAGAGGTCTGATCGCTTCCTTCTCTGCTCTGCCACCTCCAGACAGCAGCTCTAACCAGCTGCCCAGCAGCAAGAGGATGCGCACGGCTTTCACCAGCACGCAGCTGCTAGAGCTGGAGCGCGAGTTCGCTTCTAATATGTACCTGTCCCGCCTACGTCGCATCGAGATCGCGA
5	PDX1	TGCCTGACACTGACCCCAGGCGCAGCCAGGAGGGGCTTTGTGCGGGAGAGGGAGGGGGACCCCAGCTTGCCTGGGGTCCACGGGACTCTCTTCTTCCTAGTTCACTTTCTTGCTAAGGCGAAGGTCCTGAGGCAGGACGAGGGCTGAACTGCGCTGCAATCGTCCCCACCTCCAGCGAAACCCAGTTGAC
6	PDX1	TCGGCGGAGAGACCTCGAGGAGAGTATGGGGAAAGGAATGAATGCTGCGGAGCGCCCCTCTGGGCTCCACCCAAGCCTCGGAGGCGGGACGGTGGGCTCCGTCCCGACCCCTTAGGCAGCTGGACCGATACCTCCTGGATCAGACCCCACAGGAAGACTCGCGTGGGGCCCGATATGTGTACTTCAAACTCTGAGCGGCCACCCTCAGCCAACTGGCCAGTGGATGCGAATCGTGGGCCCTGAGGGGCGAGGGCGCTCGGAACTGCATGCCTGTGCACGGTGCCGGGCTCTCCAGAGTGAGGGGGCCGTAAGGAGATCTCCAAGGAAGCCGAAAAAAGCAGCCAGTTGGGCTTCGGGAAAGACTTTTCTGCAAAGGAAGTGATCTGGTCCCAGAACTCCAGGGTTGACCCCAGTACCTGACTTCTCCGGGAGCTGTCAGCTCTCCTCTGTTCTTCGGGCTTGGCGCGCTCCTTTCATAATGGACAGACACCAGTGGCCTTCAAAAGGTCTGGGGTGGGGGAACGGAGGAAGTGGCCTTGGGTGCAGAGGAAGAGCAGAGCTCCTGCCAAAGCTGAACGCAGTTAGCCCTACCCAAGTGCGCGCTGGCTCGGCATATGCGCTCCAGAGCCGGCAGGACAGCCCGGCCCTGCTCACCCCGAGGAGAAATCCAACAGCGCAGCCTCCTGCACCTCCTTGCCCCAGAGAC
7	MAB21L1	AGATCCCGGTGCATTTAAAGGCCGGCGTGATCTGCACCACGTACCTATCTCGGATTCTCAGTTTCACTTCGCTGGTGTCTGCCACCATCTTTACCACATCCCGGTAGCTACATTTGTCTACCGCTTGAGCCACCAGCGTCTGAAACCTGGACCGGATTTTGCGCGCCGAGAGGTAGCCGGAGGCGGTAATGAATTCCACCCAGAGGGACATGCTCCTCTTGCGCCCGTCGCTCAACTTCAGCACCGCGCAGCCGGGCAGTGAGCCATCGTCCACGAAGTTGAACACCCCCATTTGGTTGAGATAAAGCACCACTTCAAATTCGGT
8	RB1	ACTATGCCTTGAGGGTCAAAACGTCTGGATTTCCTGATCGATGCTGTCGTCGCTGTCCACGGAGCTACTGTCGCCGTCAGAGCGGGAAGGCACGTTCAGGGAGTAGAAGCGTGGGCTTGCAGAAAGGGACCTGTTGCTGCCTTACATGGGGGCCGGCAGGGTAGTCTTGGAAATGCCCAAGATTGCTTCCGCGCGCGTCAGTTCAGCGGACGTGTCTGCCTGGCACGAGGACCGTTCTACAAACTCGTTCCTGGAAGCCGGGCTCGCTGGAGGCGGAGCTTTGGTTTCCTTCGGGAGCTTGTGGGGAATGGTCAGCGTCTAGGCACCCCGGGCAAGGGTCTGTGGCCTTGGTGGCCACTGGCTTCCTCTAGCTGGGTGTTTTCCTGTGGGTCTCGCGCAAGGCACTTTTTTGTGGCGCTGCTTGTGCTGTGTGCGGGGTCAGGCGTCCTCTCTCCTCCCGGCGCTGGCCCTCTGGGGCAGGTCCCCGTTGGCCTCCTTGCGTGTTTGCCGCAGCTAGTACACCTGGATGGCCTCCTCAGTGCCGTCGTTGCTGCTGGAGTCTGACGCCTCGGGCGCCTGCGCCGCACTTGTGACTTGCTTTCCCCTTCTCAGGGCGCCAGCGCTCCTCTTGACCCCGCTTTTATTCTGTGGTGCTTCTGAAG
9	PCDH17	GCAAGTCGGGTAGCTACCGGGTGCTGGAGAACTCCGCACCGCACCTGCTGGACGTGGACGCAGACAGCGGGCTCCTCTACACCAAGCAGCGCATCGACCGCGAGTCCCTGTGCCGCCACAATGCCAAGTGCCAGCTGTCCCTCGAGGTGTTCGCCAACGACAAGGAGATCTGCATGATCAAGGTAGAGATCCAGGACATCAACGACAACGCGCCCTCCTTCTCCTCGGACCAGATCGAAATGGACATCTCGGAGAACGCTGCTCCGGGCACCCGCTTCCCCCTCACCAGCGCAC

SEQ ID NO	GENE NAME	SEQUENCE
		ATGACCCCGACGCCGGCGAGAATGGGCTCCGCACCTACCTGCTCACGCGCGACGATCACGGCCTCTTTGGACTGGACGTTAAGTCCCGCGGCGACGGC ACCAAGTTCCCAGAACTGGTCATCCAGAAGGCTCTGGACCGCGAGCAACAGAATCACCATACGCTCGTGCTGACTGCCCTGGACGGTGGCGAGCCTCC ACGTTCCGCCACCGTACAGATCAACGTGAAGGTGATTGACTCCAACGACAACAGCCCGGTCTTCGAGGCGCCATCCTACTTGGTGGAACTGCCCGAGA ACGCTCCGCTGGGTACAGTGGTCATCGATCTGAACGCCACCGACGCCGATGAAGGTCCCAATGGTGAAGTGCTCTACTCTTTCAGCAGCTACGTGCCT GACCGCGTGCGGGAGCTCTTCTCCATCGACCCCAAGACCGGCCTAATCCGTGTGAAGGGCAATCTGGACTATGAGGAAAACGGGATGCTGGAGATTGA CGTGCAGGCCCGAGACCTGGGGCCTAACCCTATCCCAGCCCACTGCAAAGTCACGGTCAAGCTCATCGACCGCAACGACAATGCGCCGTCCATCGGTT TCGTCTCCGTGCGCCAGGGGGCGCTGAGCGAGGCCGCCCCTCCCGGCACCGTCATCGCCCTGGTGCGGGTCACTGACCGGGACTCTGGCAAGAACGGA CAGCTGCAGTGTCGGGTCCTAGGCGGAGGAGGGACGGGCGGCGGCGGGGGCCTGGGCGGGCCCGGGGGTTCCGTCCCCTTCAAGCTTGAGGAGAACTA CGACAACTTCTACACCCTCGTGACTGACCGCCCCCTGGACCGCGAGACACAAGACGAGTACAACGTGACCATCGTCGCCCGGGACCGGCCCTCTCCTC CCCTCAACTCCACCAAGTCGTTCGCGATCAAGATTCTAGACGAGAACGACAACCCGCCTCGGTTCACCAAAGGGCTCTACGTGCTTCAGGTGCACGAG AACAACATCCCGGGAGAGTACCTGGGCTCTGTGCTCGCCCAGGATCCCGACCTGGGCCAGAACGGCACCGTATCCTACTCTATCCTGCCCTCGCACAT CGGCGACGTGTCTATCTACACCTATGTGTCTGTGAATCCCACGAACGGGGCCATCTACGCCCTGCGCTCCTTTAACTTCGAGCAGACCAAGGCTTTTG AGTTCAAGGTGCTTGCTAAGGACTCGGGGGCGCCCGCGCACTTGGAGAGCAACGCCACGGTGAGGGTGACAGTGCTAGACGTGAATGACAACGCGCCA GTGATCGTGCTCCCCACGCTGCAGAACGACACCGCGGAGCTGCAGGTGCCGCGCAACGCTGGCCTGGGCTATCTGGTGAGCACTGTGCGCGCCCTAGA CAGCGACTTCGGCGAGAGCGGGCGTCTCACCTACGAGATCGTGGACGGCAACGACGACCACCTGTTTGAGATCGACCCGTCCAGCGGCGAGATCCGCA CGCTCCACCCTTTCTCGGAGGACGTGACGCCCGTGCTGCAGCTGGTGGTGAAGGTGACCGACCACGGCAAGCCTACCCTGTCCGCAGTGGCCAAGCTC ATCATCCGCTCGGTGAGCGGATCCCTTCCCGAGGGGGTACCACGGGTGAATGGCGAGCAGCACCACTGGGACATGTCGCTGCCGCTCATCGTGACTCT GAGCACTATCTCCATCATCCTCCTA
10	KLHL1	ATGCGCCCTCTGCACCCCTAGAGCCAGAAGACGCTAGGTGGGCTGCGCGCTCTGCCAGGCGAAGGCTGGAGCGCAGACGGCAAAGCCGCGCGTTTCAG CCGTGGTCGGGTCCGCAGGACCTGGGCGTGGGACACCACCAGGCAGGAGCAGAGGCAGGACTGGGACGCCAAAAGCTGAGAATCCTCGATGCCCGCG CGAGAGCCCCGTGTTAT
11	POU4F1	TTCTGGAAACCGGGCCCCACTTGCAGGCCCGGCCACCTTGGGTTCTGGTGGCCGAAGCCGGAGCTGTGTTTCTCGCAGACTCGGGGAGCTACATTGTG CGTAGGCAATTGTTTAGTTTGAAAGGAGGCACATTTCACCACGCAGCCAGCGCCCTGCATGCAGGAGAAGCCCCCAGGGCCCAGGGTCGGCTGGCTTT AGAGGCCACTTAGGTTGTTTTAAGCACATGTGAAAGGGCAGACAGCAGGGGAGCAGGATATGGGTAAGATCTTCGGGTCTCAGAACAGGGGCTGCCCT TGGGCTGTCCCGGCGCCCTGGGCTCTGACACTGAAGGGTGGAATGGAGGAAGGAATGGAGAAAGGACGGTGGAACTTTCGCTTCCCCTCTGGGCCGCC TTCCCAGGGTCATGCCTGAGCTGCTTTGATCCCAGTGTCGCGCATCTTGGTCCGCTACCTCCCAGGCGATAGCTACTGGGCTCCTCGCTGGCCTCACT GGGGGCCATCCCGGGCAGTGGCCTGCCCTCCGAGGCCCGCGGGACCCAGCCCAGAGCTGAGGTTGGAGTTCTCCGGGCCACGTTCCGGGTCGCTTAGG CTCGGAGATTTCCCGGAGACCGTCGTCCTCCCTTTCTGCTTGGCACTGCGGAGCTCCCTCGGCCTCTCTCCTCCTCTGGTCCCTAAGGCCCGGAGTGG TTGGCGGTACTGGGGCCCGTCGTCATCTCTGCTTCTAAGGCATTCAGACTGGGCTCCAGCTGGGACCGGCAGAGGAGGTTCTCAAGGAAACTGGTGGG AAATATAGTTTTCTTTCGTCTGGTCGTTTAATTTAAATGCAACTTCCCTTGGGGACATTTTCCTGGACGTTAACCAGACCACCTTGAGATGTCGTTGA TGACCTAGAGACCCAGATGATGCGTCCCAGGAAAGTTCACTGCTGACTATTGTCACTCTTGGCGTTATATCTATAGATATAGACCTATGTACATATCT CCACCCTGATCTCTCCGTGGACATGAAACCCACCTACCTTGTGAAAGCCCTACGGGTGACACATGACTACTACGTCTCTGTCCCAACAGGGGCTGGGC CTCCCCTGCCTAATAGTTGCCAGGAGTTTCGCAGCCCAAGTGAATAATGTCTTATGGCTGAACGTGGCCAAGGACTCCTGTGATTTAGGTCCCAGGAG

SEQ ID NO	GENE NAME	SEQUENCE
		GAGCAGAGACGTCCCCGCCCCGCCTGGGCCCTGCCGCATTCAAAGCTGGAAGAAGGCGCTGATCAGAGAAGGGGCTTCCAGGTCCTGGGTTAGAACAA CAACAAACAAACGAAACTCCACAACAGACACGCCTGCCCATGACCCCACGCAAGGACATAGGAAGTTCTGTCGCCTTCCTGCTCCGCGGATAGCCGCC TGCCGTCTGCTGCCACCAGAACGCACGGACGCTCGGGGTGGAGGTAGTCAATGGGCAGCAGGGGACCCCCAGCCCCCACAAGCGCGGCTCCGAGGACC TGGAAGCGGGTGCCTGTCGCTCTCCGCAGGCTCCGCTCTGCCTCCAGGAGCAAGATCCCCAAAAGGGTCTGGAAGCTGTGGAGAAAAC
12	GPC6	TTTTTTAAACACTTCTTTTCCTTCTCTTCCTCGTTTTGATTGCACCGTTTCCATCTGGGGGCTAGAGGAGCAAGGCAGCAGCCTTCCCAGCCAGCCCT TGTTCGCTTCCCATCCTCCATCTGCCTTATAAAACTTTGCTCAGCCCAGTCCAGACGGCTGCCGCTCCTCCCCTCCGCTGCCACAACCGCGTCACC CTGGGCAGCGGCGAGGAGCGCGCCGCTGCCTCTGGCGGGCTTTCGGCTTGAGGGGCAAGGTGAAGAGCGCACCGGCCGTGGGGTTTACCGAGCTGGAT TTGTATGTTGCACCATGCCTTCTTGGATCGGGGCTGTGATTCTTCCCCTCTTGGGGCTGCTGCTCTCCCTCCCCGCCGGGGCGGATGTGAAGGCTCGG AGCTGCGGAGAGGTCCGCCAGGCGTACGGTGCCAAGGGATTCAGCCTGGCGGACATCCCCTACCAGGAGATCGCAGGTAAGCGCGGGCGCGCTGCAGG GGCAGGCTGCAGCCCTCGGCTGCCGCACGTCCCACTGGCCGCCCGGCGTCCCCTTCCTTCCCCCTGTTGCTGAGTTGGTGCTCACTTTCTGCCACCGC TATGGGACTCCGCGTCTCCGTGTTGGGCGGCGGATGCTCCTGCGGCTTCTTCGGCGGGGGAAGGTGTGCGTCTCCGCCGCCTCATTGTGTGCACACGC GGGAGCACCCTGGCTCCCGCCTCCCGCTGCTCTCGCGCCCTTCTACCCCTTAGTTGATGGCTCAGGCCCGGCTGGCCAGGGAGCCCGGGTCACTCCGG GGCGGCTGCAAGCCGCAGACGGAGAGCCGAGCCGGGCGCTCACTCCGCGTTCTGGTTCGGGCAAACTTGGAAGAACTGCGACCGCAGTTTGCCCAGCG CCACAGTCTGAGTGGCGCCTTCTCCACTCCCGCCCTTGCGCCGGCAGGGGCGGTGGAGAGACGCGGAGGGCTCCCCCAGCCCCTCTCTCCCCTATCCG TCCTTCGGGCGACAGAGCGCCCGGCGCTCGGGCCGGGGGCGGGCAAGGCTGGGAGGGACCCTCGCCGGGGACCTGGCCTCTGGACGCCGGCGTTTCAA GGCTGGTTTGGGGACTTCACGGGCTGCCTGTTTCAGATGTGGGGCGGGCTTTCCCGTTAGGGTTCCTCAGTGCTTCCCCAGTTGCTGTTGGCCACTCA GGGCCCGGGGACACCCTGCCACCCGGTCTGGAGCCGGCCTCGTCTGCCAGCGAACAGCCAACTTTAGCGGGTGGCTCAGCTGGGGATT
13	SOX21	CACTCAGTGTGTGCATATGAGAGCGGAGAGACAGCGACCTGGAGGCCATGGGTGGGGGCGGGTGGTGAAGCTGCCGAAGCCTACACATACACTTAGCT TTGACACTTCTCGTAGGTTCCAAAGACGAAGACACGGTGGCTTCAGGGAGACAAGTCGCAAGGGCGACTTTTCCAAGCGGGAGATGGTGAAGTCTTTG GACGTGTAGTGGGTAGGTGATGATCCCCGCAGCCGCCTGTAGGCCCGCAGACTTCAGAAAACAAGGGCCTTCTGTGAGCGCTGTGTCCTCCCCGGAAT CCGCGGCTTAACACATTCTTTCCAGCTGCGGGGCCAGGATCTCCACCCCGCGCATCCGTGGACACACTTAGGGTCGCCTTTGTTTTGCGCAGTGATTC AAGTTGGGTAACCCTTGCTCAACACTTGGGAAATGGGGAGAATCTCCCCCACCCGCAACCTCCCGCACCCCAGGTTCCCAAAATCTGAATCTGTATCC TAGAGTGGAGGCAGCGTCTAGAAAGCAAAGAAACGGTGTCCAAAGACCCCGGAGAGTTGAGTGAGCGCAGATCCGTGACGCCTGCGGTACGCTAGGGC ATCCAGGCTAGGGTGTGTGTGTGCGGGTCGGGGGGCGCACAGAGACCGCGCTGGTTTAGGTGGACCCGCAGTCCCGCCCGCATCTGGAACGAGCTGCT TCGCAGTTCCGGCTCCCGGCGCCCCAGAGAAGTTCGGGGAGCGGTGAGCCTAGCCGCCGCGCGCTCATGTTTATT
14	ZIC2	AGTCACTCCAGGATCAGAGGCCGCGTCGGTTCTGCTTGGGGCATGGGCAGAGGGAGGCTGCTGGGGCCAAGCCCCGGCTGGACGCGAGGGAAGAAACT CGTCCCAGGACCCGCACGCCCATACCTGGCTGTCCCAGAGCTCTTCCCTAGGCCGGCACCTTCGCTCTTCCTCTTCCCCACCCCCTAGCCCTTTTGTC TCTTTTTCAGACGGATGTTTTCAGTCTCAAGTGGTTTTATTTTCCGCACAAAACCCTGAGATCAAGGGCAGATCACAGACTGTACCGGAGGCTCGGGT TTCCCTGGACTCTGTGCTGTTCTGCGTCCCAGGGTTGGCTAGGAAGGAAGGCCTGGGCCGGCGAGGTGACGGGTCTCCCGCCCAGGTCGGCAGGACGG GGGGAGGTGTGTCCCGGTAGGTCCCTGGTGAGCTCACCCGTGGCATCGGGGACCCGCGGGAACCCACCGGGCGCCCACTAGAGACTCGGGTCCTACCC TCCCCCACACTACTCCACCGAAATGATCGGAAGGGCGCGCTAGGCCTGCTTCCAAGGGCTCAGTGATAAAGGCCTCAAAATCACACTCCATCAAGACT TGGTTGAAGCTTTGGGTAGGTTTGTTGTTGTTGTTGTTGTTTGTTTGTTTGTTTTAGCAGACACGTCCTGGAAAGAGGTCCTCAGAACCCAAAGG TTCAATAATGATTTGTGGATGGATTGATTATAGTCTGATATCGCTCTGGTTCCACAGAAACCCGGAGCTCCTTGGCCCACTGTTACCCCAGCAGACCT

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15	chr13 group-00385	TTTCCTGCCCCTCTTTTCAATGCCGCTAACGACGCCACCGTTATTCTTACCCCCGTCCCGGACGCCGATCACATTCCTGCCCAGTTGCCGCTGCTGCCCGAAC TGACTTGTTTTCTAACGACCCTCGTGACAGCCAGAGAATGTCCGTTTCTCGGAGCGCAGCACAGCCTGTCCCATCGAGAAGCCTCGGGTGAGGGGCCC GGTGGGCGCCCGGAGGCCGCTGGAGGGCTGTGGGAGGGACGGTGGCTCCCCACTCCCGTGGCGAAGGGCAGGCAAACCAGAAGCCTCTTTTGAGAGCC GTTTGGGATTGAGACGAGTAAGCCACAGCGAGTGGTTAGAAGTAGGTTAGGAAGAAGGGGAGGTAAGAAAGCCGAGTAGGGTT
16	chr13 group-00390	GTTCGGTGGACAAGGGGGCAGCGCCCACAGCAAGCCGGAAAGAGGGAGGCGCGGGGCCGCGCTTGGGGCCTGCCGCTGCACGCCAGCCTGGGCAAAGA GCTGCCACCTTCTGCGGGCGAAGCGGGTCGGGACGCAGGACGGCAGCGGGGCTGGAGGCAGCTACGTGGGTCCACACCCCCATGCCCTGCAAGGCTCC TTGGCCCTGCTTCTCCTCTGTCTCGGCGGGAGAGGAGCAGCCTCGGTTTTACAGAATTTC
17	chr13 group-00391	TGTGCCATTTAGTGAGAGGTGTTTTGGGCAAAGAATCAATTTAACTGTGACTGACCGACGGGCTTGACTGTATTAATTCTGCTACCGAAAAAAAAAAA AAAAAAAAAGCAATGAGCCGCAAGCCTTGGACTCGCAGAGCTGCCGGTGCCCGTCCGAGAGCCCCACCAGCGCGGCTCACGCCTCAGTCTC
18	chr13 group-00395	AGAGTCCCAGTTCTGCAGGCCGCTCCAGGGCTAGGGGTAGAGATGGTGGCAGGTGGTGCGTCAACTCTCTAGGGAAGAGGAACTTGCATTACAAAGAC TTGTCTTTCTGAGCTGAAGTCAAAACGGGGGCGTCAAGCGCGCTCCGTTTGGCGGCGGTGGAGGGGCCGCGCGCCCGCGCTGTCCCAGCCGGAGCTGC CCTGGCTGGTGATTGGAGGTTTAACGTCCGGAATTCAGGCGCTTCTGCAGCTCAGATTTGCCGGCCAAGGGGCCTCAGTTGCAACTTTTCAAAATGGT GTTTCTGGAAAATAACAAATTCAGACTCAACTGGTGACAGCTTTTGGCTATAGAGAATGAAACTGCTTCCCTTTGGCGGTGGAACTCTTAAACTTCGA AGAGTGAAAGAATACAATGAAATAAAATGCCATAAGATCACTGGATTTTTCAGAAAAAGGAAGACCCCAAATTACTCCCAAAATGAGGCTTTGTAAAT TCTTGTTAAAAATCTTTAAATCTCGAATTTCCCCCTACAACATCTGATGAGTGCTTTAAGAGCAAACGAGCAAATCCCACCTCGAGAATCAACAAACC CAAGCTCTGGCCAAGGCTCTCCCCGCGTTTTCTTCTCGTGACCTGGGGAATGTCCCGCCCCATCGCTCACCTGGCTCTTGTCATCTCGCTCATCTTGA AGTGACCCGTGGACAATGCTG
19	chr13 group-00399	AGCTGCCCTCTGTGGCCATGAGCGGGTGTCCAGCCCCTTCCAAGGCTGCACCGGGGAGACGCTGGTTTTCTGCTCGCTGTGACCGAACAAAGCCCCTA AGAGTCAGTGCGCGGAACAGAAGAGCCGGACCCCGACGGGCCGAGTCCCAACGTGAGGCACCCGGCAGAGAAAACACGTTCACG
20	PROZ	CCTCGGCAGCACCGGCATGGCTGGAGGCCAGTACGGCCAGGTCTGGCGGAGGGAGCGCCGTCTGGCTTGGGTCGTCCATCCTGACAGGACGCTGCAA GGGCAGGAGCCCCGCGCCCCGTGTCCTGCGCCCCCGCTCGAGGACAAGCCCCAGCCGCCGGTCTCCGCTGGGTTCCGACAG
21	CIDEA	CTTTAAGAGGCTGTGCAGGCAGACAGACCTCCAGGCCCGCTAGGGGATCCGCGCCATGGAGGCCGCCCGGGACTATGCAGGAGCCCTCATCAGGCGAG TGCCCCGCGTCCCCCTGATTGCCGTGCGCTTCCAATCGCCTTGCGTTCGGTGGCCTCATATTCCCCTGTGCGCCTCTAGTACCGTACCCCGCTCCCTT CAGCCCCCTGCTCCCCGCATTCTCTTGCGCTCCGCGACCCCGCGCACACACCCATCCGCCCCACTGGTGCCCAAGCCGTCCAGCCGCGCCCGCGGGCA

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		GAGCCCAATCCCGTCCCGCGCCTCCTCACCCTCTTGCAGCTGGGCACAGGTACCAGGTGTGGCTCTTGCGAGGTG
22	chr18 group-00091	AGACTTGCAGAACTCGGGCCCCCTGGAGGAGACCTAACCGCCACGGTCTTGGGGAGGTTCCGGAGGGCCTCGGTTGTCTGCACTCCCAACACCAAGAAACCCCTGAGACGCGAAGCTGCCAGCGTGCTGCCCTCAGAGCAGGGCGACGCAAAGCCAGCGGACCCCGGGGTGGCGGG
23	chr18 group-00094	TGCTCGGCTGGGGGGCTCGCTCCGCACTTTCGGTGCCAGAAAATGCCCAGAGGAGCGGGGCGGCCCCAGAGCCTCCTTTCGGGGCGCGAGGCCCGGCGCGTGTGTACGGAGTCCAGTCCCCCCAGGGAGTGGGGTGCCCGCACCTTCCCCTCCGCGCTCGGAGCCAC
24	KLHL14	TCTTGCACACCTGCTTGTAGTTCTGCACCGAGATCTGGTCGTTGAGCAACTGCACGCAGAGCTTGGTGACCTGGGGATGTGCAGGATCTTGCTGACCGACAGCACCTCCTCCACCGTGTCCAGGGACAGGGTCACGTTGGCCGTGTAGAGGTACTCGAGCACCAGGCGCAGCCCGATGGACGAGCAGCCCTGCAGCACCAGGTTGTTGATGGCCCGGGGGCTGGTCAGCAGCTTGTCGTCGGGGGAGGAAGAAGGAGTCCCGGGCTCCTCCTGCGGCGGCGGCTGCTGCTGCTGTGACGGCTGCTGCTGCGGCGGCTGCTGCTGGTCCTTGGGGGCCCCCAGGCCGTCCTGGCCGCCGACCCCTCCCCCGAGAGGGGGGTGGCTGGAGAAGAGCGATCGGAAGTACTGCGAGCAGGAGGCCAGCACGGCCTTGTGGCAATGGAACTGCTGGCCCTGGGCCGTCAGGGTCACGTCGCAAAACAGCTGCTTCCTCCACAGCAGGTTGAGGCCGTGCAGCAGGTTGTCGCTGTGGCTGGGGTCGAAGGTGGAGGTCCTGTCCCCGGATCTGGACATGGCGAGCTGACTCGGTGCACCTGGCTTTAAACCCTCCTCCAACCTGGCAGACAGGGGTGGGGGATGGGAGGGAGGGGAGCAGGGTGGTGGAGCGGGTGGGGTGTGGTCGGGGTGGGGAAGGGTGTGGAGGGGAGGGGAGGGCGAAGAACAAGAATCAAGGCTCAGCTTGACTCCCTCCTGGCGCGCTCCGGACCCCGACCCTAGGAGGAAAGTCCGAAGACGCTGGATCCGTGAGCGCCACCAGAAGGGCCCTGTCTGGGGTCCCGGCGCCGGTTCTGCGCCCTGCGGCTCCTCTCGCCACCTCCCACACACTTCGTCCCTCACTTTCCTAAAACCAACCACCTCAGCTCGGCTGTTGGCAGCAACAGCAGTGGCAGCAGCGACGGCAAAGTGGCGGCTGAGGCCGAGGCACCTCGTGGGCTCGTGTCCATGCCGGGCCAGATGAAGGGAAAGGCCGGGAAGTGGGGAGCCGGGGGTGCCCTGAAAGCTCAGAGGCGACCGACGGCGAAGGTTCCAGGTCAACTTGTGCCCGAAGCTTTGCTTTTCGCAGTTGGCCCAGTTTGGGGGAGGGGGTAGGAACAGGGGCCCGACCAGCGTGCGGGGTGTGCGAATCTTAGCTCTCCAAAAGCTG
25	ST8SIA3	CCTCTGTGTTAGTGCCCTCGGGAATTTGGTTGATGGGGTGTTTG
26	ONECUT2	TGATGTCGCACCTGAACGGCCTGCACCACCCGGGCCACACTCAGTCTCACGGGCCGGTGCTGGCACCCAGTCGCGAGCGGCCACCCTCGTCCTCATCGGGCTCGCAGGTGGCCACGTCGGGCCAGCTGGAAGAAATCAACACCAAAGAGGTGGCCCAGCGCATCACAGCGGAGCTGAAGCGCTACAGTATCCCCCAGGCGATCTTTGCGCAGAGGGTGCTGTGCCGGTCTCAGGGGACTCTCTCCGACCTGCTCCGGAATCCAAAACCGTGGAGTAAACTCAAATCTGGCAGGGAGACCTTCCGCAGGATGTGGAAGTGGCTTCAGGAGCCCGAGTTCCAGCGCATGTCCGCCTTACGCCTGGCAGGTAAGGCCGGGGCTAGCCAGGGGCCAGGCTGCTGGGAAGAGGGCTCCGGGTCCGGTGCTTGTGGCCCAAGTCTGCGCGCCGAGTCACTTCTCTTGATTCTTTCCTTCTCTTTCCTATACACGTCCTCTTTCTTCTCGTTTTTATTTCTTCTTCCATTTTCTCTTTCTCTTCCGCTCTTCCCCTACTTTCCCTTCTCCCTTTTCTTTTTCTTTCTTACTCTCTCCTTGTCCCTGAGCTTTCATTGACCGACCCCCCCCATTTCATTCGCCCTCCCCTCAATGTGCCAACCTTTGCCCTATTTCCGATCTTCCCAGGTACTGGGAGGCGGGATGGGGGTGTGCGTTTTCCTCTAGGAGCCCTGTCTTTCCAAGACCCACAGAAACCAGGACCTGCCCTTATTCAAAACCCCATGCACTTCAAGTCTCTTTTAGACAACACATTTCAATTTTCCGGGCTGACTAGTCTCCCTGTGCAGAGGCAGTTGAGAGGCTTTGCTCTGCAGAGGGAAAAGAGCT

SEQ ID NO	GENE NAME	SEQUENCE
		CTCTACTCTCCCACCCACCATATAGGCAAACTTATTTGGTCATTGGCTGAAGGCACAGCCTTGCCCCCGCGGGGAACCGGCGGCCAGGATACAACAGC GCTCCTGGAGCCCATCTCTGGCCTTGGCGTTGGCGCAGGGACTTTCTGACCGGGCTTGAGGGGCTCGGGCCAGCTCCAATGTCACTACCTACAGCGAG GGCAGGGTGTAAGGTTGAGAAGGTCACATTCACCGCTTTGGGAGGACGTGGGAGAAGAGACTGAGGTGGAAAGCGCTTTGCCTTGCTCACCGGCCGTC CTTGCCCCGGTCCCAGCGTTTGCTGGGATTTGCCAGGATTTGCCGGGGCTCCGGGAGACCCTGAGCACTCGCAGGAAGAGGTGCTGAGAAATTAAAAA TTCAGGTTAGTTAATGCATCCCTGCCGCCGGCTGCAGGCTCCGCCTTTGCATTAAGCGGGCGCTGATTGTGCGCGCCTGGCGACCGCGGGGAGGACTG GCGGCCCGCGGGAGGGGACGGGTAGAGGCGCGGGTTACATTGTTCTGGAGCCGGCTCGGCTCTTTGTGCCTCCTCTAGCGGCCAAGCTGCGAGGTACA GCCCTCTATTGTTCTAGGAGCACAGAAACCTCCTGTGTGGGCGGCGGGTGCGCGAGCTAGAGGGAAAGATGCAGTAGTTACTGCGACTGGCACGCAGT TGCGCGCTTTTGTGCGCACGGACCCCGCGCGGTGTGCGTGGCGACTGCGCTGCCCCTAGGAGCAAGCCACGGGCCCAGAGGGGCAAAATGTCCAGGTC CCCCCCTGCCAACGACACACTATACCCTATGCCAAGCCACGCTGGCCGACTTCCCATGCATCGGCTGCACCGCGCTATCTTTCAGCATCGCCGCCCCG TCTAGGGGAACAATTCGTGGTGGCGATGATTTGCATAGCGCGGGTCTTGGGATGCGCGCGGTTCCGAGCCAGCCTCGCACAGCTCGCTTCCGGAGCTG CGAGCTCAGGTTTCCACCCCCGATCCCCCGGGCTTTCCTCGCACCGCTGAGCCCAGCTTGTGGGGTGCACTCGACCAACGCCCGACAGGGCTGGGGAA TGTGACAGGCAGCAGGTTCACCCGGGCTTGGGGAGGGGGAGTTTCCGCTTTGACAGCATTTTCCTTTGCCGTCTGCTGGTGGATTCCTATTCCCAGTC GGTAATCGCCCCGCAGTGTTGATCTAAGAAGGTAAAGAAAACTAGGTTTCCCTGCAAAGAGCCTCCCCCAAATCGGCGGACTCCGGATACTTTGAGTG GATTTAGAAATTTATGTAATCTTTCTCCTTTAGTTTATTTTTCATCCTCTCCTACAGTTTTCTCTGATTTGCTGTTGGTTCGGGGCAAGATAAAGCAG CCAGTAGAGAGCGATAATAATAGCGGCGGGAAATGAACTGGAGACTGGCTGACAGTTCTTAACATTTTGTCATAGATCCCCCCGAATGTCCCAGGCTG TCTCTGGTGGGTTTTAGTACCCGCCGGCTTCTTGGCACCGGGACCAGAAGGAACTTCGCAGCTGGTCTTAGGGCTACAGTTAAAGGCAGGATGACA GCTATTCTCCTGCTCATCTCAGAGCGCTGCCGCCCCCTCATGCCGGTCGCGCAAAGAACACAGCTTTTAAAAAACACGTGCCTTCTGCCCATATAGGT CTGAAAGTGATGAGGAAAGTAATGCTTCGCCTATTAGCGAGTTTCAGCTTTTAAAATGATCCCAAGCGTTGCTGAGATGAGAAAGCGTGGCATCCCGG GGGTCCTCAGCCCCACCCGCGCCCATGGTGCAAGTCTGCAGGGACAGGCCCGGGACAGCACTGCCCACGCTGCTAGATTTTCCGCAGAGGATCGCTGA AGCTGCCTTCGTGGGAGACAGAATGCCTCCTCCAGCGAGTGGAAAAGGCCTGCTGAGGACCCCGCTTTGCTCGAGCATTCAAATGTGTGTCTGTTTTA TTACCCTGGGTTGAAAAGGGACAAGAGCTTTAGCCTTTTTATCTGGCCATTTTATCAGCAACTACAAGTGTGTTGAGTGGTTATTATTACATAGGAGG CTTTTCAGTTTGGGGTCAGTAGATCAGTCTCTTCAGACACTGATGCAGAAGCTGGGACTGGTAAGTAGGTATTATGTGCTCGGAGCGCTAGGGGACAG GAGCAAATGGAGAAGAAAAGCGGAGGCTTTCTCCGCCCGGAGTATCGATCGGAATCCCCGCCGGTACGCCGCAGAGGGCCCTCGCCGTTGGGCCCCGG GGGTTTAACAAGCCCAGCCGCTCCGCAGGCGGCTCGGCCGGACTCTCAGACCGGTGCCTGGAAGACACCGTCCCTGCCCCCCTCCCGCCAAACCTGCC TCTTCTCTTTCTCTCATAGGTTATAGGTTCCCTTTCTCTCTCATTTTGGCCCCGCCCCCGGGTCCTGCCAAACAGCCAAGCAGGCCGGGGTTTAGGGG GCTCAGAATGAAGAGGTCTGATTTGGCCAGCGCCGGCAAAGCTCACCCTTAGGCGAGGTCACAACAGAGGCAGGTCCTTCCTGCCCAGCCTGCCGGTG TAGTCACAGCCAAGGGTGGCACTTGAAAGGAAAAGGGAGAAAACTTCGGAGAAATTTAGATTGCCCCAACGTTAGATTTCAGAGAAATTGACTCCAAA TGCACGGATTCGTTCGGAAAGGGCGGCTAAGTGGCAGGTGGTTGCAACCCCGCCCGGTCGGGCCTTCGCAGAGGTTCCCCAAGACCAGCCCTTGCAGG GCGGTTTTCAGCAACCTGACAAGAGGCGGCCAAGACAAATTTCTGCGGGTTCGAGCACACACTCTCGGGCGTTGGGCCCCAGAGACCTCTAAACCAAG CACAAACAAGAAGGGAGTGAGAGAACCCAGGCTAGAACTTGCACGGGCATCCCACTGAGGAAAAGCGAGGCCTCGGTGGCAGGCATGTTTTCTTCCGA CGCCCGAAAATCGAGCCGAGCGCCCGACTACATTTACTGCAGAGGTTTCCGCCTCCAGTGAGCCCGGATCCCCCAGCGGCCTGCCCGGAGCTGGTCTC CAGTCCCCGCCGTAGTCCGACGCACGGCCCTCTCCTGGCAGCAAGCTCCCAGCGGCCAGTCTGAAGCCAATTCTGTTCAGGCGGCCGAGGGCCCTTAG CCAACCCACCATGATGTCGCCTGGGCCACCTGATGCCCGCAGCGGCGGGACACGGCCCGGGCAGTGCGCAGTGGCTCCTGCTAGGGGCACCGCGTGCG

SEQ ID NO	GENE NAME	SEQUENCE
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27	RAX	AACCGGAGATCTGCTTGGTGAACTGAGAGGAGTCCTTAGGAGAGCGGGGACGCCAGGGGCCGGGGGACACTTCGCTCTCGCCCTAGGGAAGGTGGTCTTGACGCTTTCTATTGAAGTCAAACTTGAAAATATCAGCTGCCGCTGGACTAT
28	chr18 group-00277	CGTGAGCAGAACGCCCGCCCTGGAGCAGTTAGGACCGAAGGTCTCCGGAGAGTCGCCGGCGGTGCCAGGTAACGCAGAGGGCTCGGGTCGGGCCCCGCTTCTGGGGCTTGGGACTCCGGGCGCGCGGAGCCAGCCCTCTGGGGCGAAATCCCCGGGCGGCGTGCGCGGTCCCTCTCCGCGCTGTGCTCTCCCAGCAACTCCCTGCCACCTCGACGAGCCTACCGGCCGCTCCGAGTTCGACTTCCTCGGACTTAGTGGGAGAAGGGGTTGGAAATGGGCTGCCGGGACTGGGGGAGCTGCTCTCTGGAAGCAGGGAAGCTGGGGCGCACCGGGGCAGGT
29	NETO1	TAGAAGAGGAAGACTCCTCTGGCCCCACTAGGTATCATCCGCGCTCTCCCGCTTTCCACCTGCGCCCTCGCTTGGGCCAATCTCTGCCGCACGTGTCCATCCCTGAACTGCACGCTATCCTCCACCCCCGGGGGGTTCCTGCGCACTGAAAGACCGTTCTCCGGCAGGTTTTGGGATCCGGCGACGGCTGACCGCGCGCCGCCCCCACGCCCGGTTCCACGATGCTGCAATACAGAAAGTTTACGTCGGCCCCGACCCGCGCGGGACTGCAGGGTCCGCCGGAGCGCGGCGCAGAGGCTTTTCCTGCGCGTTCGGCCCCGGGAAAGGGGCGGGAGGGCTGGCTCCGGGAGCGCACGGGCGCGGCGGGGAGGGTACTCACTGTGAAGCACGCTGCGCCCATGGATCATGTCTGTGCGTTACACCAGAGGCTCCGGGCTCCACTAATTCCATTTAGAGACGGGAAGACTTCCAGTGGCGGGGGGAGGACAGGGTCGAGAGGTGTTAAAGACGCAAAGCAAGAAGGAAATAAAGGGGGGCCGAGAGGGAGACCGAGAGGAAGGGGGAGCTCCGAGCCCACGCTGCAGCCAGATCCGGATGAGTCCGTCCTCCGCCCCGGGCGGGCTCTCGCTCTCGCTGGCCCTCAGCGCCGCGCAGCCAGCAGCATCCCCACCGTGACGCTCGCATCACACCCGGGCGCCGGCCGCCACCATCCGCGCCGCCGCCGTCAGGACCCTCCTCCCGGGCATCGTCGCCGCCGCGGGTCGGGAGGACGCGGCGCGCGGGAGGCGGCGGTCGCAGGGCGAGCCCCGGGACGCCCCGAGCCGGGGCCGGGGCCGGGGAGAGGGCGCAGCGAGGTGGGGGCCAGTCCAGACCGACGGCAGCGACGGAGCGGGCGGCGGCGGCGGCGCCGGCGGCGGCGGGGTGGCTCAGTCCCCAGTCTCAGACGCGCCGCGCAGCAGGTCGGAGCAGCCTCCCCGGGAGGATGTCCAGCGGCAGCGCTCCTCGCTCCAGCCCTTGGGGATCTTCCGCTGAGGCATTGAAGGCAGGAAGAAGGGGTCCGTCATCGGCTCGCCGGGCTGCGCGCCACCTCTGCTATCTTGCGGAAAGAGGAGCGGGTGGGTGGGCGTCTGGGAGGCGGGCTGGAGGGCGGTGCAGGGGAGCGGGGCGGCCGGGGGGGGGGCCGGGGGGCGGGGAAGGGAGGGAGGAGAAAGGAGCCGGAAGAGGGCAGAGTTACCAAATGGGCTCCTTAGTCATGGCTTGGGGCTCCACGACCCTCCTGGAAGCCCGGAGCCTGGGTGGGATAGCGAGGCTGCGCGCGGCCGGCGCCCCGGGGCTGGTGCGCGGCAGAATGGGGCCGCGGCGGCGGCAGCAAGGACATCCCAGCCGCGCGGATCTGGGGGAGGGGCGGGGAGGGGGTGAGGACCCGGCTGGGATCCGCGGCTCGGCCCGCCAGGCGCAGAGAGAGGATGCAGCCGCAAATCCCGAGCCGGATCCTCGTGCCGGACGGAAGGCGTGGAAGCGGGAGGGGCCTTCGTGTGAAAATCCCTTGTGGGGTTTGGTGTTTCACTTTTTAAAGGTTAGACCTTGCGGGCTCTCTGCCTCCCACCCCTTCTTTTCCATCCGCGTAAAGGAACTGGGCGCCCCCTCTCCCTCCCTCCCTGGGG

SEQ ID NO	GENE NAME	SEQUENCE
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30	MBP	AAGAAACAGCTCATTTCGGAGCTGAGGACAAGGCGTGGGAAGAAGACGCGTTTGGTTTCACCCAGGCGGGTGGCGGCAAAGCTGTGGGATGCGCGCTGCACACTCCTTCCCTCATCCCGTTCCCACCTTCCACACACACCTGCGCCACCTCCCACATCTCCTGATTGCCTGTTCATCACCATGCCAAACCCAACATGAGGAGAACGCCACTGACGCTGGGTGCGCCGGCTTTCCCAGCCCTCGTGCATAACGGGGAGGGAGATGCAGAAGTTTTTTCCAACATCGGTGCAAAGGGGAAGCTGAGGTTTTCCTAT
31	NFATC 1	TCTGTCAGCTGCTGCCATGGGGCAGCGGGAAGGCCCTGGAGGGTGCCTGGGCTGTGTCTGGTCCCGGCCACGCGTCCCTGCAGCGTCTGAGACCTTGTGGAACACACTTGACCCGGCGCTGGGACGGGGTCGGCCCACACGCACCGCCAGCCCGCAGGAGTGAGGTGCAGGCTGCCGCTGGCTCCTTAGGCCTCGACAGCTCTCTTGAGGTCGGCCCTCCTCCCCTCCCGAGAGCTCAGCAGCCGCAGACCCAGGCAGAGAGAGCAAAGGAGGCTGTGGTGGCCCCCGACGGGAACCTGGGTGGCCGGGGGACACACCGAGGAACTTTCCGCCCCCCGACGGGCTCTCCCACCGAGGCTCAGGTGCTCGTGGGCAGCAAGGGGAAGCCCCATGGCCATGCCGCTTCCCTTTCACCCTCAGCGACGCGCCCTCCTGTGCCCGCGGGGAACAAGACGGCTCTCGGCGGCCATGCAGGCGGCCTGTCCCACGAACACGATGGAGACCTCAGACGCCGTCCCCACCCTGTCACTGTCACCATCACCCATCCTGTCCCCTCACGCCTCCCCACATCCCATCATTACTAC
32	chr18 group-00430	GAAGTAGAATCACAGTAAATGAGGAGTTAGGGAATTTAGGGTAGAGATTAAAGTAATGAACAGAGGAGGAGGCCTGAGACAGCTGCAGAGAGACCCTGTGTTCCCTGTGAGGTGAAGCGTCTGCTGTCAAAGCCGGTTGGCGCTGAGAAGAGGTACCGGGGGCAGCACCCGCCTCCTGGGAGAGGGATGGGCCTGCGGGCACCTGGGGGAACCGCACGGACACAGACGACACTATAAACGCGGGCGAGACATCAGGGACCGGGAAACAGAAGGACGCGCGTTTCGAGCAGCTGCCCAGTGGGCCACAAGCCCCGCCACGCCACAGCCTCTTCCCCTCAGCACGCAGAGA
33	OLIG2	TACTCCGGCGACGGGAGGATGTTGAGGGAAGCCTGCCAGGTGAAGAAGGGGCCAGCAGCAGCACAGAGCTTCCGACTTTGCCTTCCAGGCTCTAGACTCGCGCCATGCCAAGACGGGCCCCTCGACTTTCACCCCTGACTCCCAACTCCAGCCACTGGACCGAGCGCGCAAAGAACCTGAGACCGCTTGCTCTCACCGCCGCAAGTCGGTCGCAGGACAGACACCAGTGGGCAGCAACAAAAAAAGAAACCGGGTTCCGGGACACGTGCCGGCGGCTGGACTAACCTCAGCGGCTGCAACCAAGGAGCGCGCACGTTGCGCCTGCTGGTGTTTATTAGCTACACTGGCAGGCGCACAACTCCGCGCCCCGACTGGTGGCCCCACAGCGCGCACCACACATGGCCTCGCTGCTGTTGGCGGGGTAGGCCCGAAGGAGGCATCTACAAATGCCCGAGCCCTTTCTGATCCCCACCCCCCCGCTCCCTGCGTCGTCCGAGTGACAGATTCTACTAATTGAACGGTTATGGGTCATCCTTGTAACCGTTGGACGACATAACACCACGCTTCAGTTCTTCATGTTTTAAATACATATTTAACGGATGGCTGCAGAGCCAGCTGGGAAACACGCGGATTGAAAAATAATGCTCCAGAAGGCACGAGACTGGGGCGAAGGCGAGAGCGGGCTGGGCTTCTAGCGGAGACCGCAGAGGGAGACATATCTCAGAACTAGGGGCAATAACGTGGGTTTCTCTTTGTATTTGTTTATTTTGTAACTTTGCTACTTGAAGACCAATTATTTACTATGCTAATTTGTTTGCTTGTTTTTAAAACCGTACTTGCACAGTAAAAGTTCCCCAACAACGGAAGTAACCCGACGTTCCTCACACTCCCTAGGAGACTGTGTGCGTGTGTGCCCGCGCGTGCGCTCACAGTGTCAAGTGCTAGCATCCGAGATCTGCAGAAACAAATGTCTGAATTCGAAATGTATGGGTGTGAGAAATTCAGCTCGGGGAAGAGATTAGGGACTGGGGGAGACAGGTGGCTGCCTGTACTATAAGGAACCGCCAACGCCAGCATCTGTAGTCCAAGCAGGGCTGCTCTGTAAAGGCTTAGCAATTTTTCTGTAGGCTTGCTGCACACGGTCTCTGGCTTTTCCCATCTGTAAAATGGGTGAATGCATCCGTACCTCAGCTACCTCCGTGAGGTGCTTCTCCAGTTCGGGCTTAATTCCTCATCGTCAAGAGTTTTCAGGTTTCAGAGCCAGCCTGCAATCGGTAAAACATGTCCCAACGCGGTCGCGAGTGGTTCCATCTCGCTGTCTGGCCCACAGCGTGGAGAAGCCTTGCCCAGGCCTGAAACTTCTCTTTGCAGTTCCAGAAAGCAGGCGACTGGGACGGAAGGCTCTTTGCTAACCTTTTACAGCGGAGCCCTGCTTGGACTACAGATGCCAGCGTTGCCCCTGCCCCAAG

SEQ ID NO	GENE NAME	SEQUENCE
		GCGTGTGGTGATCACAAAGACGACACTGAAAATACTTACTATCATCCGGCTCCCCTGCTAATAAATGGAGGGGTGTTTAACTACAGGCACGACCCTGC CCTTGTGCTAGCGCGGTTACCGTGCGGAAATAACTCGTCCCTGTACCCACACCATCCTCAACCTAAAGGAGAGTTGTGAATTCTTTCAAAACACTCTT CTGGAGTCCGTCCCCTCCCTCCTTGCCCGCCCTCTACCCCTCAAGTCCCTGCCCCCAGCTGGGGGCGCTACCGGCTGCCGTCGGAGCTGCAGCCACGG CCATCTCCTAGACGCGCGAGTAGAGCACCAAGATAGTGGGGACTTTGTGCCTGGGCATCGTTTACATTTGGGGCGCCAAATGCCCACGTGTTGATGAA ACCAGTGAGATGGGAACAGGCGGCGGGAAACCAGACAGAGGAAGAGCTAGGGAGGAGACCCCAGCCCCGGATCCTGGGTCGCCAGGGTTTTCCGCGCG CATCCCAAAAGGTGCGGCTGCGTGGGGCATCAGGTTAGTTTGTTAGACTCTGCAGAGTCTCCAAACCATCCCATCCCCCAACCTGACTCTGTGGTGGC CGTATTTTTTACAGAAATTTGACCACGTTCCCTTTCTCCCTTGGTCCCAAGCGCGCTCAGCCCTCCCTCCATCCCCCTTGAGCCGCCCTTCTCCTCCC CCTCGCCTCCTCGGGTCCCTCCTCCAGTCCCTCCCCAAGAATCTCCCGGCCACGGGCGCCCATTGGTTGTGCGCAGGGAGGAGGCGTGTGCCCGGCCT GGCCAGTTTCATTGACCCGAATTAGCCCGGATGACATCACCTTCCCAGCCCCCCGCGCGCCCAGCTCATTCGCCAGCCAGCCCCTCCACCACACCGCA CATTGTTCCCCGCCCCCGCCCCCGCCACCGCTGCCGCCGTCGCCGCTGCCACCGGGCTATAAAAACCGGCCGAGCCCCTAAAGGTGCGGATGCTTATT ATAGATCGACGCGACACCAGCGCCCGGTGCCAGGTTCTCCCCTGAGGCTTTTCGGAGCGAGCTCCTCAAATCGCATCCAGAGTAAGTGTCCCCGCCCC ACAGCAGCCGCAGCCTAGATCCCAGGGACAGACTCTCCTCAACTCGGCTGTGACCCAGAATGCTCCGATACAGGGGGTCTGGATCCCTACTCTGCGGG CCATTTCTCCAGAGCGACTTTGCTCTTCTGTCCTCCCCACACTCACCGCTGCATCTCCCTCACCAAAAGCGAGAAGTCGGAGCGACAACAGCTCTTTC TGCCCAAGCCCCAGTCAGCTGGTGAGCTCCCCGTGGTCTCCAGATGCAGCACATGGACTCTGGGCCCCGCGCCGGCTCTGGGTGCATGTGCGTGTGCG TGTGTTTGCTGCGTGGTGTCGATGGAGATAAGGTGGATCCGTTTGAGGAACCAAATCATTAGTTCTCTATCTAGATCTCCATTCTCCCCAAAGAAAGG CCCTCACTTCCCACTCGTTTATTCCAGCCCGGGGGCTCAGTTTTCCCACACCTAACTGAAAGCCCGAAGCCTCTACAATGCCACCCGCACCCCGAGGG TCACCAACGCTCCCTGAAATAACCTGTTGCATGAGAGCAGAGGGGAGATAGAGAGAGCTTAATTATAGGTACCCGCGTGCAGCTAAAAGGAGGGCCAG AGATAGTAGCGAGGGGGACGAGGAGCCACGGGCCACCTGTGCCGGGACCCCGCGCTGTGGTACTGCGGTGCAGGCGGGAGCAGCTTTTCTGTCTCTCA CTGACTCACTCTCTCTCTCTCTCCCTCTCTCTCTCTCTCATTCTCTCTCTTTTCTCCTCCTCTCCTGGAAGTTTTCGGGTCCGAGGGAAGGAGGACCC TGCGAAAGCTGCGACGACTATCTTCCCCTGGGGCCATGGACTCGGACGCCAGCCTGGTGTCCAGCCGCCCGTCGTCGCCAGAGCCCGATGACCTTTTT CTGCCGGCCCGGAGTAAGGGCAGCAGCGGCAGCGCCTTCACTGGGGGCACCGTGTCCTCGTCCACCCCGAGTGACTGCCC
34	SIM2	TTAATTCGAAAATGGCAGACAGAGCTGAGCGCTGCCGTTCTTTTCAGGATTGAAAATGTGCCAGTGGGCCAGGGGCGCTGGGACCCGCGGTGCGGAAG ACTCGGAACAGGAAGAAATAGTGGCGCGCTGGGTGGGCTGCCCCGCCGCCCACGCCGGTTGCCGCTGGTGACAGTGGCTGCCCGGCCAGGCACCTCCG AGCAGCAGGTCTGAGCGTTTTTGGCGTCCCAAGCGTTCCGGGCCGCGTCTTCCAGAGCCTCTGCTCCCAGCGGGGTCGCTGCGGCCTGGCCCGAAGGA TTTGACTCTTTGCTGGGAGGCGCGCTGCTCAGGGTTCTG
35	SIM2	CCGGTCCCCAGTTTGGAAAAAGGCGCAAGAAGCGGGCTTTTCAGGGACCCCGGGGAGAACACGAGGGCTCCGACGCGGGAGAAGGATTGAAGCGTGCA GAGGCGCCCCAAATTGCGACAATTTACTGGGATCCTTTTGTGGGGAAAGGAGGCTTAGAGGCTCAAGCTATAGGCTGTCCTAGAGCAACTAGGCGAGA ACCTGGCCCCAAACTCCCTCCTTACGCCCTGGCACAGGTTCCCGGCGACTGGTGTTCCCAAGGGAGCCCCCTGAGCCTACCGCCCTTGCAGGGGGTCG TGCTGCGGCTTCTGGGTCATAAACGCCGAGGTCGGGGGTGGCGGAGCTGTAGAGGCTGCCCGCGCAGAAAGCTCCAGGATCCCAATATGTG
36	DSCR6	GCGCAGGTCCCCCCAGTCCCCGAGGGAGTGCGCCCGACGGAAACGCCCCTAGCCCGCGGGCCTCGCTTTCCTCTCCCGGGTTCCTGGGTCACTTCCCG CTGTCTC
37	DSCAM	TTCCCTCGCGGCTTTGGAAAGGGGGTGCAAATGCACCCTTCTGCGGGCCCGCTACCCGCTGCAACACCTGTGTTTCCTTTCTGGGCACCTTCTAGGTT TCTAGATATTGCTGTGAATACGGTCCTCCGCTGTACAGTTGAAAACAAA

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38	chr21 group-00165	TGGGAATTTAGGTCGGGCACTGCCGATATGTCGCCTTCCACAAGGCGGGCCCGGGCCTCTGCTGACCGTGCACCGGTCCTGGGGCTGGGTAATTCTGCAGCAGCAGCGCAGCCCATGCCGGGGAATTTGCGGGCAGAGGAGACAGTGAGGCCCGCGTTCTGTGCGGGAACTCCCGAGCTCACAGAGCCCAAGACCACACGGCTGCATCTGCTTGGCTGACTGGGCCAGGCCCACGCGTAGTAACCCGGACGTCTCTCTCTCACAGTCCCCTTGCGTCTGGCCAGGGAGCTGCCAGGCTGCACCCCGCGGTGGGGATCGGGAGAGGGGCAGTGTCGCCCATCCCCGGAAGGCTGAGCCTGGTGCAG
39	PRMT2	CGGTTTTCTCCTGGAGGACTGTGTTCAGACAGATACTGGTTTCCTTATCCGCAGGTGTGCGCGGCGCTCGCAAGTGGTCAGCATAACGCCGGGCGAATTCCGAAAGCCCCGTGCCTCCCGTGCACCACCCACTTCGAAGCACTTGCCAGAACTCCTTCTTCCCACCCCCCGACGCTTCCCTCCCTCTGTCCTTCCACCCACAAAAAGCCCAGACCCTAACCCGCTCCTTTCTCCCGCCGCGTCCATGCAGAACTCCGCCGTTCCTGGGAGGGGAAGCCCGCGAGGCGTCGGGAGAGGCACGTCCTCCGTGAGCAAAGAGCTCCTCCGAGCGCGCGGCGGGGACGCTGGGCCGACAGGGGACCGCGGGGGCAGGGCGGAGAGGACCCGCCCTCGAGTCGGCCCAGCCCTAACACTCAGGAC
40	SIX2	AGGGAATCGGGCTGACCAGTCCTAAGGTCCCACGCTCCCCTGACCTCAGGGCCCAGAGCCTCGCATTACCCCGAGCAGTGCGTTGGTTACTCTCCCTGGAAAGCCGCCCCCGCCGGGGCAAGTGGGAGTTGCTGCACTGCGGTCTTTGGAGGCCTAGGTCGCCCAGAGTAGGCGGAGCCCTGTATCCCTCCTGGAGCCGGCCTGCGGTGAGGTCGGTACCCAGTACTTAGGGAGGGAGGACGCGCTTGGTGCTCAGGGTAGGCTGGGCCGCTGCTAGCTCTTGATTTAGTCTCATGTCCGCCTTTGTGCCGGCCTCTCCGATTTGTGGGTCCTTCCAAGAAAGAGTCCTCTAGGGCAGCTAGGGTCGTCTCTTGGGTCTGGCGAGGCGGCAGGCCTTCTTCGGACCTATCCCCAGAGGTGTAACGGAGACTTTCTCCACTGCAGGGCGGCCTGGGGCGGGCATCTGCCAGGCGAGGGAGCTGCCCTGCCGCCGAGATTGTGGGGAAACGGCGTGGAAGACACCCCATCGGAGGGCACCCAATCTGCCTCTGCACTCGATTCCATCCTGCAACCCAGGAGAAACCATTTCCGAGTTCCAGCCGCAGAGGCACCCGCGGAGTTGCCAAAAGAGACTCCCGCGAGGTCGCTCGGAACCTTGACCCTGACACCTGGACGCGAGGTCTTTCAGGACCAGTCTCGGCTCGGTAGCCTGGTCCCCGACCACCGCGACCAGGAGTTCCTTCTTCCCTTCCTGCTCACCAGCCGGCCGCCGGCAGCGGCTCCAGGAAGGAGCACCAACCCGCGCTGGGGGCGGAGGTTCAGGCGGCAGGAATGGAGAGGCTGATCCTCCTCTAGCCCCGGCGCATTCACTTAGGTGCGGGAGCCCTGAGGTTCAGCCTGACTTTC
41	SIX2	CACTACGGATCTGCCTGGACTGGTTCAGATGCGTCGTTTAAAGGGGGGGCTGGCACTCCAGAGAGGAGGGGGCGCTGCAGGTTAATTGATAGCCACGGAAGCACCTAGGCGCCCCATGCGCGGAGCCGGAGCCGCCAGCTCAGTCTGACCCCTGTCTTTTCTCTCCTCTTCCCTCTCCCACCCCTCACTCCGGGAAAGCGAGGGCCGAGGTAGGGGCAGATAGATCACCAGACAGGCGGAGAAGGACAGGAGTACAGATGGAGGGACCAGGACACAGAATGCAAAAGACTGGCAGGTGAGAAGAAGGGAGAAACAGAGGGAGAGAGAAAGGGAGAAACAGAGCAGAGGCGGCCGCCGGCCCGGCCGCCCTGAGTCCGATTTCCCTCCTTCCCTGACCCTTCAGTTTCACTGCAAATCCACAGAAGCAGGTTTGCGAGCTCGAATACCTTTGCTCCACTGCCACACGCAGCACCGGGACTGGGCGTCTGGAGCTTAAGTCTGGGGGTCTGAGCCTGGGACCGGCAAATCCGCGCAGCGCATCGCGCCCAGTCTCGGAGACTGCAACCACCGCCAAGGAGTACGCGCGGCAGGAAACTTCTGCGGCCCAATTTCTTCCCCAGCTTTGGCATCTCCGAAGGCACGTACCCGCCCTCGGCACAAGCTCTCTCGTCTTCCACTTCGACCTCGAGGTGGAGAAAGAGGCTGGCAAGGGCTGTGCGCGTCGCTGGTGTGGGGAGGGCAGCAGGCTGCCCCTCCCCGCTTCTGCAGCGAGTTTTCCCAGCCAGGAAAAGGGAGGGAGCTGTTTCAGGAATTTCAGTGCCTTCACCTAGCGACTGACACAAGTCGTGTGTATAGGAAG
42	SOX14	GGAGCCTGAAGTCAGAAAAGATGGGGCCTCGTTACTCACTTTCTAGCCCAGCCCCTGGCCCTGGGTCCCGCAGAGCCGTCATCGCAGGCTCCTGCCCAGCCTCTGGGGTCGGGTGAGCAAGGTGTTCTCTTCGGAAGCGGGAAGGGCTGCGGGTCGGGGACGTCCCTTGGCTGCCACCCCTGATTCTGCATCCTTTTCGCTCGAATCCCTGCGCTAGGCATCCTCCCCGATCCCCCAAAAGCCCAAGCACTGGGTCTGGGTTGAGGAAGGGAACGGGTGCCCAGGCCGGACAGAGGCTGAAAGGAGGCCTCAAGGTTCCTCTTTGCTACAAAGTGGAGAAGTTGCTCTACTCTGGAGGGCAGTGGCCTTTTCCAAACTTTTCCACTTAGGTCCGTAAGAAAAGCAATTCATACACGATCAGCGCTTTCGGTGCGAGGATGGAAAGAAACTTC

SEQ ID NO	GENE NAME	SEQUENCE
43	TLX3	TTTTCCTGTTACAGAGCTGAGCCCACTCATGTGGTGCCAAGTAGCGACTATCTCTCGGCCACCTCCACCCAGAGCAATGTGGGCGCCCCCAGCGGGTGGGAGCGATTGCCGAGCGGCGCAAGGGCGTTTAACGCCTAACCCCCTCCTCCTGGGTTGCCAAGCCGCTAGGTCGCCGTTTCCAACGTGGCTGCGCGGGACTGAAGTCCGACGACTCCTCGTCCTCAGTAGGAGACACACCTCCCACTGCCCCCAGCCACGCGAGCTATGGGCAGAATCGGGGCAACGGTAATATCTGGATGGGGCAGGCTCCCCTGAGGCTGTGCTTAAGAAAAAAGGAATCTGGAGTAGCCTGAGGGGCCCCACGAGGGGGCCTCCTTTGCGATCGTCTCCCAGCCTTAGGCCAAGGCTACGGAGGCAGGCGGCCGAGTGTTGGCGCCCAGCCCGGCCGAGGACTGGATGGAGGACGAGAAGCAGCCTGCCTCTGGGCGACAGCTGCGGACGCAGCCTCGCCGCCTCGCCGCCTCAGCCTCGGTCCCAGCGTCTCTAAAGCCGCGCCCATTTTACAGATGCAGGGCAGGGAGACAAGAGGCATCTCCGGGGGCCGAGTAGAATGATGGCGCGGGTTCTCCCGGCGCCCTGATTTCGAGGCTGCGCCCGGGGCCCTACATGCAGGCGGGGAGGCCTGGGCCGAAGGCGTCTGCAAGGAGGGGCGAGTCTGCCCGGTCCGGGCAGGGAGTGAGGCCACAGTCAGTTCTCCCTAGGAGGCCGCGCAGCGGGTAGGGTATGGCACTGCCCGACGCCAACCGCGACCTGCCCCAATCAGACCCCTCACCAGAGAACCCCAAAACTCTCGCCCCGCCCCCTCGCTTCCCCCTAGTGCCAATGGTTTCCGGTCATCCGTTCCCAGTCCAGCCCCGGGTAGGGAGCTCTGATTTGCAATGCACAGCACTTGCGAGGTTCGAATGCCCCCGCAATTTGCAGATGGAAATACTAAGCCTAGGCCGGGCGTGGTGGCTCAAGCCTATCATCTCAGCCCTTTGGGAGGCCAAGCCGGGAGGATTGTTTGAGCCCAAGAATTCAAAACCAGCCTGAGCAACATAGCGACCCCGTCTCTACAAAATAAAATAAAATAAATTATCCGGGCGTGGTGGCACGCGCCTGTGGTTCCAGCTACTCCGGAGGCTGAGGTGGGAGGATCGCTTGAGTCCGGGAGGTCGAGGCTACAGTGAGCCGTGATCGCACCACTGCACTCCAGCCTGGGCGACAGAGTGAGACCTTGTCTCAAAAAAGGAAAAAAAGAAAAAGAAAGTAAGCTTCAAAGAAGCTCTGATAATAGTTCTGGGTCGTGCAGCGGTGGCGGCCCCGCGCTCTCGCCCCTAAAGCAAGCGCTCTTTGTACTGGGTGGAGGAGCTTTGAGTAGTGAGGGTGGAGATGCAGCTTCGGGGTGGCGCAGCCACCCTGACACTAGGCCCGGCGTCGCAGTGGCACAGAAGAGTCTGCCGCTCTGACTTGGGCTCTGAGTTCCAAGGCGCCCCGGCACTTCTACCCTCCCAGGCTTGCGCGCTGGCGCCTTTGCCATCCGTGCCGAAGTGGGGAGACCTAGCCGCGACCACCACGAGCGCAGCGGTGACACCCAGAGGTCCCACCGGGCCCCTGGGCAGGGTAACCTTAGCCTGTCCGCTTCGGCAGCTTTGCGAAGAGTGGCGCGCAGCTAGGGCTGAGGCTCTTGCGGACCTGCGGTCGAAGCAGGCGGCTGAGCCAGTTCGATCGCCAAGGCCTGGGCTGCCGACAGTGGTGCGCGCTCTGTTCCGCCGCGGCCGGGCCAGGCGCTCTGGAATAGCGATGGGGGACACGGCCTCCAACTTTCTGCAGAGACCATCGGGCAGCTCCGGGCCTAAGCAGCGACCTCACCGAAGGTTCCTGGGAACCTTTGCCAAAATCCCAGCCTCTGCCTCGGTCCAGCTAAACCGTGTGTAAACAAGTGCACCAAG
44	FOXP4	ATAAAGGACCGGGTAATTTCGCGGAATGCGGATTTTGAGACAGGCCCAGACGGCGGCGGATTCCCTGTGTCCCCCAACTGGGGCGATCTCGTGAACACACCTGCGTCCCACCCCGATCCTAGGTTGGGGGAAAGGGTATGGGAACCCTGAGCCCAGAGCGCGCCCCGCTCTTTCCTTTGCTCCCCGGCTTCCCTGGCCAGCCCCCTCCCGGCTGGTTTCCTCGCTCACTCGGCGCCTGGCGTTTCGGGCGTCTGGAGATCACCGCGTGTCTGGCACCCCAACGTCTAGTCTCCCCGCAGGTTGACCGCGGCGCCTGGAGCCGGGAATAGGGGTGGGGAGTCCGGAGAACCAAACCCGAGCCTGAAGTTGCCATTCGGGTGACTCCCGAGAAAGCCCGGGAGCATTTTGGCCAATGCGGGTTTTTACCTGAACTTCAGCATCTTCACC
45	FOXP4	AATTGGAAAACCCTGGTATTGTGCCTGTTTGGGGGAAGAAAACGTCAATAAAAATTAATTGATGAGTTGGCAGGGCGGGCGGTGCGGGTTCGCGGCGAGGCGCAGGGTGTCATGGCAAATGTTACGGCTCAGATTAAGCGATTGTTAATTAAAAAGCGACGGTAATTAATACTCGCTACGCCATATGGGCCCGTGAAAAGGCACAAAAGGTTTCTCCGCATGTGGGGTTCCCCTTCTCTTTTCTCCTTCCACAAAAGCACCCCAGCCCGTGGGTCCCCCCTTTGGCCCCAAGGTAGGTGGAACTCGTCACTTCCGGCCAGGGAGGGGATGGGGCGGTCTCCGGCGAGTTCCAAGGGCGTCCCTCGTTGCGCACTCGCCCGCCCAGGTTCTTTGAA
46	chr7	GGGAAGCGATCGTCTCCTCTGTCAACTCGCGCCTGGGCACTTAGCCCCTCCCGTTTCAGGGCGCCGCCTCCCCGGATGGCAAACACTATAAAGTGGCGGCGAATAAGGTTCCTCCTGCTGCTCTCGGTTTAGTCCAAGATCAGCGATATCACGCGTCCCCCGGAGCATCGCGTGCAGGAGCCATGGCGCGGGAGCT

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	group-00267	ATACCACGAAGAGTTCGCCCGGGCGGGCAAGCAGGCGGGGCTGCAGGTCTGGAGGATTGAGAAGCTGGAGCTGGTGCCCGTGCCCCAGAGCGCTCACGGCGACTTCTACGTCGGGGATGCCTACCTGGTGCTGCACACGGCCAAGACGAGCCGAGGCTTCACCTACCACCTGCACTTCTGGCTCGGTAAGGGACGGCGGGCGGCGGGACCCCGACGCACCAAGGCCGGCGAGGGGAGGGCGTAGGGGTCTGAGATTTGCAGGCGTGGGAGTAAAGGGGACCGCAAACTGAGCTAG
47	NPY	CTCAGGGGCGGGAAGTGGCGGGTGGGAGTCACCCAAGCGTGACTGCCCGAGGCCCCTCCTGCCGCGGCGAGGAAGCTCCATAAAAGCCCTGTCGCGACCCCGCTCTCTCCACCCCATCCGCTGGCTCTCACCCCTCGGACACGCTCGCCCCGACACCATAGTACTTGCCCCCCAGCCACGCCCCCCCGCCAGCCACCCTGAGTGCTACGACCCGTCTGTCTAGGGGTGGGAGCGAACGGGGCGCCCGCGAACTTGCTAGAGACGCAGCCTCCCGCTCTGTGGAGCCCTGGGGCCCTGGGATGATCGCGCTCCACTCCCCAGCGGACTATGCCGGCTCCGCGCCCCGACGCGGACCAGCCCTCTTGGCGGCTAAATTCCACTTGTTCCTCTGCTCCCCTCTGATTGTCCACGGCCCTTCTCCCGGGCCCTTCCCGCTGGGCGGTTCTTCTGAGTTACCTTTTAGCAGATATGGAGGGAGAACCCGGGACCGCTATCCCAAGGCAGCTGGCGGTCTCCCTGCGGGTCGCCGCCTTGAGGCCCAGGAAGCGGTGCGCGGTAGGAAGGTTTCCCCGGCAGCGCCATCGAGTGAGGAATCCCTGGAGCTCTAGAGCCCCGCGCCCTGCCACCTCCCTGGATTCTTGGGCTCCAAATCTCTTTGGAGCAATTCTGGCCCAGGGAGCAATTCTCTTTCCCCTTCCCCACCGCAGTCGTCACCCCGAGGTGATCTCTGCTGTCAGCGTTGATCCCCTGAAGCTAGGCAGACCAGAAGTAACAGAGAAGAAACTTTTCTTCCCAGACAAGAGTTTGGGCAAGAAGGCAGAAAAGTGACCCAGCAGGAAGAACTTCCAATTCGGTTTTGAATGCTAAACTGGCGCGGCCCCCACCTTGCACTCTCGCCGCGCGCTTCTTGGTCCCTGAGACTTCGAACGAAGTTGCGCGAAGTTTTCAGGTGGAGCAGAGGGGCAGGTCCCGACCGGACGGCGCCCGGAGCCCGCAAGGTGGTGCTAGCCACTCCTGGGTTCTCTCTGCGGGACTGGGACGAGAGCGGATTGGGGGTCGCGTGTGGTAGCAGGAGGAGGAGCGCGGGGGGCAGAGGAGGGAGGTGCTGCGCGTGGGTGCTCTGAATCCCCAAGCCCGTCCGTTGAGCCTTCTGTGCCTGCAGATGCTAGGTAACAAGCGACTGGGGCTGTCCGGACTGACCCTCGCCCTGTCCCTGCTCGTGTGCCTGGGTGCGCTGGCCGAGGCGTACCCCTCCAAGCCGGACAACCCGGGCGAGGACGCACCAG
48	SHH	TGGAGAACCTTGGGCTCTGTGGCCTCAAAGGTAGGGGTGATTTCGAGGGGCCGGCACCTCACAGGGCAGGTTCCACCGCGGAAACGCAGTCATCGCCCAGCGACCCTGCTCCTGGCCCTCAGCCTCCCCCAGGTTTCTTTTTCTCTTGAATCAAGCCGAGGTGCGCCAATGGCCTTCCTTGGGTCGGATCCGGGGGGCCAGGGCCAGCTTACCTGCTTTCACCGAGCAGTGGATATGTGCCTTGGACTCGTAGTACACCCAGTCGAAGCCGGCCTCCACCGCCAGGCGGGCCAGCATGCCGTACTTGCTGCGGTCGCGGTCAGACGTGGTGATGTCCACTGCGCGGCCCTCGTAGTGCAGAGACTCCTCTGAGTGGTGGCCATCTTCGTCCCAGCCCTCGGTCACCCGCAGTTTCACTCCTGGCCACTGGTTCATCACCGAGATGGCCAAAGCGTTCAACTTGTCCTTACACCTCTGCGAAGACAAGGGGACCCCCACCGACGGACACGTTAGCCTGGGCAACCGCCACCCCTCCCGGCCCCTCCATCAGCCT
49	OSR2	TCTCACGACCCATCCGTTAACCCACCGTTCCCAGGAGCTCCGAGGCGCAGCGGCGACAGAGGTTCGCCCCGGCCTGCTAGCATTGGCATTGCGGTTGACTGAGCTTCGCCTAACAGGCTTGGGGAGGGTGGGCTGGGCTGGGCTGGGCTGGGCTGGGTGCTGCCCGGCTGTCCGCCTTTCGTTTTCCTGGGACCGAGGAGTCTTCCGCTCCGTATCTGCCTAGAGTCTGAATCCGACTTTCTTTCCTTTGGGCACGCGCTCGCCAGTGGAGCACTTCTTGTTCTGGCCCCGGGCTGATCTGCACGCGGACTTGAGCAGGTGCCAAGGTGCCACGCAGTCCCCTCACGGCTTTCGGGGGGTCTTGGAGTCGGGTGGGGAGGGAGACTTAGGTGTGGTAACCTGCGCAGGTGCCAAAGGGCAGAAGGAGCAGCCTTGGATTATAGTCACGGTCTCTCCCTCTCTTCCCTGCCATTTTTAGGGCTTTCTCTACGTGCTGTTGTCTCACTGGGTTTTTGTCGGAGCCCCACGCCCTCCGGCCTCTGATTCCTGGAAGAAAGGGTTGGTCCCCTCAGCACCCCCAGCATCCCGGAAAATGGGGAGCAAGGCTCTGCCAGCGCCCATCCCGCTCCACCCGTCGCTGCAGCTCACCAATTACTCCTTCCTGCAGGCCGTGAACACCTTCCCGGCCACGGTGGACCACCTGCAGGGCCTGTACGGTCTCAGCGCGGTACAGACCATGCACATGAACCACTGGACGCTGGGGTATCCCAAT

SEQ ID NO	GENE NAME	SEQUENCE
50	GLIS3	TGGTTTCCTTTCGCTTCTCGCCTCCCAAACACCTCCAGCAAGTCGGAGGGCGCGAACGCGGAGCCAGAAACCCTTCCCCAAAGTTTCTCCCGCCAGGT ACCTAATTGAATCATCCATAGGATGACAAATCAGCCAGGGCCAAGATTTCCAGACACTTGAGTGACTTCCCGGTCCCCGAGGTGACTTGTCAGCTCCA GTGAGTAACTTGGAACTGTCGCTCGGGGCAAGGTGTGTGTCTAGGAGAGAGCCGGCGGCTCACTCACGCTTTCCAGAGAGCGACCCGGGCCGACTTCA AAATACACACAGGGTCATTTATAGGGACTGGAGCCGCGCGCAGGACAACGTCTCCGAGACTGAGACATTTTCCAAACAGTGCTGACATTTTGTCGGGC CCCATAAAAAATGTAAACGCGAGGTGACGAACCCGGCGGGGAGGGTTCGTGTCTGGCTGTGTCTGCGTCCTGGCGGCGTGGGAGGTTATAGTTCCAGA CCTGGCGGCTGCGGATCGCCGGGCCGGTACCCGCGAGGAGTGTAGGTACCCTCAGCCCGACCACCTCCCGCAATCATGGGGACACCGGCTTGGATGAG ACACAGGCGTGGAAAACAGCCTTCGTGAAACTCCACAAACACGTGGAACTTGAAAAGACAACTACAGCCCCGCGTGTGCGCGAGAGACCTCACGTCAC CCCATCAGTTCCCACTTCGCCAAAGTTTCCCTTCAGTGGGGACTCCAGAGTGGTGCGCCCCATGCCCGTGCGTCCTGTAACGTGCCCTGATTGTGTAC CCCTCTGCCCCGCTCTACTTGAAATGAAAACACAAAAACTCTTCCGAATTACCCAACTTTAAACCCCCGTTATCTCTCTTCTACACTGCCCCGCTCTTA GGCCACTGACAGAAACATGGTTTGAACCCTAATTGTTGCTATCAGTCTCAGTCAGCGCAGGTCTCTCAGTGACCTGTGACGCCGGGAGTTGAGGTGCG CGTATCCTTAAACCCGCGCGAACGCCACCGGCTCAGCGTAGAAAACTATTTGTAATCCCTAGTTTGCGTCTCTGAGCTTTAACTCCCCCACACTCTCA AGCGCCCGGTTTCTCCTCGTCTCTCGCCTGCGAGCAAAGTTCCTATGGCATCCACTTACCAGGTAACCGGGATTTCCACAACAAAGCCCGGCGTGCGG GTCCCTTCCCCCGGCCGGCCAGCGCGAGTGACAGCGGGCGGCCGGCGCTGGCGAGGAGTAACTTGGGGCTCCAGCCCTTCAGAGCGCTCCGCGGGCTG TGCCTCCTTCGGAAATGAAAACCCCCATCCAAACGGGGGGACGGAGCGCGGAAACCCGGCCCAAGTGCCGTGTGTGCGCGCGCGTCTG
51	PRMT8	GAAAGCCATCCTTACCATTCCCCTCACCCTCCGCCCTCTGATCGCCCACCCGCCGAAAGGGTTTCTAAAAATAGCCCAGGGCTTCAAGGCCGCGCTTC TGTGAAGTGTGGAGCGAGCGGGCACGTAGCGGTCTCTGCCAGGTGGCTGGAGCCCTGGAAGCGAGAAGGCGCTTCCTCCCTGCATTTCCACCTCACCC CACCCCCGGCTCATTTTTCTAAGAAAAAGTTTTTGCGGTTCCCTTTGCCTCCTACCCCCGCTGCCGCGCGGGGTCTGGGTGCAGACCCCTGCCAGGTT CCGCAGTGTGCAGCGGCGGCTGCTGCGCTCTCCCAGCCTCGGCGAGGGTTAAAGGCGTCCGGAGCAGGCAGAGCGCCGCGCGCCAGTCTATTTTTACT TGCTTCCCCCGCCGCTCCGCGCTCCCCCTTCTCAGCAGTTGCACATGCCAGCTCTGCTGAAGGCATCAATGAAAACAGCAGTAG
52	TBX3	ATCGAAAATGTCGACATCTTGCTAATGGTCTGCAAACTTCCGCCAATTATGACTGACCTCCCAGACTCGGCCCCAGGAGGCTCGTATTAGGCAGGGAG GCCGCCGTAATTCTGGGATCAAAAGCGGGAAGGTGCGAACTCCTCTTTGTCTCTGCGTGCCCGGCGCGCCCCCCTCCCGGTGGGTGATAAACCCACTC TGGCGCCGGCCATGCGCTGGGTGATTAATTTGCGAACAAACAAAAGCGGCCTGGTGGCCACTGCATTCGGGTTAAACATTGGCCAGCGTGTTCCGAAG GCTTGT
53	chr12 group-00801	ATCAACATCGTGGCTTTGGTCTTTTCCATCATGGTGAGTGAATCACGGCCAGAGGCAGCCTGGGAGGAGAGACCCGGGCGGCTTTGAGCCCCTGCAGG GGAGTCCGCGCGCTCTCTGCGGCTCCCTTCCTCACGGCCCGGCCCGCGCTAGGTGTTCTTTGTCCTCGCACCTCCTCCTCACCTTTCTCGGGCTCTCA GAGCTCTCCCCGCAATCATCAGCACCTCCTCTGCACTCCTCGTGGTACTCAGAGCCCTGATCAAGCTTCCCCCAGGCTAGCTTTCCTCTTCTTTCCAG CTCCCAGGGTGCGTTTCCTCTCCAACCCGGGGAAGTTCTTCCGTGGACTTTGCTGACTCCTCTGACCTTCCTAGGCACTTGCCCGGGGCTTCTCAACC CTCTTTTCTAGAGCCCCAGTGCGCGCCACCCTAGCGAGCGCAGTAAGCTCATACCCCGAGCATGCAGGCTCTACGTTCCTTTCCCTGCCGCTCCGGGG GCTCCTGCTCTCCAGCGCCCAGGACTGTCTCTATCTCAGCCTGTGCTCCCTTCTCTCTTTGCTGCGCCCAAGGGCACCGCTTCCGCCACTCTCCGGGG GGTCCCCAGGCGATTCCTGATGCCCCCTCCTTGATCCCGTTTCCGCGCTTTGGCACGGCACGCTCTGTCCAGGCAACAGTTTCCTCTCGCTTCTTCCT ACACCCAACTTCCTCTCCTTGCCTCCCTCCGGCGCCCCCTTTTTAACGCGCCCGAGGCTGGCTCACACCCACTACCTCTTTAGGCCTTTCTTAGGCTC CCCGTGTGCCCCCCTCACCAGCAAAGTGGGTGCGCCTCTCTTACTCTTTCTACCCAGCGCGTCGTAGTTCCTCCCCGTTTGCTGCGCACTGGCCCTAA CCTCTCTTCTCTTGGTGTCCCCCAGAGCTCCCAGGCGCCCCTCCACCGCTCTGTCCTGCGCCCGGGGCTCTCCCGGGAATGAACTAGGGGATTCCACG

SEQ ID NO	GENE NAME	SEQUENCE
		CAACGTGCGGCTCCGCCCGCCCTCTGCGCTCAGACCTCCCGAGCTGCCCGCCTCTCTAGGAGTGGCCGCTGGGGCCTCTAGTCCGCCCTTCCGGAGCTCAGCTCCCTAGCCCTCTTCAACCCTGGTAGGAACACCCGAGCGAACCCCACCAGGAGGGCGACGAGCGCCTGCTAGGCCCTCGCCTTATTGACTGCAGCAGCTGGCCCGGGGGTGGCGGCGGGGTGAGGTTCGTACCGGCACTGTCCCGGGACAACCCTTGCAGTTGC
54	PAX9	ACAAATAAAACACCCTCTAGCTTCCCCTAGACTTTGTTTAACTGGCCGGGTCTCCAGAAGGAACGCTGGGGATGGGATGGGTGGAGAGAGGGAGCGGCTCAAGGACTTTAGTGAGGAGCAGGCGAGAAGGAGCACGTTCAGGCGTCAAGACCGATTTCTCCCCCTGCTTCGGGAGACTTTTGAACGCTCGGAGAGGCCCCGCCATCTCACCACTTTACTTGCCCCTACCGCCCTCCCCGCACCGCAGCAATCACGCAGCGCGCTCCCATTCGACACTCACGCTTTCGCCCCCGTTCCCCTATGTTCAGGGACCATATGGTTTGGGGACAGCCCCAGTAGTTAGTAGGGGACGGGTGCGTTCGCCCAGTCCCCGGATGCGTAGGGAGGCCCAGTGGCAGGCAGCTGTCCCAAGCAGCGGGTGCGCGTCCCTGCGCGCTGTGTGTTCATTTTGCAGAGCCAGCCTTCGGGGAGGTGAACCAGCTGGGAGGAGTGTTCGTGAACGGGAGGCCGCTGCCCAACGCCATCCGGCTTCGCATCGTGGAACTGGCCCAACTGGGCATCCGACCGTGTGACATCAGCCGCCAGCTACGGGTCTCGCACGGCTGCGTCAGCAAGATCCTGGCGCGATACAACGAGACGGGCTCGATCTTGCCAGGAGCCATCGGGGGCAGCAAGCCCCGGGTCACTACCCCCACCGTGGTGAAACACATCCGGACCTACAAGCAGAGAGACCCCGGCATCTTCGCCTGGGAGATCCGGGACCGCCTGCTGGCGGACGGCGTGTGCGACAAGTACAATGTGCCCTCCGTGAGCTCCATCAGCCGCATTCTGCGCAACAAGATCGGCAACTTGGCCCAGCAGGGTCATTACGACTCATACAAGCAGCACCAGCCGACGCCGCAGCCAGCGCTGCCCTACAACCACATCTACTCGTACCCCAGCCCTATCACGGCGGCGGCCGCCAAGGTGCCCACGCCACCCGGGGTGC
55	SIX1	AGGAGGCGCAACGCGCTGCCAGGGCGGCTTTATCCTGCCGCCACAGGGCGGGGACCAGCCCGGCAGCCGGGTGTCCAGCGCCGCTCACGTGCCTCGCCTGGAGCTTAGCTCTCAGACTCCGAAGAGGGCGACTGAGACTTGGGCCTGGGAGTTGGCTTCGGGGTACCCAAGGCGACGACAGCTGAGTTGTACCACGAAGCTCAGGCCGAGGCCTCCTCCCTTGTCTGGCCTTCGAATCCATACTGGCAGCCTCTCCTCTCAGGCACTCCGCGGGCCGGGCCACTAGGCCCCCTGCTCCTGGAGCTGCGCTATGATCCGGGTCTTGAGATGCGCGCGATTCTCTCTGAACCGGTGGAGAGGAGGCTCTGCCCCGCGCGGAGCGAGGACAGCGGCGCCCGAGCTTCCCGCGCCTCTCCAGGGCCCAATGGCAAGAACAGCCTCCGAAGTGCGCGGATGACAGGAAAAGATCTTCAGTTCTTCTGCCGCTAGAGAAGTGCGGGATACAAGCCTCTATTGGATCCACAACCTGGAGTCCTGCCTTCGGA
56	ISL2	ATCTGCGTGCCCTTTTCTGGGCGAGCCCTGGGAGATCCAGGGAGAACTGGGCGCTCCAGATGGTGTATGTCTGTACCTTCACAGCAAGGCTTCCCTTGGATTTGAGGCTTCCTATTTTGTCTGGGATCGGGGTTTCTCCTTGTCCCAGTGGCAGCCCCGCGTTGCGGGTTCCGGGCGCTGCGCGGAGCCCAAGGCTGCATGGCAGTGTGCAGCGCCCGCCAGTCGGGCTGGTGGGTTGTGCACTCCGTCGGCAGCTGCAGAAAGGTGGGAGTGCAGGTCTTGCCTTTCCTCACCGGGCGGTTGGCTTCCAGCACCGAGGCTGACCTATCGTGGCAAGTTTGCGGCCCCCGCAGATCCCCAGTGGAGAAAGAGGGCTCTTCCGATGCGATCGAGTGTGCGCCTCCCCGCAAAGCAATGCAGACCCTAAATCACTCAAGGCCTGGAGCTCCAGTCTCAAAGGTGGCAGAAAAGGCCAGACCTAACTCGAGCACCTACTGCCTTCTGCTTGCCCCGCAGAGCCTTCAGGGACTGACTGGGACGCCCCTGGTGGCGGGCAGTCCCATCCGCCATGAGAACGCCGTGCAGGGCAGCGCAGTGGAGGTGCAGACGTACCAGCCGCCGTGGAAGGCGCTCAGCGAGTTTGCCCTCCAGAGCGACCTGGACCAACCCGCCTTCCAACAGCTGGTGAGGCCCTGCCCTACCCGCCCCGACCTCGGGACTCTGCGGGTTGGGGATTTAGCCACTTAGCCTGGCAGAGAGGGGAGGGGGTGGCCTTGGGCTGAGGGGCTGGGTACAGCCCTAGGCGGTGGGGGAGGGGGAACAGTGGCGGGCTCTGAAACCTCACCTCGGCCCATTACGCGCCCTAAACCAGGTCTCCCTGGATTAAAGTGCTCACAAGAGAGGTCGCAGGATTAACCAACCCGCTCCCCCGCCCTAATCCCCCCCTCGTGCGCCTGGGGACCTGGCCTCCTTCTCCGCAGGGCTTGCTCTCAGCTGGCGGCCGGTCCCCAAGGGACACTTTCCGACTCGGAGCACGCGGCCCTGGAGCACCAGCTCGCGTGCCTCTTCACCTGCCTCTTCCCGGTGTTTCCGCCGCCCCAGGTCTCCTTCTCCGAGTCCGGCTCCCTAGGCAACTCCTCCGGCAGCGACGTGACCTCCCTGTCCTCGCAGCTCCCGGACACCCCCAACAGTATGGTGCCGAGTCCCGTGGAGACGTGAGGGGGACCCCTCCCTGCCAGCCCGCGGACCTCGCATGCTCCCTGCATGAGACTCAC

SEQ ID NO	GENE NAME	SEQUENCE
		CCATGCTCAGGCCATTCCAGTTCCGAAAGCTCTCTCGCCTTCGTAATTATTCTATTGTTATTTATGAGAGAGTACCGAGAGACACGGTCTGGACAGCCCAAGGCGCCAGGATGCAACCTGCTTTCACCAGACTGCAGACCCCTGCTCCGAGGACTCTTAGTTTTTCAAAACCAGAATCTGGACTTACCAGGGTTAGCTCTGCCCTCTCCTCTCCTCTCTACGTGGCCGCCGCTCTGTCTCTCCACGCCCCACCTGTGT
57	DLX4	AGGTCTCTTCAGACTGCCCATTCTCCGGGCCTCGCTGAATGCGGGGGCTCTATCCACAGCGCGCGGGGCCGAGCTCAGGCAGGCTGGGGCGAAGATCTGATTCTTTCCTTCCCGCCGCCAAACCGAATTAATCAGTTTCTTCAACCTGAGTTACTAAGAAAGAAAGGTCCTTCCAAATAAAACTGAAAATCACTGCGAATCACAATACTATACTACAAGTTCGTTTTCGCCCCCGCTCGCTCGCCATCCAGCACAAACGCCACCGATAATCCCCGACGCCCCCCCACTCAGCAGCACTATGGTCGCGGTGGAATCTCTGTTCCGCTGGCACATCCGCGCAGGTGCGGCTCTGAGTGCTGGCTCGGGGTTACAGACCTCGGCATCCGGCTGCAGGGGCAGACAGAGACCTCCTCTGCTAGGGCGTGCGGTAGGCATCGTATGGAGCCCAGAGACTGCCGAGAGCACTGCGCACTCACCAAGTGTTAGGGGTGCCCGTGATAGACCGCCAGGGAAGGGGCTGGTTCGGAGGGAATTCCCGCTACCGGGAAGGTCGGAACTCGGGGTGATCAAACAAGGAATGCATCTCACCTCCGTGGGTGCTTGTGCTGCGCAAGGAATTATTACCGGAGCGGTTGCGATGGCCTTTGCCCGGCGACCCAAGAAGAGTAAGCAAACTACCGTCCACCCAGCGGATCAGGTCCAAT
58	CBX4	GATGTCCTGTTTCTAGCAGCCTCCAGAGCCAAGCTAGGCGAGAGGCGTAGGAGGCAGAGAGAGCGGGCGCGGGAGGCCAGGGTCCGCCTGGGGGCCTGAGGGGACTTCGTGGGGTCCCGGGAGTGGCCTAGAAACAGGGAGCTGGGAGGGCCGGGAAGAGCTTGAGGCTGAGCGGGGGACGAACGGGCAGCGCAAAGGGGAGATGAACGGAATGGCCGAGGAGCCACGCATTCGCCTTGTGTCCGCGGACCCTTGTTCCCGACAGGCGACCAAGCCAAGGCCCTCCGGACTGACGCGGCCTGAGCAGCAGCGAGTGTGAAGTTTGGCACCTCCGGCGGCGAGACGGCGCGTTCTGGCGCGCGGCTCCTGCGTCCGGCTGGTGGAGCTGCTGCGCCCTATGCGGCCTGCCGAGGGCGCCGCCGAGGGCCCGCGAGCTCCGTGGGGTCGGGGTGGGGGGACCCGGGAGCGGACAGCGCGGCCCGAGGGGCAGGGGCAGGGGCGCGCCTGGCCTGGGGTGTGTCTGGGCCCCGGCTCCGGGCTCTTGAAGGACCGCGAGCAGGAGGCTTGCGCAATCCCTTGGCTGAGCGTCCACGGAGAAAGAAAAAGAGCAAAAGCAGAGCGAGAGTGGAGCGAGGGATGGGGGCGGGCAAAGAGCCATCCGGGTCTCCACCACCGCCCTGACACGCGACCCGGCTGTCTGTTGGGGACCGCACGGGGGCTCGGGCGAGCAGGGGAGGGAGGAGCCTGCGCGGGGCTCGTGTTCGCCCAGGAATCCCGGAGAAGCTCGAAGACGGTCTGGTGTTGAACGCACACGTGGACTCCATTTCATTACCACCTTGCAGCTCTTGCGCCACGGAGGCTGCTGCTGCCCGGCGGCTGCTACCCACCGAGACCCACGTGGCCCCTCCCCAGGGGTGTAGGGGTGACGGTTGTCTTCTGGTGACAGCAGAGGTGTTGGGTTTGCGACTGATCTCTAACGAGCTTGAGGCGCAAACCTAGGATTCCCTGAGTGTTGGGGTGCGGCGGGGGGGCAAGCAAGGTGGACGACGCCTGCCTGGTTTCCCTGACTAGTTGCGGGGGGTGGGGGCCGGCTCTCAGGGGCCACCAGAAGCTGGGTGGGTGTACAGGAAAATATTTTTCTCCTGCCGTGTTTGGCTTTTTCCTGGCATTTTTGCCCAGGGCGAAGAACTGTCGCGCGGGGCAGCTCCACCGCGGAGGGAGAGGGGTCGCGAGGCTGGCGCGGGAAGCGCTGTAGGTGGCAGTCATCCGTCCACGCCGCACAGGCCGTCTGCGCCGTCGGACCATCGGGAGGTCTGCAGCAACTTTGTCCCGGCCAGTCCCCTTGTCCGGGAAGGGGCTGAGCTTCCCGACACTCTACCCTCCCCCTCTTGAAAATCCCCTGGAAAATCTGTTTGCAATGGGTGTTTCCGCGGCGTCCAGGTCTGGGCTGCCGGGGGAGGCCGAGCGGCTGCTGCAGCCTCCCTGCTGCCAGGGGCGTCGGACTCCGCTTCGCTCACTACGCCCAGGCCCCTCAGGGGCCCACGCTCAGGACTTCGGGGCCACACAGCAGGACCCGGTGCCCCGACGACGAGTTTGCGCAGGACCCGGGCTGGGCCAGCCGCGGAGCTGGGGAGGAAGGGGCGGGGGTCGGTGCAGCGGATCTTTTCTGTTGCTGCCTGTGCGGCGGCAGGAAGCGTCTTGAGGCTCCCCAAGACTACCTGAGGGGCCGCCCAAGCACTTCAGAAGCCCAAGGAGCCCCCGGCCACCCCCGCTCCTGGCCTTTTTGCCAACGACTTTGAAAGTGAAATGCACAAGCACCAGCAATTGACTTCCCTTCCGTGGTTATTTATTTTGTCTTTGTGGATGGTGGGCAGATGGGGAGAGAGGCCCCTACCTAACCTCGGTGGCTGGTCCCTAGACCACCCCTGCCAGCCGGTGTGGGGAGGAGCTCAGGTCCGCGGGAGAGCGAATGGGCGCCAGGAGGTGGGACAGAATCCTGGGAAGGTACAGCGGACGCCCTGGAAGCTCCCCTGATGCCCCAGAGGGCCCTTCCTGGGAAACCTCCCGGGGGGGTGCCCCATACCATCCCACCCGGCTGTCTTGGCCCCTCCCAGGGAGCCGCAGGAGAAACTAGCCCTACACCTGGGATTCCCAG

SEQ ID NO	GENE NAME	SEQUENCE
		AGCCTTCTGCTGGGGCTCCTGCCCCCGACTTCGGATAACCAGCTCCGCACAGGTCCCCGAGAAGGGCCGCTGGCCTGCTTATTTGATACTGCCCCCTCCCAGACAGGGGCTGGTCGAGCCCCTGGTTCTGCTGCCAGACTGAAGCCTTCCAGACGCCACCTCGGTTTGGGCCCCCAGGGCCCTCAGGGGCCCCAGGAGAGGAGAGCTGCTATCTAGCTCAGCCACAGGCTCGCTCCTGGTGGGGGCCAGGCTGAAGGAGTGGACCCTGGAGAGGTCGGGAACCTTTTAACAGCCGTGGGCTGGAGGGTGGCTACTAAGTGTTCGGTCTGGGAAGAGGCATGACCCGCACCATCCCGGGGAAATAAACGACTTCTTAAGGGAATCTTCTCGCTGAGCGGGTGCTCTGGGCCAGGAGATTGCCACCGCCAGCCCACGGAACCCAGATTTGGGCTCTGCCTTGAGCGGGCCGCCTGTGGCTTCCCGGGTCGCTCCCCCGACTCAGAAAGCTCTCAAGTTGGTATCGTTTTCCCGGCCCTCGGAGGTGGATTGCAGATCACCGAGAGGGGATTTACCAGTAACCACTACAGAATCTACCCGGGCTTTAACAAGCGCTCATTTCTCTCCCTTGTCCTTAGAAAAACTTCGCGCTGGCGTTGATCATATCGTACTTGTAGCGGCAGCTTAGGGGCAGCGGAACTGGTGGGGTTGTGCGTGCAGGGGGAGGCTGTGAGGGAGCCCTGCACTCCGCCCCTCCACCCTTCTGGAGGAGTGGCTTTGTTTCTAACGGTCCCCCCCCAACCCCCCGCTCCCCACTTCAATCTTTCTGCTCTTTGTCCCACCGCCCCGTCAAAGCTCCCCTTTCATTTGCTCCCCCAAGCCTCCCACGCCCCCGAGTCCCACCCTAGCGGGCCGCGCGGCACTGCAGCCGGGGGTTCCTGCGGACTGGCCCGACAGGGTGCGCGGACGGGGACGCGGGCCCCGAGCACCGCGACGCCAGGGTCCTTTGGCAGGGCCCAAGCACCCCT
59	EDG6	TGGCGGCCGGCGGCACAGCCGGCTCATTGTTCTGCACTACAACCACTCGGGCCGGCTGGCCGGGCGCGGGGGGCCGGAGGATGGCGGCCTGGGGGCCCTGCGGGGGCTGTCGGTGGCCGCCAGCTGCCTGGTGGTGCTGGAGAACTTGCTGGTGCTGGCGGCCATCACCAGCCACATGCGGTCGCCACGCTGGGTCTACTATTGCCTGGTGAACATCACGCTGAGTGACCTGCTCACGGGCGCGGCCTACCTGGCCAACGTGCTGCTGTCGGGGGCCCGCACCTTCCGTCTGGCGCCCGCCCAGTGGTTCCTACGGGAGGGCCTGCTCTTCACCGCCCTGGCCGCCTCCACCTTCAGCCTGCTCTTCACTGCAGGGGAGCGCTTTGCCACCATGGTGCGGCCGGTGGCCGAGAGCGGGGCCACCAAGACCAGCCGCGTCTACGGCTTCATCGGCCTCTGCTGGCTGCTGGCCGCGCTGCTGGGGATGCTGCCTTTGCTGGGCTGGAACTGCCTGTGCGCCTTTGACCGCTGCTCCAGCCTTCTGCCCCTCTACTCCAAGCGCTACATCCTCTTCTGCCTGGTGATCTTCGCCGGCGTCCTGGCCACCATCATGGGCCTCTATGGGGCCATCTTCCGCCTGGTGCAGGCCAGCGGGCAGAAGGCCCCACGCCCAGCGGCCCGCCGCAAGGCCCGCCGCCTGCTGAAGACGGTGCTGATGATCCTGCTGGCCTTCCTGGTGTGCTGGGGCCCACTCTTCGGGCTGCTGCTGGCCGACGTCTTTGGCTCCAACCTCTGGGCCCAGGAGTACCTGCGGGGCATGGACTGGATCCTGGCCCTGGCCGTCCTCAACTCGGCGGTCAACCCCATCATCTACTCCTTCCGCAGCAGGGAGGTGTGCAGAGCCGTGCTCAGCTTCCTCTGCTGCGGGTGTCTCCGGCTGGGCATGCGAGGGCCCGGGGACTGCCTGGCCCGGGCCGTCGAGGCTCACTCCGGAGCTTCCACCACCGACAGCTCTCTGAGGCCAAGGGACAGCTTTC
60	chr13 group-00005	TAGTAAGGCACCGAGGGGTGGCTCCTCTCCCTGCAGCGGCTGTCGCTTACCATCCTGTAGACCGTGACCTCCTCACACAGCGCCAGGACGAGGATCGCGGTGAGCCAGCAGGTGACTGCGATCCTGGAGCTGGTCGCAGCAGGCCATCCTGCACGCGGTGGAGGCGCCCCCTGCAGGCCGCAGCGCATCCCCAGCTTCTGGACGCACTGTGAGCGGTTATGCAGCAGCACGCTCATATGAGATGCCCCGCAGGGTGCTATGCAGGCCCACGTCCCCACAAAGCCCATGGCAGGCGCCCGGGTGCCGGAGCACGCACTTGGCCCCATGGATCTCTGTGCCCAGGGCTCAGCCAGGCATCTGGCCGCTAAAGGTTT
61	CRYL1	TCTCATCTGAGCGCTGTCTTTCACCAGAGCTCTGTAGGACTGAGGCAGTAGCGCTGGCCCGCCTGCGAGAGCCCGACCGTGGACGATGCGTCGCGCCCTTCCCATCGCGGCCTGGGCGGGCCCGCCTGCCCTCGGCTGAGCCCGGTTTCCCTACCCCGGGGCACCTCCCCTCGCCCGCACCCGGCCCCAGTCCCTCCCAGGCTTGCGGGTAGAGCCTGTCTTTGCCCAGAAGGCCGTCTCCAAGCT
62	IL17D	CAGTCCCCGAGGCCCTCCCCGGTGACTCTAACCAGGGATTTCAGCGCGCGGCGCGGGGCTGCCCCCAGGCGTGACCTCACCCGTGCTCTCTCCCTGCAGAATCTCCTACGACCCGGCGAGGTACCCCAGGTACCTGCCTGAAGCCTACTGCCTGTGCCGGGGCTGCCTGACCGGGCTGTTCGGCGAGGAGGACGTGCGCTTCCGCAGCGCCCCTGTCTACAT

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63	IRS2	AGAGAGACATTTTCCACGGAGGCCGAGTTGTGGCGCTTGGGGTTGTGGGCGAAGGACGGGGACACGGGGGTGACCGTCGTGGTGGAGGAGAAGGTCTC GGAACTGTGGCGGCGGCGGCCCCCCTGCGGGTCTGCGCGGATGACCTTGGCGCCGCGGTGGGGGTCCGGGGGCTGGCTGGCCTGCAGGAAGGCCTCGA CTCCCGACACCTGCTCCATGAGGCTCAGCCTCTTCACGCCCGACGTCGGGCTGGCCACGCGGGCAGCTTCTGGCTTCGGGGGGGCCGCGATAGGTTGC GGCGGGGTGGCGGCCACACCAAAAGCCATCTCGGTGTAGTCACCATTGTCCCCGGTGTCCGAGGACAACGATGAGGCGGCGCCCGGGCCCTGGGCGGT GGCAACGGCCGAGGCGGGGGGCAGGCGGTACAGCTCCCCCGGGGCCGGCGGCGGTGGCGGCGGCTGCAGAGACGACGACGGGGACGCGGACGGACGCG GGGGCAACGGCGGATACGGGGAGGAGGCCTCGGGGGACAGGAGGCCGTCCAAGGAGCCCACGGGGTGGCCGCTCGGGGCGCCCGGCTTAGGAGACTTG GGGGAGCTGAAGTCGAGGTTCATGTAGTCGGAGAGCGGAGACCGCTGCCGGCTGTCGCTGCTGGTGCCCGGGGTGCCTGAGCCCAGCGACGAGGCCGG GCTGCTGGCGGACAAGAGCGAGGAGGACGAGGCCGCCGACGCCAGCAGGGGAGGCGCGGGCGGCGACAGGCGGGCCCCGGGCTCGCCAAAGTCGATGT TGATCTACTCGCCCGGCCCTCTTCGCCTCCCGTCGCCACTGCGTACTCCTGCATGCTCGCCAGGCTGCGCACCCCCTCCACGGACACGCCGCTGCGCCCTC ACCGCCCGGCCGCGCTGGCCCAAGAAGCCCTCCGGGCGGCCGCCGCTAGGCCGCACGGGCGAAGGCACTACAGGGTGAGGGGGCTGCGTGGGGCCGGC CCCGAAGGCGCTGGCCGCCTGGCTGGGCCCTGGCGTGGCCTGAGGCTCCAGACGCTCCTCCTCCAGGATGCGCCCCACGGGGGAGCTCATGAGCACGT ACTGGTCGCTGTCCCCGCCACAGGTGTAGGGGGCCTTGTAGGAGCGGGGCAAGGAGCTGTAGCAGCAGCCGGGAACGCCCCTGAGCGGCTCCCCGCCG GGGTGCAGGGCTGCGGAGAAGAAGTCGGGCGGGGTGCCCGTGGTGACCGCGTCGCTGGGGGACACGTTGAGGTAGTCCCCGTTGGGCAGCAGCTTGCC ATCTGCATGCTCCATGGACAGCTTGGAACCGCACCACATGCGCATGTACCCACTGTCCTCGGGGGAGCTCTCGGCGGGCGAGCTGGCCTTGTAGCCGC CCCCGCTCGCCGGGAATGTCCTGCCCGCCGCAGAGGTGGGTGCTGGCCCCGCAGGCCCCGCAGAAGGCACGGCGGCGGCGGCGGCGGCGGCCCTG GGCTCCAAGATCTGCTTGGGGCGGACACGCTGGCGGGCTCATGGCATGTAGTCGTCGCTCCTGCAGCTGCCGCTCCCACTGCCCGCGAGGGCCGC GCCGGGCGTCATGGGCATGTAGCCGTCGTCTGCCCCCAGGTTGCTGCTGGAGCTCCTGTGGGAGCCGATCTCGATGTCTCCGTAGTCCTCTGGGTAGG GGTGGTAGGCCACCTTGGGAGAGGACGCGGGGCAGGACGGGCAGAGGCGGCCCGCGCTGCCCGAGAAGGTGGCCCGCATCAGGGTGTATTCATCCAGC GAGGCAGAGGAGGGCTGGGGCACCGGCCGCTGCCGGGCTGGCGTGGTCAGGGAGTAGGTCCTCTTGCGCAGCCCTCGGTCCAGGTCCTGGGCCGCGTC CCCCGAGACCCGGCGGTAGGAGCGGCCACAGTGGCTCAGGGGCCTGTCCATGGTCATGTACCCGTAGAACTCACCGCCGCCGCCGCCGTCTCGGGCCG GGGGCGTCTCCGCGATGGACTCGGGCGTGTTGCTTCGGTGGCTGCAGAAGGCGCGCAGGTCGCCTGGGCTGGAGCCGTACTCGTCCAGGGACATGAAG CCGGGGTCGCTGGGGGAGCCCGAGGCGGAGGCGCTGCCGCTGGAGGGCCGCTGGCCGGGGCCGTGGTGCAGCGGATGCGGCAGAGGCGGGTGCGGGCC GGGCGGCGGCGGCTAGGAGCCCGAGCCGTGGCCGCTGCTGGACGACAGGGAGC
64	chr13 group-00350	TAACCTAAAGAATGAAGTCATGCCCCGGCCTGCACCCGGGAAACTGCACACAGCGAAAGATCGCCACTGAGATAAAGAGCTGAAAGCTATTCCCCAAT TCAGCTGTTTCAGCCGTGCGGTCTCACAATGGGCTCACAGACGGCAGCATC
65	MCF2L	GTTTCCACAATCCACCTCGTAGCTGGGGCGTGCCGCTTGCCTCGGCTTGTCCCGGCAGAACACTCTTACCTTTAATGGCGACTGAAAAGTTGCCACGA GTTCCTGATCATTGTGGTAGGTGCTGCGTGAAGCTGAGACGTGCGTGAGCCACATCCCAGGGGGCTTTGAGCCCCCACCGCGGCGGCGGCTGAGGGGA GGCTTGTCGTACTCGCACAGGAGGACACAGGGCTGCAGTGTTCACTCCAGGGCCTCTTATCATTGGGATCTGAGGAATTTTCCGAGAGGAAGTGCGAA TTAACAATGATGAAAGGTTTGTGAGTGAGTGACAGGCACGTTCTATTGAGCACTGCATGGGGCATTATGTGCCACCAGAGACGGGGGCAGAGGTCAAG AGCCCTCGAGGGCTGGGAGAGTTCGGAGGATAGAAGTCATCAGAGCACAATGAAGCCAGACCCTGCAGCCGCCTTCCCCTTCGGGGGCTTCCTTAGAA TGCAGCATTGCGGGGACTGAGCTGTCCCAGGTGAAGGGGGGCCGTCACGGTGTGTGGACGCCCCTCGGCTCAGCCCTCTAAGAGACTCGGCAGCCAGG

SEQ ID NO	GENE NAME	SEQUENCE
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66	F7	TCCAGCTGCAGCGAGGGCGGCCAGGCCCCCTTCTCCGACCTGCAGGGGTAGCGCGGCCTCGGCGCCGGAGACCCGCGCGCTGTCTGGGGCTGCGGTGGCGTGGGGAGGGCGCGGCCCCCGGACGCCCCGAGGAAGGGGCACCTCACCGCCCCCACCCAGAGCGCCTGGCCGTGCGGGCTGCAGAGGACCCCTCCGGGGCACAGCCAGGTTCCACGGAAGACCCCGCCCCCGCTGGCCCTTCCCCGGACACTCCAGAG
67	chr18 group-00039	ACTTACTGCTTCCAAAAGCGCTGGGCACAGCCTTATATGACTGACCCCGCCCCCGAGTCCCAGGCCGCCCCATGCAACCGCCCAACCGCCCAACCGCCACTCCAAAGGTCACCAACCACTGCTCCAGGCCACGGGCTGCCTCTCCCCACGGCTCTAGGGCCCTTCCCCTCCACCGCAGGCTGAC
68	C18orf1	TGCCACACCCAGGTACCGCCCGCCCGCGCGAGAGCCGGGCAGGTGGGCCGCGGATGCTCCCAGAGGCCGGCCCAGCAGAGCGATGGACTTGGACAGGCTAAGATGGAAGTGACCTGAG
69	CD33L3	TCGCCAGCGCAGCGCTGGTCCATGCAGGTGCCACCCGAGGTGAGCGCGGAGGCAGGCGACGCGGCAGTGCTGCCCTGCACCTTCACGCACCCGCACCGCCACTACGACGGGCCGCTGACGGCCATCTGGCGCGCGGGCGAGCCCTATGCGGGCCCGCAGGTGTTCCGCTGCGCTGCGGCGCGGGGCAGCGAGCTCTGCCAGACGGCGCTGAGCCTGCACGGCCGCTTCCGGCTGCTGGGCAACCCGCGCCGCAACGACCTCTCGCTGCGCGTCGAGCGCCTCGCCCTGGCTGACGACCGCCGCTACTTCTGCCGCGTCGAGTTCGCCGGCGACGTCCATGACCGCTACGAGAGCCGCCACGGCGTCCGGCTGCACGTGACAGGCGAGGCGGCGTGGGAGCGGGTCCCCGGCCTCCCTTCCCGCCCTCCCGCCTGCCCCGCCCCAAGGGCTACGTGGGTGCCAGGCGCTGTGCTGAGCCAGGAAGGGCAACGAGACCCAGCCCTCTCCTCTACCCCAGGGATCTCACACCTGGGGGTAGTTTAGGACCACCTGGGAGCTTGACACAAATGCAGAATCCAGGTCCCAGGAAGGGCTGAGGTGGGCCCGGGAATAGGCATTGCCGTGACTCTCGTAGAGTGACTGTCCCCAGTGGCTCTCAGACGAAGAGGCGAGAAAGACAAGTGAATGGCAATCCTAAATATGCCAAGAGGTGCAATGTGGTGTGTGCTACCAGCCCGGAAAGACACTCGCAGCCCCTCTACCCAGGGGTGCACAGACAGCCCACCAAGTAGTGCCTAGCACTTTGCCAGACCCTGATATACAAAGATGCCTGAACCAGGGTCCCGTCCCTAGAGCAGTGGCTCTCCACTCTAGCCCCCACCCTGCTCTGCGACAATAATGGCCACTTAGCATTTGCTAGGGAGCCGGGACCTAGTCCAAGCACCCACAAGCATGAATTTGCCAAATCTTTTCAGCAACCTCTTAAGGCAACTGCTATCATGATCCTCACTTTACACATGGAGAAGCAGAAGCAGAGATGATAGAATCTTTCGCCCAAGGCCACATCTGTATTGGGACGGGGGCAGCCTGGCACCCAAGTGCCCATTCCTCCCTTCTGACCAGCCCCCACCCCTCCGGCTCTGGCGTCCAAAGGGCTAAGGGGAGGGGTGCCCTTGTGACAGTCACCCGCCTTCTCCCCTGCAGCCGCGCCGCGGATCGTCAACATCTCGGTGCTGCCCAGTCCGGCTCACGCCTTCCGCGCGCTCTGCACTGCCGAAGGGGAGCCGCCGCCCGCCCTCGCCTGGTCCGGCCCGGCCCTGGGCAACAGCTTGGCAGCCGTGCGGAGCCCGCGTGAGGGTCACGGCCACCTAGTGACCGCCGAACTGCCCGCACTGACCCATGACGGCCGCTACACGTGTACGGCCGCCAACAGCCTGGGCCGCTCCGAGGCCAGCGTCTACCTGTTCCGCTTCCATGGCGCCAGCGGGGCCTCGACGGTCGCCCTCCTGCTCGGCGCTCTCGGCTTCAAGGCGCT
70	TNFRSF11A	ATGAACTTCAAGGGCGACATCATCGTGGTCTACGTCAGCCAGACCTCGCAGGAGGGCGCGGCGGCGGCTGCGGAGCCCATGGGCCGCCCGGTGCAGGAGGAGACCCTGGCGCGCCGAGACTCCTTCGCGGGGAACGGCCCGCGCTTCCCGGACCCGTGCGGCGGCCCCGAGGGGCTGCGGGAGCCGGAGAAGGCCTCGAGGCCGGTGCAGGAGCAAGGCGGGGCCAAGGCTTGAGCGCCCCCCATGGCTGGGAGCCCGAAGCTCGGAGC
71	ZNF236	TCAGTGTTATGTGGGGAGCGCTAGATCGTGCACACAGTAGGCGTCAGGAAGTGTTTTCCCCAGTAATTTATTCTCCATGGTACTTTGCTAAAGTCATGAAATAACTCAGATTTTGTTTTCCAAGGAAGGAGAAAGGCCCAGAATTTAAGAGCAGGCAGACACACAACCGGGCACCCCCAGACCCTGGCCCTTCCAG

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72	chr18 group-00342	GTGGAAACACAACCTGCCTTCCATTGTCTGCGCCTCCAAAACACACCCCCCGCGCATCCGTGAAGCTGTGTGTTTCTGTGTTACTACAGGGGCCGGCTGTGGAAATCCCACGCTCCAGACCGCGTGCCGGGCAGGCCCAGCC
73	OLIG2	TCCACACCTCGGGCAGTCACTAGGAAAAGGGTCGCCAACTGAAAGGCCTGCAGGAACCAGGATGATACCTGCGTCAGTCCCGCGGCTGCTGCGAGTGCGCGCTCTCCTGCCAGGGGGACCTCAGACCCTCCTTTACAGCACACCGAGGGCCCTGCAGACACGCGAGCGGGCCTTCAGTTTGCAAACCCTGAAAGCGGGCGCGGTCCACCAGGACGATCTGGCAGGGCTCTGGGTGAGGAGGCCGCGTCTTTATTTGGGGTCCTCGGGCAGCCACGTTGCAGCTCTGGGGGAAGACTGCTTAAGGAACCCGCTCTGAACTGCGCGCTGGTGTCCTCTCCGGCCCTCGCTTCCCCGACCCCGCACAGGCTAACGGGAGACGCGCAGGCCCACCCCACCGGCTGGAGACCCCGGCACGGCCCGCATCCGCCAGGATTGAAGCAGCTGGCTTGGACGCGCGCAGTTTTCCTTTGGCGACATTGCAGCGTCGGTGCGGCCACAATCCGTCCACTGGTTGTGGGAACGGTTGGAGGTCCCCCAAGAAGGAGACACGCAGAGCTCTCCAGAACCGCCTACATGCGCATGGGGCCCAAACAGCCTCCCAAGGAGCACCCAGGTCCATGCACCCGAGCCCAAAATCACAGACCCGCTACGGGCTTTTGCACATCAGCTCCAAACACCTGAGTCCACGTGCACAGGCTCTCGCACAGGGGACTCACGCACCTGAGTTCGCGCTCACAGATC
74	RUNX1	CTGCCCTCGCGGATCTCCCCCGGCCTCGCCGGCCTCCGCCTGTCCTCCCACCACCCTCTCCGGGCCAGTACCTTGAAAGCGATGGGCAGGGTCTTGTTGCAGCGCCAGTGCGTAGGCAGCACGGAGCAGAGGAAGTTGGGGCTGTCGGTGCGCACCAGCTCGCCCGGGTGGTCGGCCAGCACCTCCACCATGCTGCGGTCGCCGCTCCTCAGCTTGCCGGCCAGGGCAGCGCCGGCGTCCGGGGCGCCCAGCGGCAACGCCTCGCTCATCTTGCCTGGGCTCAGCGCGGTGGAAGGCGGCGTGAAGCGGCGGCTCGTGCTGGCATCTACGGGGATACGCATCACAACAAGCCGATTGAGTTAGGACCCTGCAAACAGCTCCTACCAGACGGCGACAGGGGCGCGGATCTTCAGCAAGCAGCTCCCGGGAGACCAACATACACGTTCAGGGGCCTTTATTACTGCGGGGGTGGGGGGGGCGGGGGTGGTTAGGGGAGGAGGGAGACTAAGTTACTAACAGTCCAGGAGGGGAAAACGTTCTGGTTCTGCGGATCGGCCTCTGACCCAGGATGGGCTCCTAGCAACCGATTGCTTAGTGCATTAAAAAGTGGAGACTATCTTCCACGAATCTTGCTTGCAGAGGTTAAGTTCTGTCTTTGGCTGTTAGAAAAGTTCCTGAAGGCAAAATTCTCATACACTTCCTAAAATATTTATGCGAAGAGTAAAACGATCAGCAAACACATTATTTGGAAGTTCCAGTAGTTAATGCCTGTCAGTTTTTGCAGGTGAGTTTTGTCTAAAGTCCCAACAGAACACAATTATCTCCCGTAACAAGGCCACTTTTATCATGCAAAACTGGCTTCAGTCCCGAAAAGCAAGAGCTGAGACTTCCAAAGGTAGTGCTACTAATGTATGTGCACGTATATATAAATATATACATATGCTCTACTTCATAAAATATTTACAATACAATCTGTGGAGAATTTAAACACAACAGAAATCCATTAATGTACGCTGCAGATTTTTTAAGTAGCCTTGAAAATCAGCTTCAGTAGTTGGAGCAGTGCTGAGCTAGAAGTACTTGTCATGTTCTCTGTTCTCTCAATGAATTCTGTCAAAACGCTCAGTGCAGAAAATTCAGCGTTTCAGAGATCTTCAGCTAATCTTAAAACAACAATCATAAGAAGGCCCAGTCGATGACACTCAGGGTTCTACAGCTCTCCCACATCTGTGAACTCGGGTTTGGGGATGTTGGTTAAGTTTGTGGCTGGTCCTCTGGTTTGTTGGGAGTTGAGCAGCCGCAGAGTCACACACATGCAAACACGCACTCTTCGGAAGGCAGCCACTGTCTACATCAGCTGGGTGACTCAGCCCTGACTCGGGCAGCAGCGAGACGATACTCCTCCACCGTCGCCCAGCACCCGCCGGTTAGCTGCTCCGAGGCACGAACACCCACGAGCGCCGCGTAACCGCAGCAGGTGGAGCGGGCCTTGAGGGAGGGCTCCGCGGCGCAGATCGAAACAGATCGGGCGGCTCGGGTTACACACGCACGCACATCCTGCCACGCACACTGCCACGCACACGCAACTTCACGGCTCGCCTCGGACCACAGAGCACTTTCTCCCCCTGTTGTAAAAGGAAAACAATTGGGGAAAAGTTCGCAGCCAGGAAAGAAGTTGAAAACATCCAGCCAAGAAGCCAGTTAATTCAAAAGGAAGAAAGGGGAAAAACAAAAAAAAACAACAAAAAAAGGAAGGTCCAACGCAGGCCAAGGAGAAGCAGCAGAGGTTGACTTCCTTCTGGCGTCCCTAGGAGCCCCGGAAAGAAGTGCCTGGCGGCGCAGGGCCGGGCAGCGTGGTGCCCTGGCTGGGTCCGGCCGCGGGGCGCCCGTCCCGCCCGCGCCCGCTGGCTCTATGAATGAGAGTGCCTGGAAATGAACGTGCTTTTACTGTAAGCC

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75	AIRE	TTCGGAAGTGAGAGTTCTCTGAGTCCCGCACAGAGCGAGTCTCTGTCCCCAGCCCCCAAGGCAGCTGCCCTGGTGGGTGAGTCAGGCCAGGCCCGGAG ACTTCCCGAGAGCGAGGGAGGGACAGCAGCGCCTCCATCACAGGGAAGTGTCCCTGCGGGAGGCCCTGGCCCTGATTGGGCGCCGGGGCGGAGCGGCC TTTGCTCTTTGCGTGGTCGCGGGGGTATAACAGCGGCGCGCGTGGCTCGCAGACCGGGGAGACGGGCGGGCGCACAGCCGGCGCGGAGGCCCCACAGC CCCGCCGGGACCCGAGGCCAAGCGAGGGGCTGCCAGTGTCCCGGGACCCACCGCGTCCGCCCCAGCCCCGGGTCCCCGCGCCCACCCCATGGCGACGG ACGCGGCGCTACGCCGGCTTCTGAGGCTGCACCGCACGGAGATCGCGGTGGCCGTGGACAG
76	SUMO	ACGCACACTGGGGGTGTGATGGAAAGGGGGACGCGATGGATAGGGGTGGGCGCACACTGGGGGACGCGACGGGGAGGGGTGAGCACACACTGGGGGTG TGATGGAGAGGGCGACGCAATAGGGAGGGGTGGGCGCACACCAGGGACGCGATGATGGGGACGGGTGGGCGCACACCAGGTGGCATGATGGGGAGGAG

SEQ ID NO	GENE NAME	SEQUENCE
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77	C21orf 70	CCACAGGGTGGGGTGCGCCCACCTGCCCTGTCCATGTGGCCTTGGGCCTGCGGGGGAGAGGGAATCAGGACCCACAGGGCGAGCCCCCTCCGTAGCCCGCGGCACCGACTGGATCTCAGTGAACACCCGTCAGCCCATCCAGAGGCTAGAAGGGGGA
78	C21orf 123	TTGAGGTCTCTGTGCATGCTTGTGCGTACCCTGGACTTTGCCGTGAGGGGTGGCCAGTGCTCTGGGTGCCTTTGCCAGACAACTGGTCTGCCGGGCCGAGCATTCATGCTGGTC
79	COL18 A1	TGACGCGCCCCTCTCCCCGCAGCTCCACCTGGTTGCGCTCAACAGCCCCCTGTCAGGCGGCATGCGGGGCATCCGCGGGGCCGACTTCCAGTGCTTCCAGCAGG
80	PRRT3	AACACACTGTCTCGCACTAGGTGCTCGCGGAAGAGCGCGGCGTCGATGCTGCGGCTCAGGTTGATGGGCGATGGCGGCCGCAGATCCAGCTCGCTCAGCGATGGCGCCGGTCCCACACCGTTGCGGGACAGTCCCGGGCCACCCTGGGGTCCGCGACCCAACGACGCAGCCGAGCCCCAGGCGCCTGAACTGGGCGTGGCCAGCTGCCCACTCTCCGCCGGGTTGCGGATGAGGCTCTTGCTGATGTCCAAGCTGCCTGCACCAACGTTGCTGGGCCCTGCATAGCAGTTATTGGGTCGCTCCGGCACCTCGCTCTTTCCTGACGGCGCCGGGCACGCCAGACGCATCAGCTTAGCCCAGCAAGCGTGCTCCGTGGGCGGCCTGGGTCTCGCGGCAGCCACCGCGGCCAACGCCAGGGCGAGCGCCCATGTCAGCTCCAGGAGGCGCAGCCAGAAGTGGACACCCCACCAGGCCCACGAGAAGCGGCCCACGCGGCCTGGGCCCGGGTACAGCCAGAGCGCAGCCGCCAGCTGCAAGCCGCTAGCCAGCAGCCCCAGCGCGCCCGCCACAGCCAACAGCCGAGGGCCCGGGCTGGCATCCCAGCCCCGTGGGCCGTCCAGCAGGCGGCGACGGCACAGGCAGAGCGTGCCCAGAGCCAC
81	MGC29 506	GTCTCCACGAAGCCCCGCGCGGCCTGCAGGGGGCCCAGCGACTCGTCCAGGGAACCGGTGCGCAGGACCAGCCGGGGGCGCGGCGCGCCGGCCGCCCTTGGGGGACTCTGGGGCCGGGGGCGCAGCTCGATCTGACGCTTGGGCACTGTCCGGGGCCTGGCGGGCGCGGCGCCCTCCTCCAGAGCCACCTCCACACACTCGAACTGCGCTGGGGCGGCAGGACTTGGCCCACGGGGCCGCAGCTCTAGGTAGGTGGCCCAGCGGGAGCCACCATCGGGGACCTGGGACTGGCGTGGGACCGCGGCGGGAGACGCTGGCCCCGGCGGCAAGGGGCTGATGAAGGCCGGCTCCGTGAACTGTTGTTGCGCCTCGCGATCGTCTGCGCCGGAGCAGCCGAACAGGGGTCCGACGCCGAAGATGACTTCCATCTCCCCCGACGGCAGCGTGCGCAGCTGGGGCTGGGGTGGCCGTGGGCCGGAACCTGGGCCTCGCGGGAAACCCGAGCCGGGCCCGTGCCGCTGGCGGCTATTCTGGGCGCTGACGGACAGGCGAGGCTGCGCGCCCGCCCCCCGCCCAGGAGCCACCCAGGGCCAATTCGCTGGGCCTTTCGCGTCCGGCCCAACGTCCGGGGGCTCCGGAGAACCTGGAGCCGTGTAGTAGGAGCCTGACGAACCGGAGGAGTCCTGGCGCCGCGCGGGGGCCGTGGGCAGCTGCCTCGGGATCCCAGGCAGGGCTGGCGGGGCGAGCGCGGTCAGCATGGTGGGGCCGGACGCCGTGCACTATCTCCCTCGCATTCGCCTCCGCTGGTGGCGC
82	TEAD3	CTGGAGAGAACTATACGGGCTGTGGGAGTCACCGGGCGACTATCACCGGGCCTCCTTTCCACATCCTCCTCCGGGAAGGGACCCCGTTCCGGGCCTCGACCGGCGCAGACTGGGCTGACCCACTTTCTTGGGCCCACTGAGTCACCTCGAAACCTCCAGGCCGGTAGCGGGGAGGAGAGGAGGAGCAGGCGGGGGTGCCAAGGTGTGGGCTGCGCCCTGGTTAGGGGGCGAGCCCGGCTTGTTTATGAGGAGGAGCGCGGAGGAGGATCCAGACACACAGGCTTGCGCGCCCAGACTCGCCCGGCCAGCGGCTGGCGGCCTCCGACGTCACCAAACCGGTTGGGTGAGAGGGCAGAGAGCAGGGGGAAGGGCCGCAGTCCCGCCCGCGCCCCCCGGCACGCACCGTACATCTTGCCCTCGTCTGACAGGATGATCTTCCG

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84	CENTG 1	TCTTCTTTCCGCCCCTAGGGGGCACAAGCGGGCATGTCCAAGCGCCTAGGAGCCCGTACCGCTGGGGACCTCCCCTTCCGCGAACCCCGAGCGGGTAG ACCCAGAGCAATCCGAGTGTGGAAACAATGGAGAGGGGGCGTGTTGAGCTGGGGTCTCCATGCCTCGTTGGGGAGAGGGAGGTGAGTTTGTGTCTTCT GGAAGGCGTGGGGGCTGTGCCCTCGTGGGGGTAGGAAGTGCTCCCGTGGGGCGGGGTGCGGATCGGAGAGGTGAGTGGGTGCGTCTGTCCAGCGGTCC GCCCGGTGTGGTCGTGCCCGGCCCGCGTGGGGATGGGGGTGTCTCTCCCGCTGGGCAACTATACCAGCGCAACCGGGGCGTCGGCGCGGCCCACGCTA GCGGCGCTGCTCCGGCGGCGGGGGCTGGGCGTGGCGGTGATGCTGGGCGTGGTGGCCGCGCTGGGCGTGGTGGCCGCGCTGCCGCCCTCACCCGGGCA GCCGTGCTGGAGAAGGATGTCGGCGCACAGCTGGCTTCCAGCCTGGCGGGCGTAGAACAGCGCCGTGCGGCCCTGGGCGTCACGGGCCGCCACGTCCG CGCCGTACTAGAGGGCGGAAACGGCCGCGTGACCGCGCGTCCCCAGGGCGCCCACACCCGGCGCCGCCTCCCCCACATGGCCAAGCCTACTTCCGGGG TCCCTCTGGGAATTTCGGGCTTTCCCGCGCCAGGCGTTTTCCGAGATGAAGCCTCAAAGACCCCCTTTCCTCCCCCCAGCTCACGTACCCACAGCAGC AGTTGCGTGATGACGACGTGGGCGAGCTCGGCCGCCAGGTGGAGTGGGGAGCGCAGCTGTGGGTCCTCTACGCTGGTGTCGAGCGGCCCGTGTCGCGC ATGGGCCAAAAGCAGGAGAACGGTAGCCACGTCCTGGGCCTGCACGGCGGCCCACAGCTGGCGGCCCAGCGGCTCCTCCGAGGTGCTCAGCGGCGCCA GGAACAGTAGCTGCTCGTACTTGGCGCGAATCCACGACTCGCGCTCCTCCCTGCAAGACCAGGGATCAACGGAAAAGGCTCTAGGGACCCCCAGCCAG GACTTCTGCCCCTACCCACGGGACCGTCTCAGGTTCGCACACCCTCAGCAACCCTCCCCCCGCTCTGTTCCCTCACGCTTACCGCGAAGAGTCCCGCG AGGGCTTGGCACGGCCTCGCGTGTCGCTTTCCCACACGCGGTTGGCCGTGTCGTTGCCAATAGCCGTCAGCACCAGGGTCAGCTCCCGTGGCCAGTCG TCCAAGTCCAGCGAGCGAACGCGGGACAGGTGTGTGCCCAGGTTGCGGTGGATGCCAGAACACTCGATGCAGATGAGGGCGCCCAGGTTCAAGCTGGC CCACGTGGGGTCTGCGGAAGGAGCGTAGAGGTCGGCTCCCAGCCGGGCAGCACAGGCACCCCGGCATTCACTACACTCCCTAGCCCCTCCGCTGCCTC CTGGCACTCACTGGGGGCCCCGCAGTCCACGCAGATTGAATTCCCCTTGGCGTTCCGGATCGCCTGGAT
85	CENTG 1	AGCCAGGTCCAGCCCCCGCGCCTGACACCGGCCGGACGTTCCCGGGGCGCCGCAGCTGCGGCGGGAACTCTGGGATCCGGAGCCATCTGCTCCCACCC GCTCCGGAGCCAAACCCCGGGGGCCGCCTCCGCTCCCGGACCCGCCTCCTCTCCCGGGAGTGTGAGCCGAACCAACAGTCTCCTGCCTATCTCCTCCA GTAGGAAAATAGTAATAATAATAGACACCCTGCCCCCGTAAAAAACACTACCTTCCCCGTACCGCCTCCCAAGTCTCCCGGGGTACGGATTGCCTTTG CAGCAGTTCCGCCCCACCTGACTCACTCCAGGGTCAGCCCCGGGTGGGTTTCAATGCGGCTCTGGGGAGGGGGTGGGCAGTGGGGGAAGTGAGGCTTC CTATCCGCCCCCTCTCACTTCACATTTAAATATTCTGCACGTTCCAGCCCCCGCGGACTCGCGTACCGCCCAATCCGCCTTCACCGCACGAAAAACAT CACTAGCCTGCTCTCAGCCCAGGGGACGACTAGTCCCTGGCGAGAAGCTGCCTGCAAGGTCACTGTCATGCCACCTGCCCCAAGTGCTCAGGGGAAAC TGAGGCTTCCTCATCCCCTTCACCTTCAACGTCGCTCTAAACACGGCAAAGCCCCGTTTCCATGCTCCCAGAGTTCAGCTGAGGCTGGAAGTGGGGTC CTGGGCTTCTCTGGGAGCAATTTTCTAGTCACTCTGATCAAGGACGTTACTTTCCCAGAAAGCTCTGAGGCTGAGTCCCTCTGAAATCAAGTCCTTTC TCCTGTCGCACAATGTAGCTACTCGCCCCGCTTCAGGACTCCTATTCTTTGCCCCAATCCTTGACAGAGGGGTGAGCTTGGTTCATCCGCCCACCCCA GAGAAAAGCTTCCCTAGTTTCCTGGACCTCGCTCCTCCACCCCAAGCTGAGCATTCCAGGTACCCTTCCCTCCCTGTTCTCAAGCCCTGACTCAACTC ACTAGGGGAAGCGCGGAGCTCGGCGCCCAGCAGCTCCCTGGACCCGCTGCCAGAAGACAGGCTGGGGGGTCCGGGAAGGGGCCCGGAGCCAGGAGGCC CTCCTGTGCTCTTGGTGAAGATGCCGCTGATAAACTTGAGCATCTTGCGGTCACGAGTGGATGCTCGGCCCCCCTCCCGGCCCCGTTTCAGCCCCGGA GCTGGAGGCTCCAGAGTGATTGGAGGTGCAGGCCCGGGGGGCTGCGCGGAAGCAGCGGTGACAGCAGTGGCTGGACTCGGAGTTGGTGGGAGGGTTAG

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86	chr18 group-00304	AGGAGCGCAAGGCTTGCAGGGCATGCTGGGAGAGCGCAGGGAACGCTGGGAGAGCGCGGGAAATACTGGGATTGGCTCCCGAGGGCTGTGAGGAGGGCACGAGGGGACACTCCGATGAAGGCAGGGCACGCGGGGCGAGCCGGGAGCGTCTCCTGAGGGCAGCGAGGAGGGAGCTGAGGCACGCGGGCTCTCAATCGACGCCCCACAGAGACCAAGAGGCCTGGCCTTGGGGGGCAGCTGCTTGAAGGAGGCAGAGCGGAAGCGAGGGAGACTGCTGGAGGCCCTGCCGCCCACCCGCCCTTTCCTCCCCCTGAGGAGACGCCTGACGCATCTGCAGTGCAGGAGGCCGTGGGCGTTAGAAGTGTTGCTTTTCCAGTTTGTAAGACCATTTTCCTGATTCTCTTCCCCACGGTTGCGGAGGAGCAGGTCAGGGCCGCCATGAGGGCAGGATC
87	TSHZ1	TCGACCGCTACTATTATGAAAACAGCGACCAGCCCATTGACTTAACCAAGTCCAAGAACAAGCCGCTGGTGTCCAGCGTGGCTGATTCGGTGGCATCACCTCTGCGGGAGAGCGCACTCATGGACATCTCCGACATGGTGAAAAACCTCACAGGCCGCCTGACGCCCAAGTCCTCCACGCCCTCCACAGTTTCAGAGAAGTCCGATGCTGATGGCAGCAGCTTTGAGGAGGC
88	CTDP1	TGTGCCGTCGCACACAGACGCCCTCAACGTCGGAGAGCTGTGAGCGGGGCCGTGCTCTTGGGATGGGAGCCCCCGGGAGAGCTGCCCGCCAACACCACTCCGACGTGATCCATGCTGGACATAAAGTGCTCTTCCCTCCGCTAGTCATCGGCCGAGCGGGCCCCTCGCTCCTGGGTGTAAGTTCTTTCTGTGCGTCCTTCTCCCATCTCCGTGCAGTTCAG
89	KCNG2	CCATGCGCCGCTGCGCGCGCGAGTTCGGGCTGCTGCTGCTGTTCCTCTGCGTGGCCATGGCGCTCTTCGCGCCACTGGTGCACCTGGCCGAGCGCGAGCTGGGCGCGCGCCGCGACTTCTCCAGCGTGCCCGCCAGCTATTGGTGGGCCGTCATCTCCATGACCACCGTGGGCTACGGCGACATGGTCCCGCGCAGCCTGCCCGGGCAGGTGGTGGCGCTCAGCAGCATCCTCAGCGGCATCCTGCTCATGGCCTTCCCGGTCACCTCCATCTTCCACACCTTTTCGCGCTCCTACTCCGAGCTCAAGGAGCAGCAGCAGCGCGCGGCCAGCCCCGAGCCGGCCCTGCAGGAGGACAGCACGCACTCGGCCACAGCCACCGAGGACAGCTCGCAGGGCCCCGACAGCGCGGGCCTGGCCGACGACTCCGCGGATGCGCTGTGGGTGCGGGCAGGGCGCTGACGCCTGCGCCGCCCAC

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The entirety of each patent, patent application, publication and document referenced herein hereby is incorporated by reference. Citation of the above patents, patent applications, publications and documents is not an admission that any of the foregoing is pertinent prior art, nor does it constitute any admission as to the contents or date of these publications or documents.

Modifications may be made to the foregoing without departing from the basic aspects of the invention. Although the invention has been described in substantial detail with reference to one or more specific embodiments, those of ordinary skill in the art will recognize that changes may be made to the embodiments specifically disclosed in this application, yet these modifications and improvements are within the scope and spirit of the invention.

The invention illustratively described herein suitably may be practiced in the absence of any element(s) not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising," "consisting essentially of," and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and use of such terms and expressions do not exclude any equivalents of the features shown and described or portions thereof, and various modifications are possible within the scope of the invention claimed. The term "a" or "an" can refer to one of or a plurality of the elements it modifies (e.g., "a reagent" can mean one or more reagents) unless it is contextually clear either one of the elements or more than one of the elements is described. The term "about" as used herein refers to a value within 10% of the underlying parameter (i.e., plus or minus 10%), and use of the term "about" at the beginning of a string of values modifies each of the values (i.e., "about 1, 2 and 3" refers to about 1, about 2 and about 3). For example, a weight of "about 100 grams" can include weights between 90 grams and 110 grams. Further, when a listing of values is described herein (e.g., about 50%, 60%, 70%, 80%, 85% or 86%) the listing includes all intermediate and fractional values thereof (e.g., 54%, 85.4%). Thus, it should be understood that although the present invention has been specifically disclosed by representative embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and such modifications and variations are considered within the scope of this invention.

Certain embodiments of the invention are set forth in the claims that follow.

The claims defining the invention are as follows:

1. A method for determining the amount of fetal nucleic acid in a sample comprising:

a) contacting nucleic acid present in a sample from a pregnant female, which nucleic acid comprises fetal nucleic acid and maternal nucleic acid, the combination of the fetal nucleic acid and the maternal nucleic acid comprising total nucleic acid in the sample, with a reagent that digests nucleic acid at non-methylated sites, thereby enriching methylated nucleic acid; and
b) determining the amount of fetal nucleic acid in (a) by analyzing the amount of fetal nucleic acid at a plurality of loci selected from SEQ ID NOs:1-59.

2. The method of claim 1, comprising determining the amount of total nucleic acid present in the sample.

3. The method of claim 2, wherein determining the amount of fetal nucleic acid in the sample comprises comparing the amount of fetal nucleic acid in (b) to the total amount of nucleic acid.

4. The method of any one of claims 1 to 3, comprising after (a) introducing one or more competitors at known concentrations.

5. The method of claim 4, comprising determining the absolute amount of fetal nucleic acid in the sample.

6. The method of any one of claims 1-5, wherein the reagent that digest nucleic acid at non-methylated sites is a methylation-sensitive restriction enzyme.

7. The method of any one of claims 1 to 6, wherein the plurality of loci are selected from loci of SEQ ID NO:1-33 and 36-59.

8. The method of any one of claims 1 to 7, wherein the plurality of loci comprises a locus of SEQ ID NO:42.

9. The method of any one of claims 1 to 7, wherein the plurality of loci comprises a locus of SEQ ID NO:52.

10. The method of any one of claims 1 to 9, wherein the amount of fetal nucleic acid is indicative of a fetal trait.

11. The method of claim 10, wherein the fetal trait is a fetal aneuploidy.

12. The method of claim 10 or 11, wherein the method requires a concentration of fetal nucleic acid to meet certain clinical sensitivity or specificity requirements.

13. The method of any one of claims 1 to 12, wherein determining the amount of fetal nucleic acid comprises use of one or more processes selected from mass spectrometry methods, RT-PCR, digital PCR, array-based methods, sequencing methods, nanopore-based methods, nucleic acid-bound bead-based counting methods, competitor-based methods and methods for separating nucleic acid using agents that bind nucleic acid based on methylation status.

14. The method of claim 13, wherein determining the amount of fetal nucleic acid comprises use of a mass spectrometry method.

15. The method of claim 13, wherein determining the amount of fetal nucleic acid comprises use of a sequencing method.

16. The method of claim 15, wherein the sequencing method comprises sequencing by synthesis.

17. The method of any one of claims 1 to 16, wherein the digestion efficiency of the reagent that digests nucleic acid at non-methylated sites is determined.

18. The method of any one of claims 1 to 16, comprising determining the presence or absence of Y-chromosome nucleic acid in a sample from a pregnant female.

19. The method of claim 18, wherein the amount of Y-chromosome nucleic acid present in a sample from a pregnant female is determined for a male fetus.

20. The method of claim 19, wherein the amount of fetal nucleic acid is compared to the amount of Y-chromosome nucleic acid.

21. The method of any one of claims 1 to 20, wherein the amount of fetal nucleic acid at 10 or more loci is determined.

22. The method of claim 19, wherein the amount of Y-chromosome nucleic acid for a male fetus and the digestion efficiency of the reagent that digests nucleic acid at non-methylated sites are determined.

23. The method of claim 22, wherein two or more assays are used to determine the total amount of nucleic acid, one or more assays are used to determine the amount of Y-chromosome nucleic acid for a male fetus, and one or more assays are used to determine the digestion efficiency of the reagent that digests nucleic acid at non-methylated sites.

24. The method of claim 23, wherein the amount of fetal nucleic acid at 3 or more loci is determined.

25. The method of any one of claims 1 to 24, wherein the amount of fetal nucleic acid is determined by an amplification reaction that generates amplicons larger than the average length of the digested maternal nucleic acid, thereby further enriching the fetal nucleic acid.

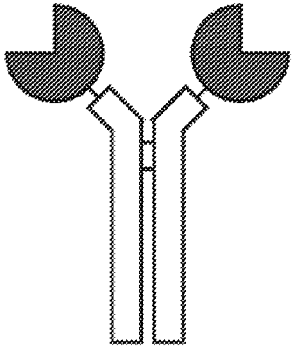
26. The method of any one of claims 1 to 25, wherein the amount of fetal nucleic acid from a target chromosome is determined and the amount of fetal nucleic acid from a reference chromosome is determined.

27. The method of claim 26, further comprising determining the presence or absence of a fetal aneuploidy by comparing the amounts of fetal nucleic acid from the target chromosome and the reference chromosome.

28. The method of claim 1, substantially as hereinbefore described with reference to any of the Examples and/or Figures.

FIGURE 1

Design of the recombinant MBD-Fc protein



Methyl-CpG-binding domain of human MBD2

- binds double-stranded mCpG
  (only when methylated on both strands!)
- strongest affinity of known MBDs
- affinity depends on salt concentration

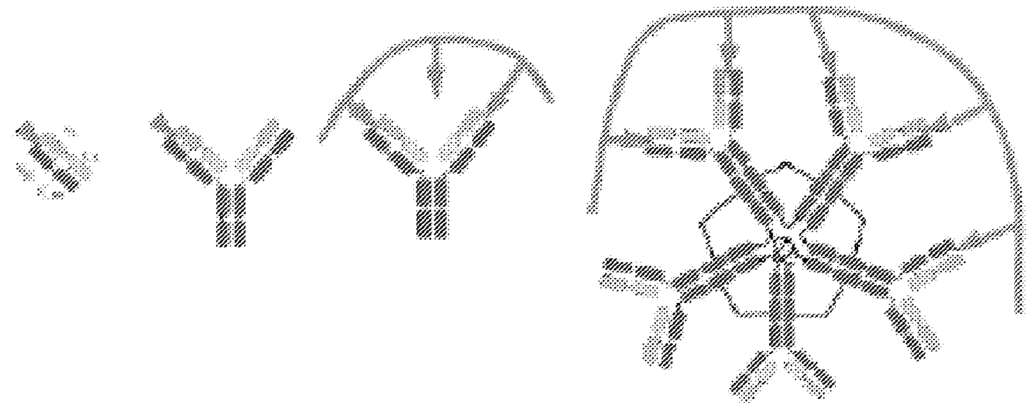
Fc-tail of human IgG1

- easy purification procedure
- relatively strong binding to protein A/G

FIGURE 2

MBD Protein

Affinity and avidity



Fab	IgG	IgG	IgM	
1	1	2	5	effective valence
1	1	100	$10^4 - 10^5$	relative equilibrium constant

monovalent affinity

multivalent avidity

FIGURE 3

Fractionating DNA
Based on Methylation

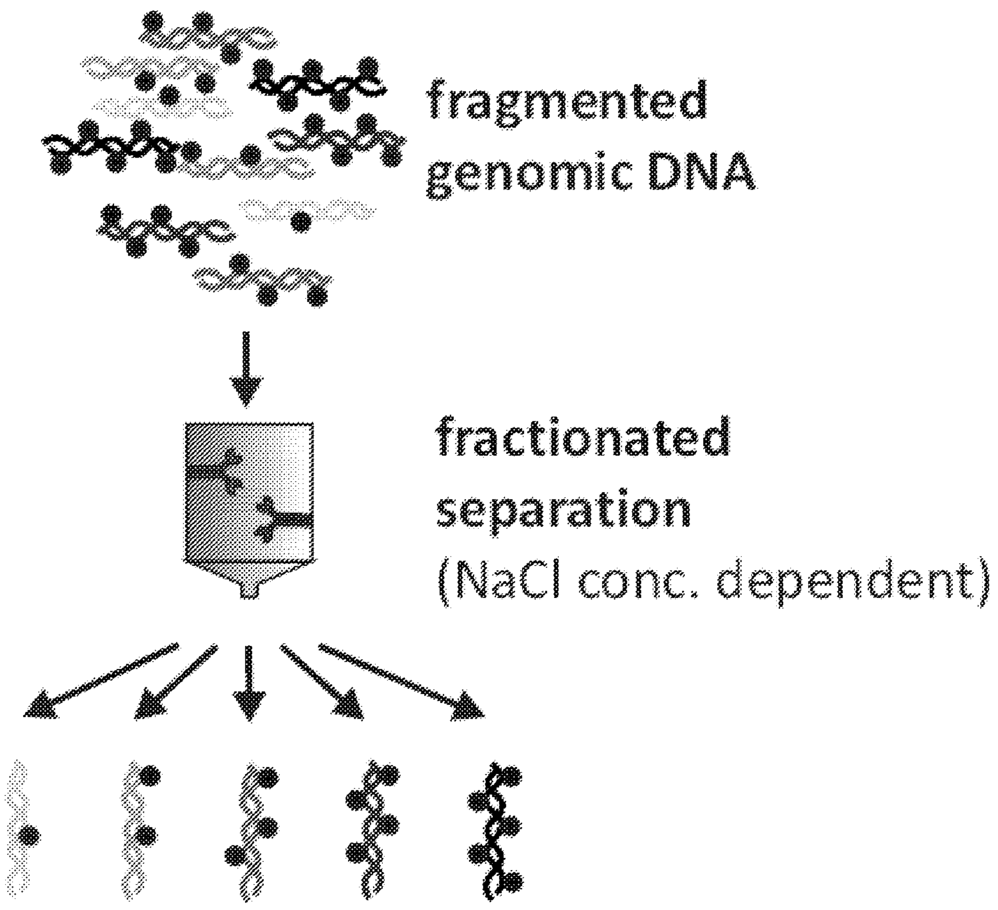


FIGURE 4

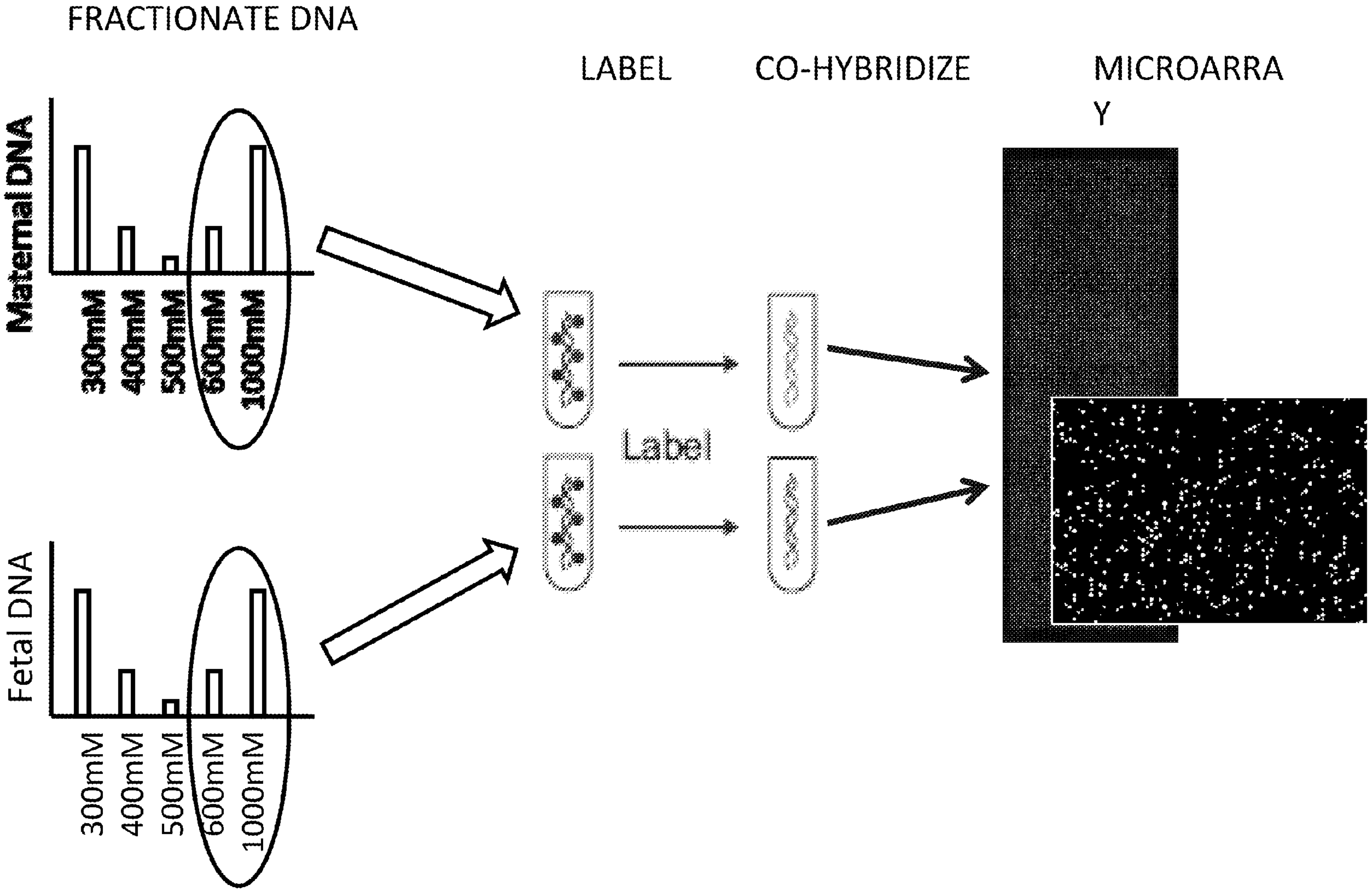

FRACTIONATE DNA
LABEL
CO-HYBRIDIZE
MICROARRA
Y
Maternal DNA
300mM
400mM
500mM
600mM
1000mM
Fetal DNA
300mM
400mM
500mM
600mM
1000mM
Label

FIGURE 5

EpiTYPER Validation Method

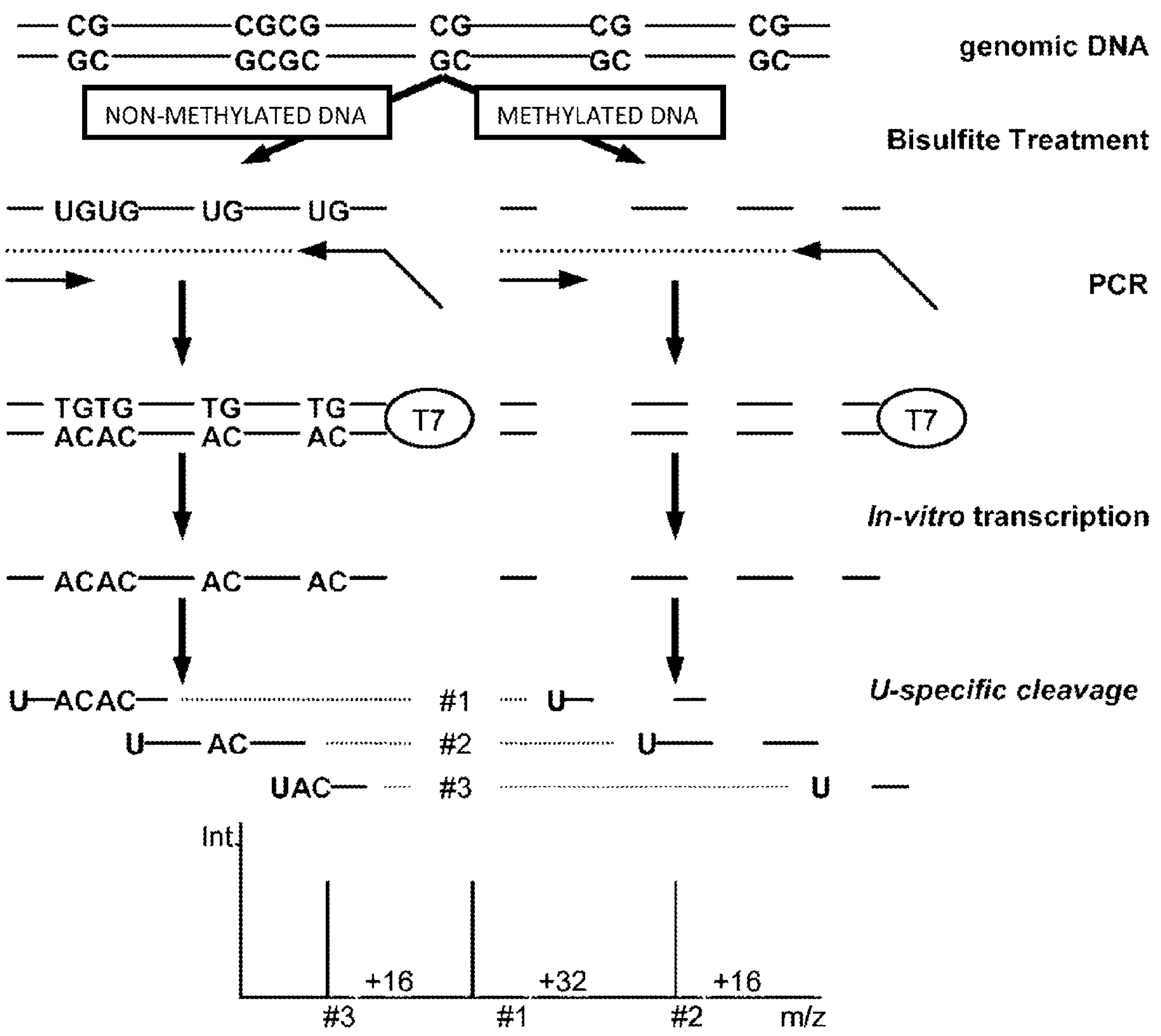


FIGURE 6

EpiTYPER Results

Quantitation of Methylation Ratios

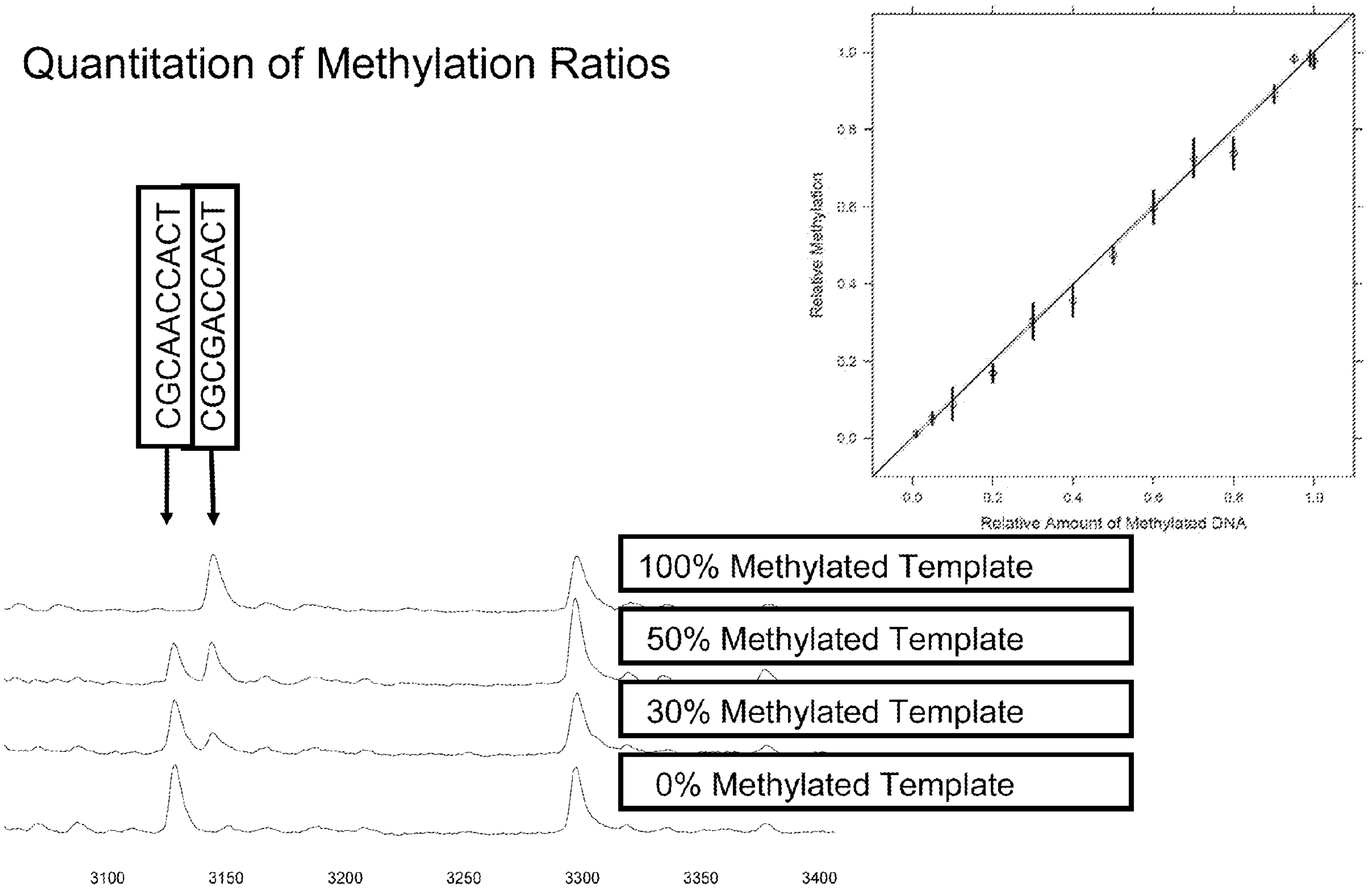


FIGURE 7

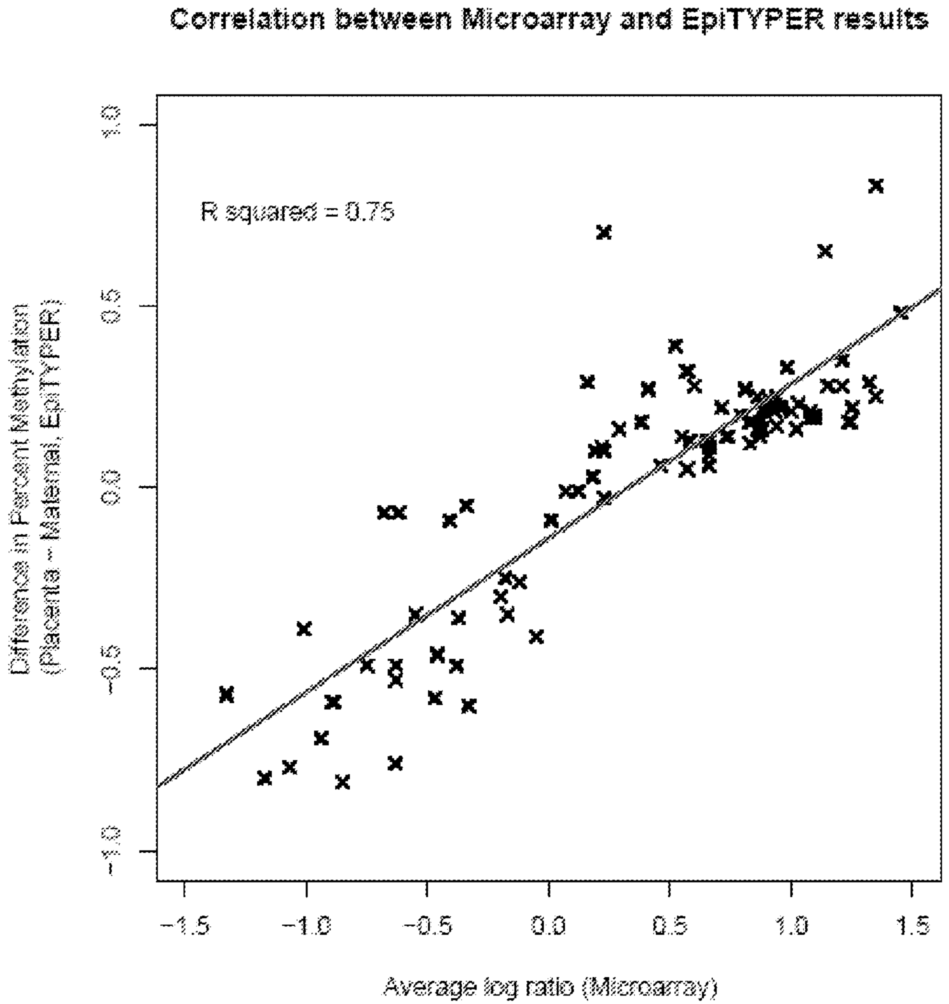

Correlation between Microarray and EpiTYPER results
R squared = 0.75
Difference in Percent Methylation
(Placenta − Maternal, EpiTYPER)
1.0
0.5
0.0
−0.5
−1.0
−1.5
−1.0
−0.5
0.0
0.5
1.0
1.5
Average log ratio (Microarray)

FIGURE 8

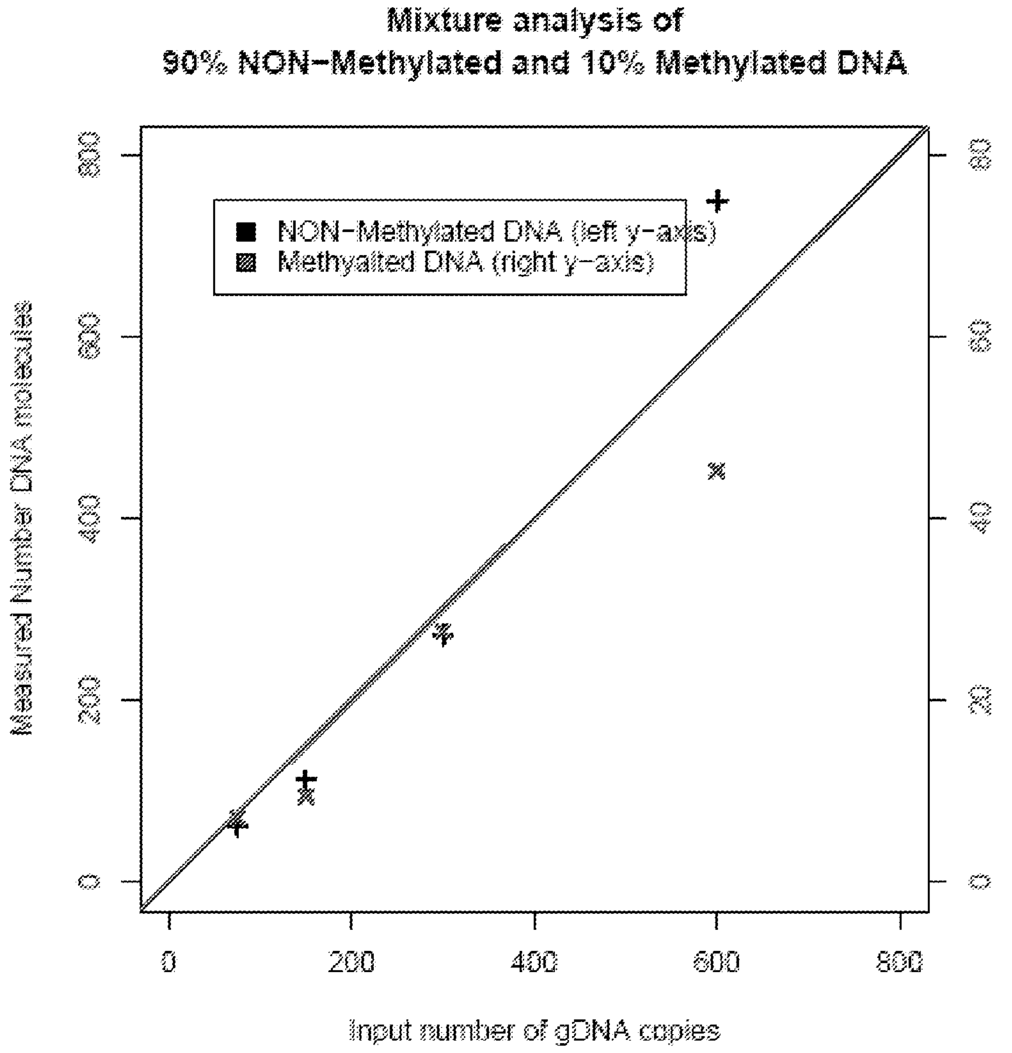

Mixture analysis of
90% NON−Methylated and 10% Methylated DNA
■ NON−Methylated DNA (left y−axis)
▨ Methyalted DNA (right y−axis)
Measured Number DNA molecules
0
200
400
600
800
0
20
40
60
80
0
200
400
600
800
Input number of gDNA copies

FIGURE 9A

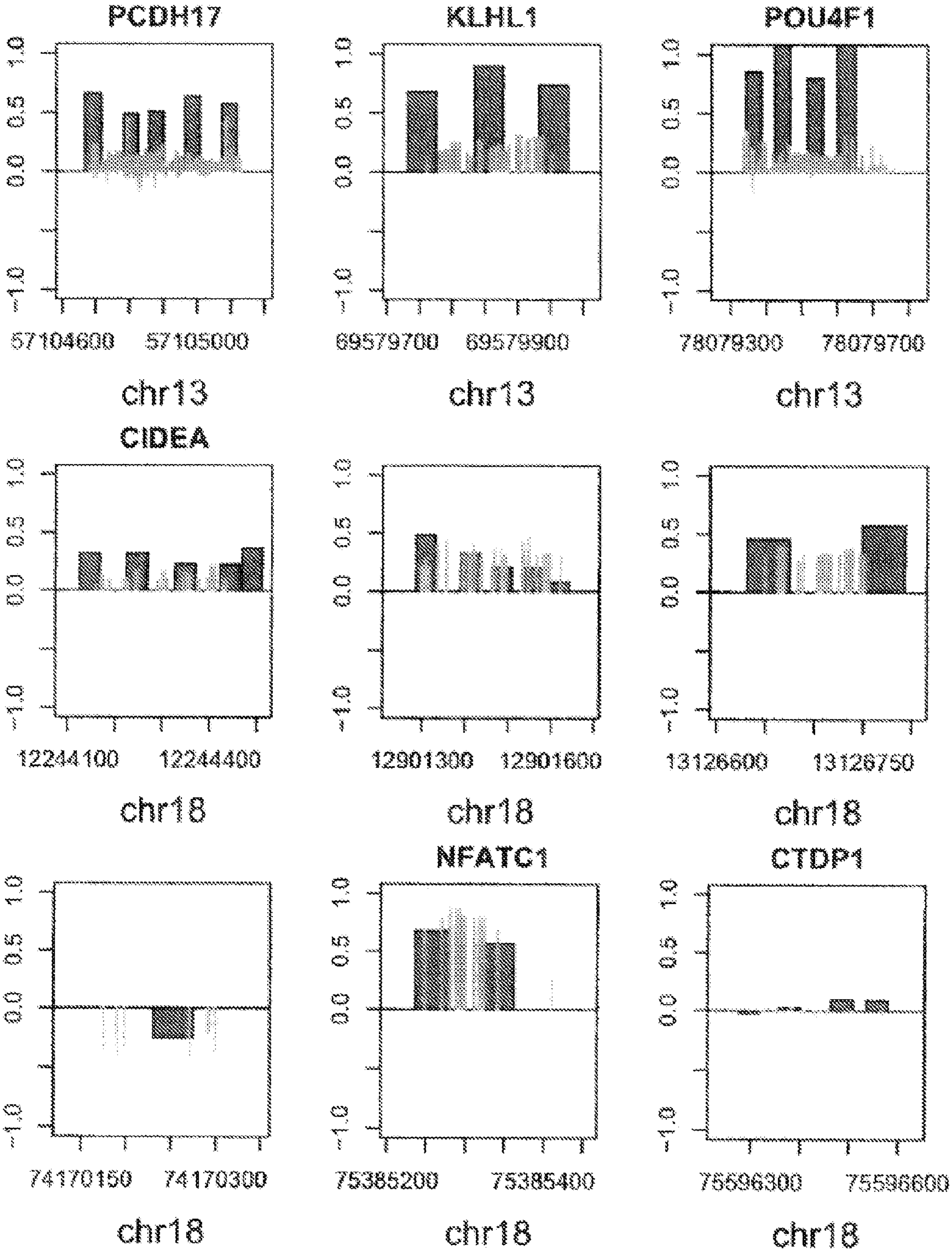

PCDH17
1.0
0.5
0.0
-1.0
57104600
57105000
chr13
KLHL1
1.0
0.5
0.0
-1.0
69579700
69579900
chr13
POU4F1
1.0
0.5
0.0
-1.0
78079300
78079700
chr13
CIDEA
1.0
0.5
0.0
-1.0
12244100
12244400
chr18
1.0
0.5
0.0
-1.0
12901300
12901600
chr18
1.0
0.5
0.0
-1.0
13126600
13126750
chr18
1.0
0.5
0.0
-1.0
74170150
74170300
chr18
NFATC1
1.0
0.5
0.0
-1.0
75385200
75385400
chr18
CTDP1
1.0
0.5
0.0
-1.0
75596300
75596600
chr18

FIGURE 9B

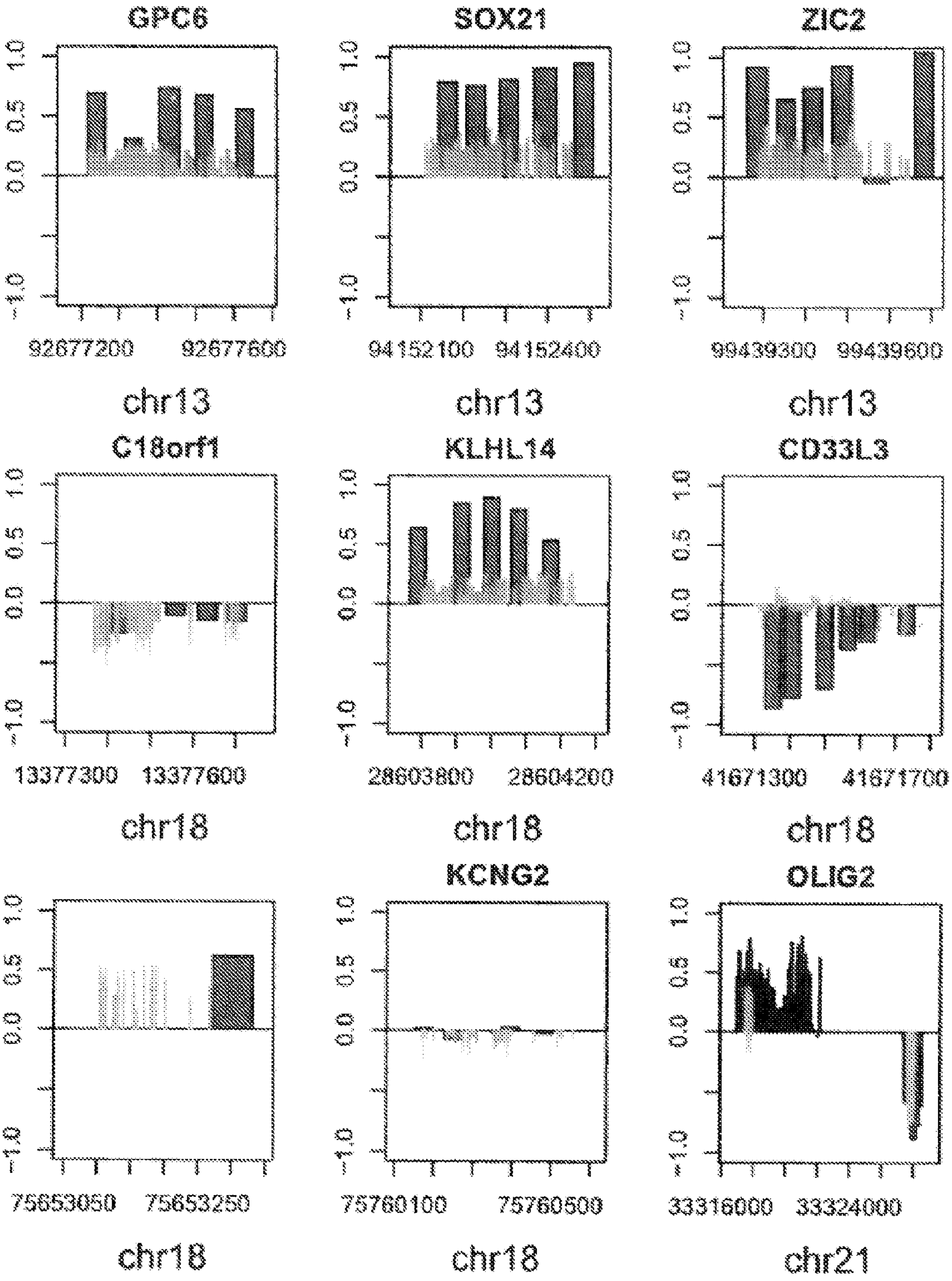

GPC6
SOX21
ZIC2
1.0
0.5
0.0
-1.0
92677200
92677600
94152100
94152400
99439300
99439600
chr13
chr13
chr13
C18orf1
KLHL14
CD33L3
13377300
13377600
28603800
28604200
41671300
41671700
chr18
chr18
chr18
KCNG2
OLIG2
75653050
75653250
75760100
75760500
33316000
33324000
chr18
chr18
chr21

FIGURE 9C

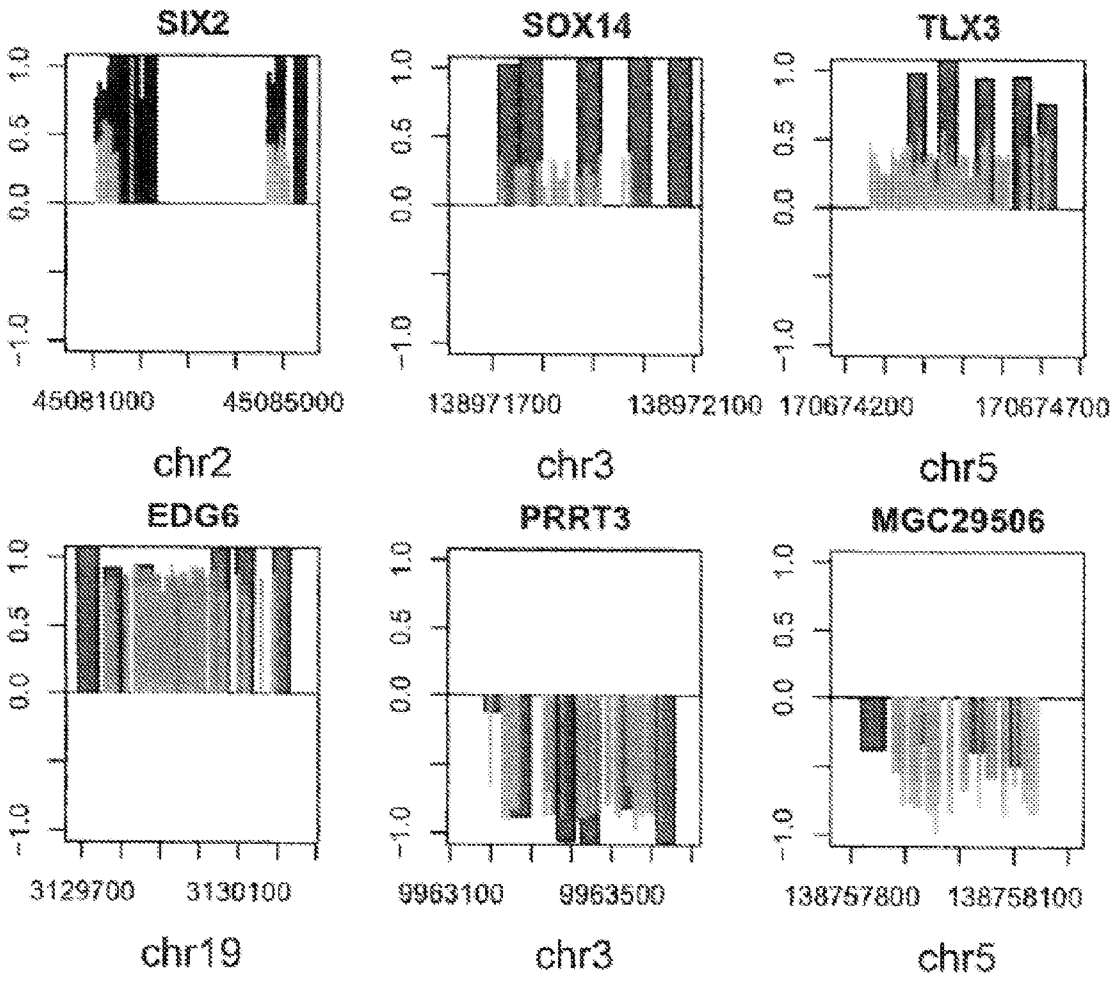

SIX2
1.0
0.5
0.0
−1.0
45081000
45085000
chr2
SOX14
1.0
0.5
0.0
−1.0
138971700
138972100
chr3
TLX3
1.0
0.5
0.0
−1.0
170674200
170674700
chr5
EDG6
1.0
0.5
0.0
−1.0
3129700
3130100
chr19
PRRT3
1.0
0.5
0.0
−1.0
9963100
9963500
chr3
MGC29506
1.0
0.5
0.0
−1.0
138757800
138758100
chr5

FIGURE 9D

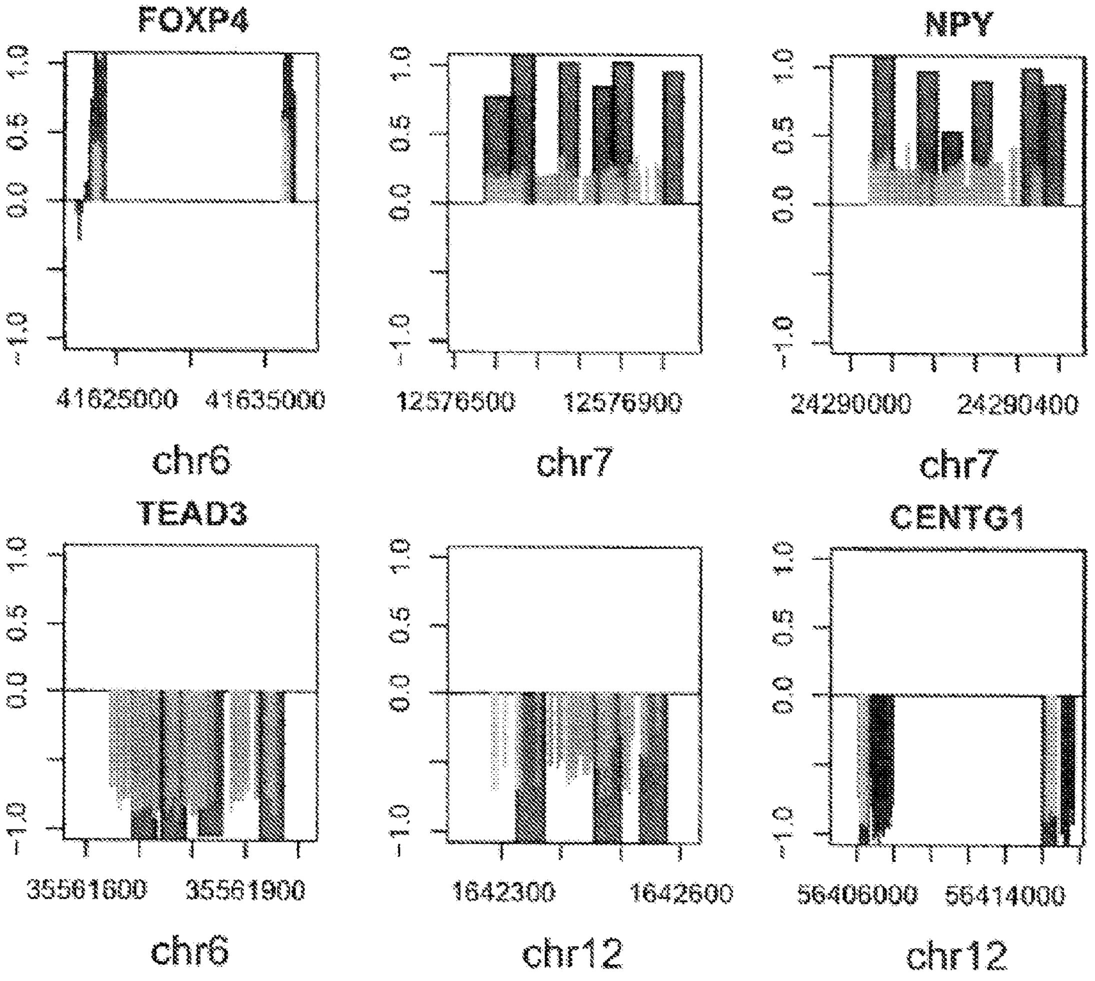

FOXP4
1.0
0.5
0.0
-1.0
41625000
41635000
chr6
1.0
0.5
0.0
-1.0
12576500
12576900
chr7
NPY
1.0
0.5
0.0
-1.0
24290000
24290400
chr7
TEAD3
1.0
0.5
0.0
-1.0
35561600
35561900
chr6
1.0
0.5
0.0
-1.0
1642300
1642600
chr12
CENTG1
1.0
0.5
0.0
-1.0
56406000
56414000
chr12

FIGURE 9E

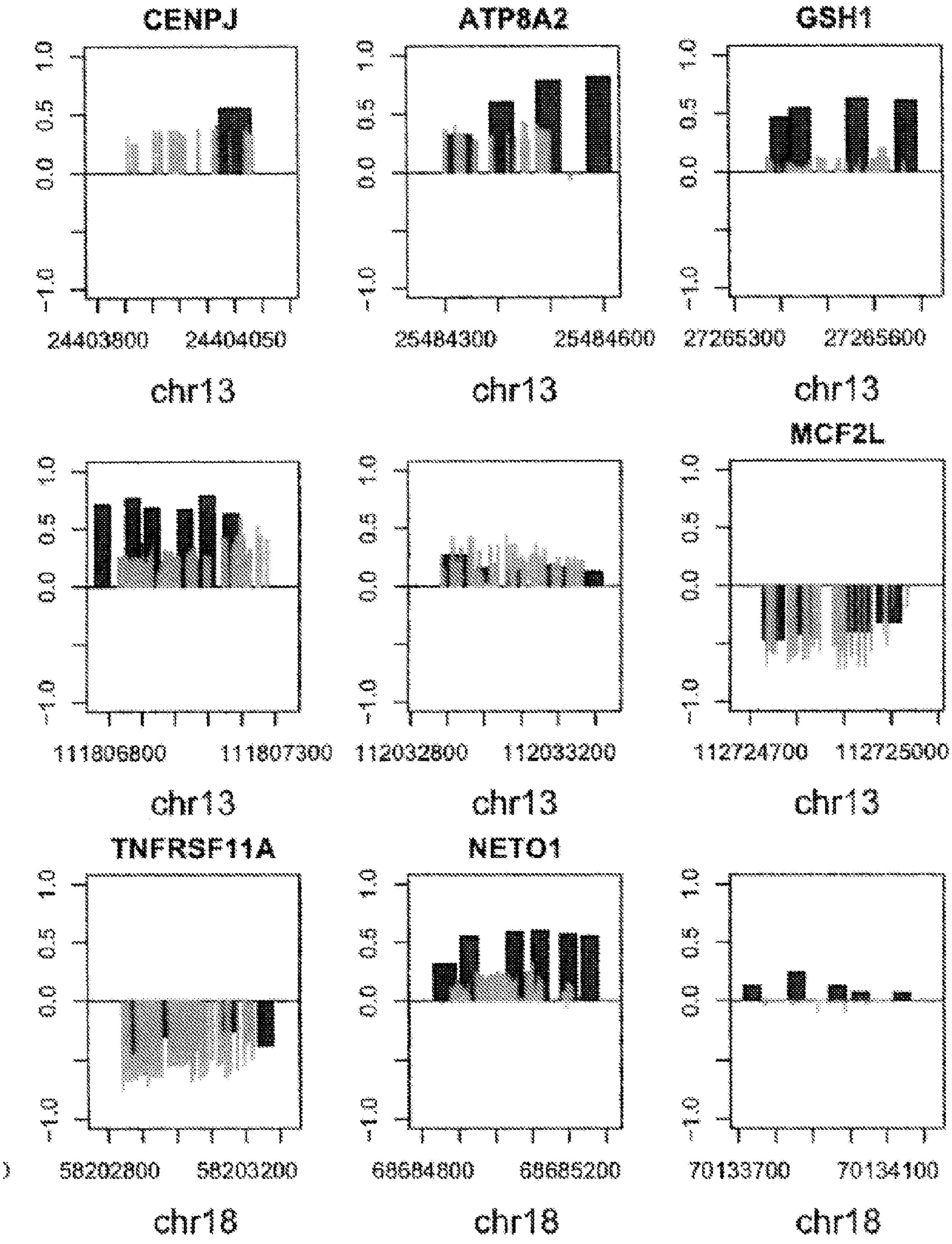

CENPJ
ATP8A2
GSH1
1.0
0.5
0.0
-1.0
24403800
24404050
25484300
25484600
27265300
27265600
chr13
chr13
chr13
MCF2L
111806800
111807300
112032800
112033200
112724700
112725000
chr13
chr13
chr13
TNFRSF11A
NETO1
58202800
58203200
68684800
68685200
70133700
70134100
chr18
chr18
chr18

FIGURE 9F

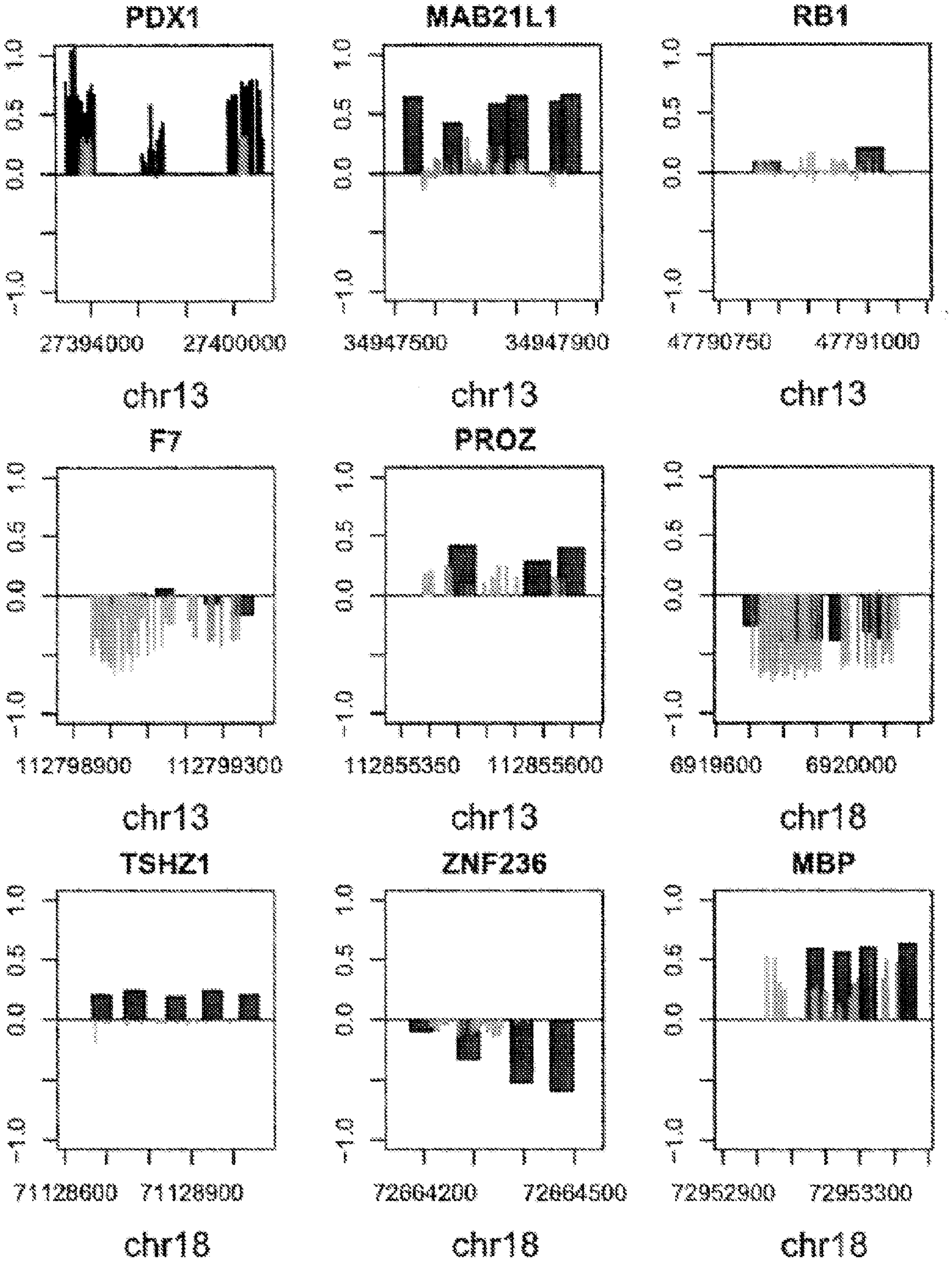

PDX1
MAB21L1
RB1
1.0
0.5
0.0
-1.0
27394000
27400000
34947500
34947900
47790750
47791000
chr13
chr13
chr13
F7
PROZ
112798900
112799300
112855350
112855600
6919600
6920000
chr13
chr13
chr18
TSHZ1
ZNF236
MBP
71128600
71128900
72664200
72664500
72952900
72953300
chr18
chr18
chr18

FIGURE 9G

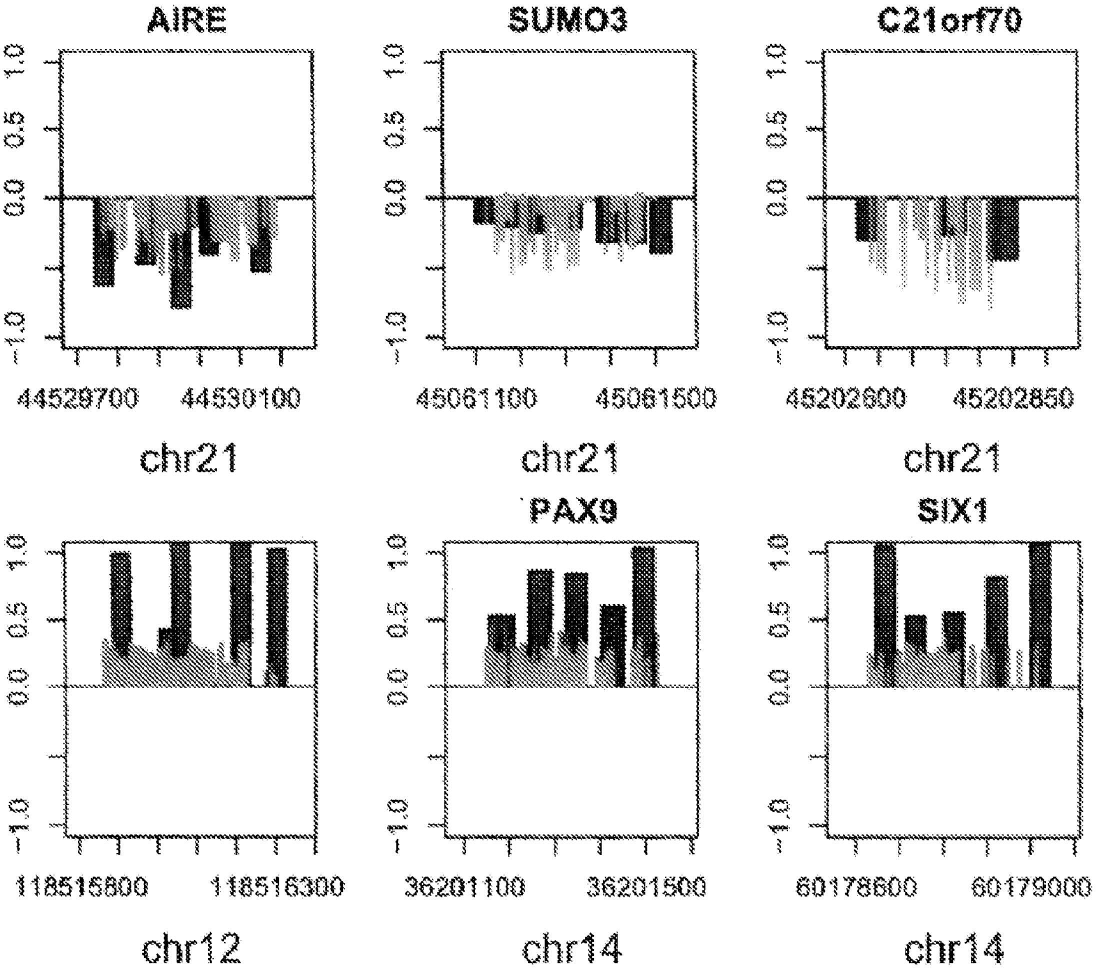

AIRE
SUMO3
C21orf70
1.0
0.5
0.0
−1.0
44529700
44530100
45061100
45061500
45202800
45202850
chr21
chr21
chr21
PAX9
SIX1
118515800
118516300
36201100
36201500
60178600
60179000
chr12
chr14
chr14

FIGURE 9H

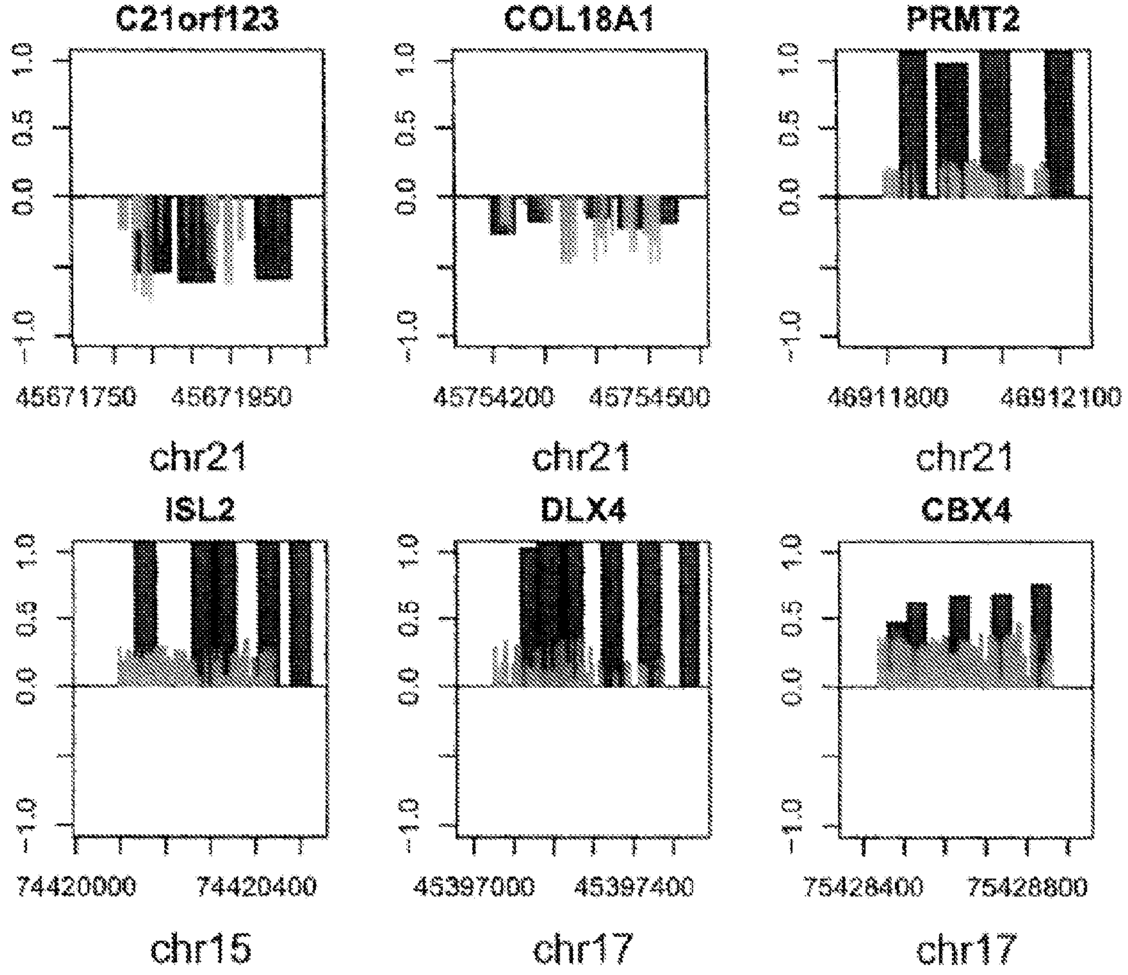

C21orf123
COL18A1
PRMT2
−1.0
0.0
0.5
1.0
45671750
45671950
45754200
45754500
46911800
46912100
chr21
chr21
chr21
ISL2
DLX4
CBX4
74420000
74420400
45397000
45397400
75428400
75428800
chr15
chr17
chr17

FIGURE 9I

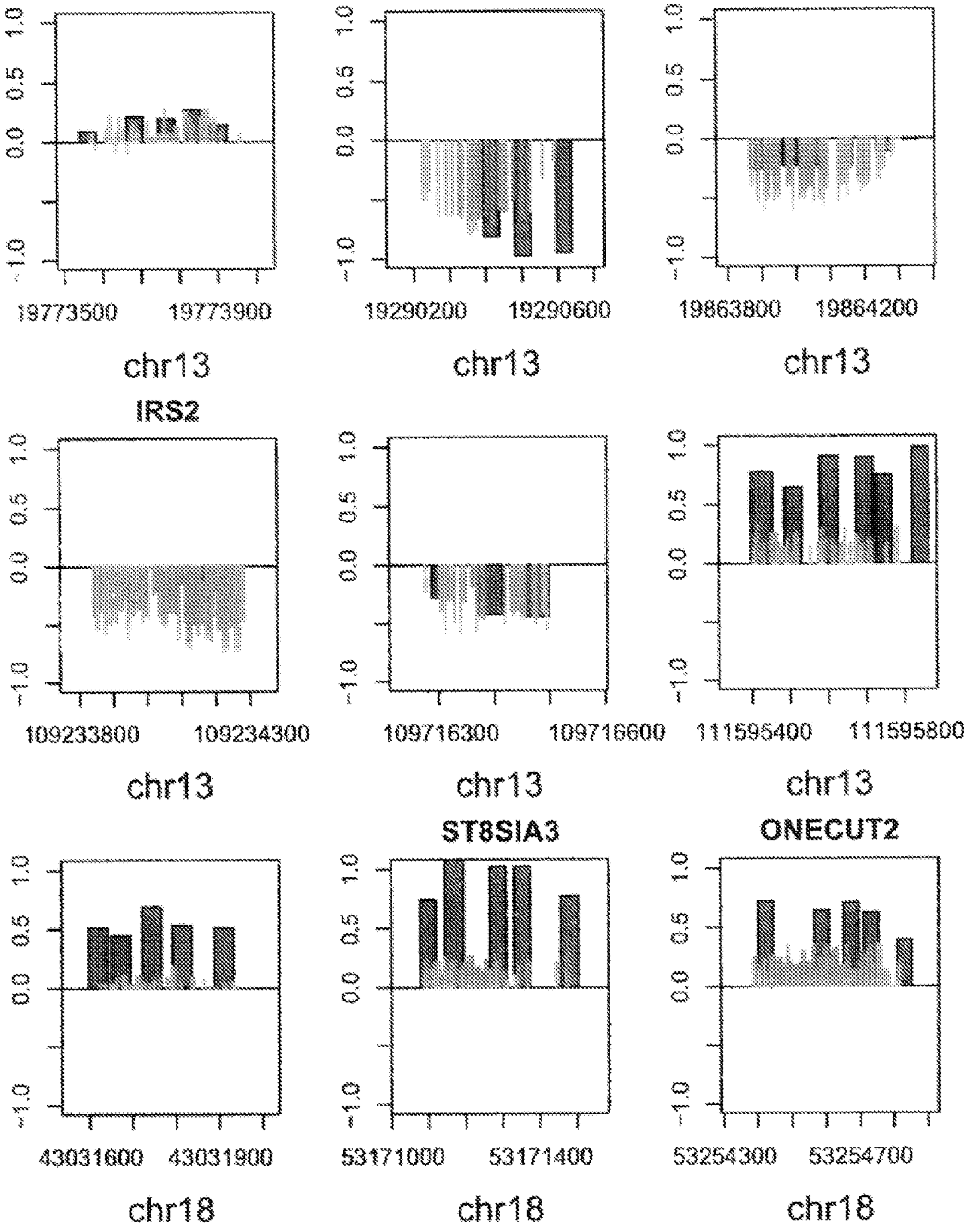

1.0
0.5
0.0
-1.0
19773500
19773900
chr13
19290200
19290600
chr13
19863800
19864200
chr13
IRS2
109233800
109234300
chr13
109716300
109716600
chr13
111595400
111595800
chr13
43031600
43031900
chr18
ST8SIA3
53171000
53171400
chr18
ONECUT2
53254300
53254700
chr18

FIGURE 9J

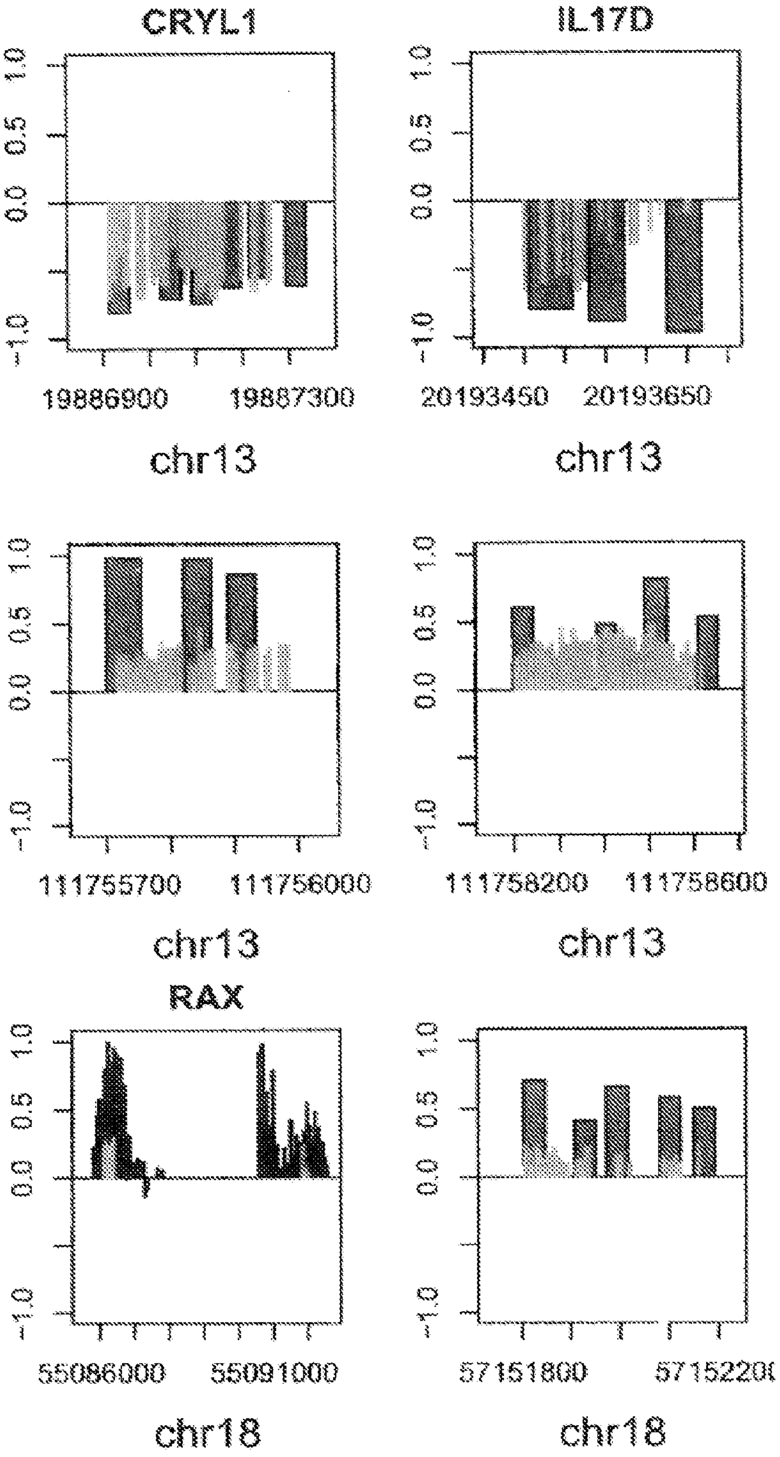

CRYL1
1.0
0.5
0.0
-1.0
19886900
19887300
chr13
IL17D
1.0
0.5
0.0
-1.0
20193450
20193650
chr13
1.0
0.5
0.0
-1.0
111755700
111756000
chr13
1.0
0.5
0.0
-1.0
111758200
111758600
chr13
RAX
1.0
0.5
0.0
-1.0
55086000
55091000
chr18
1.0
0.5
0.0
-1.0
57151800
5715220
chr18

FIGURE 9K

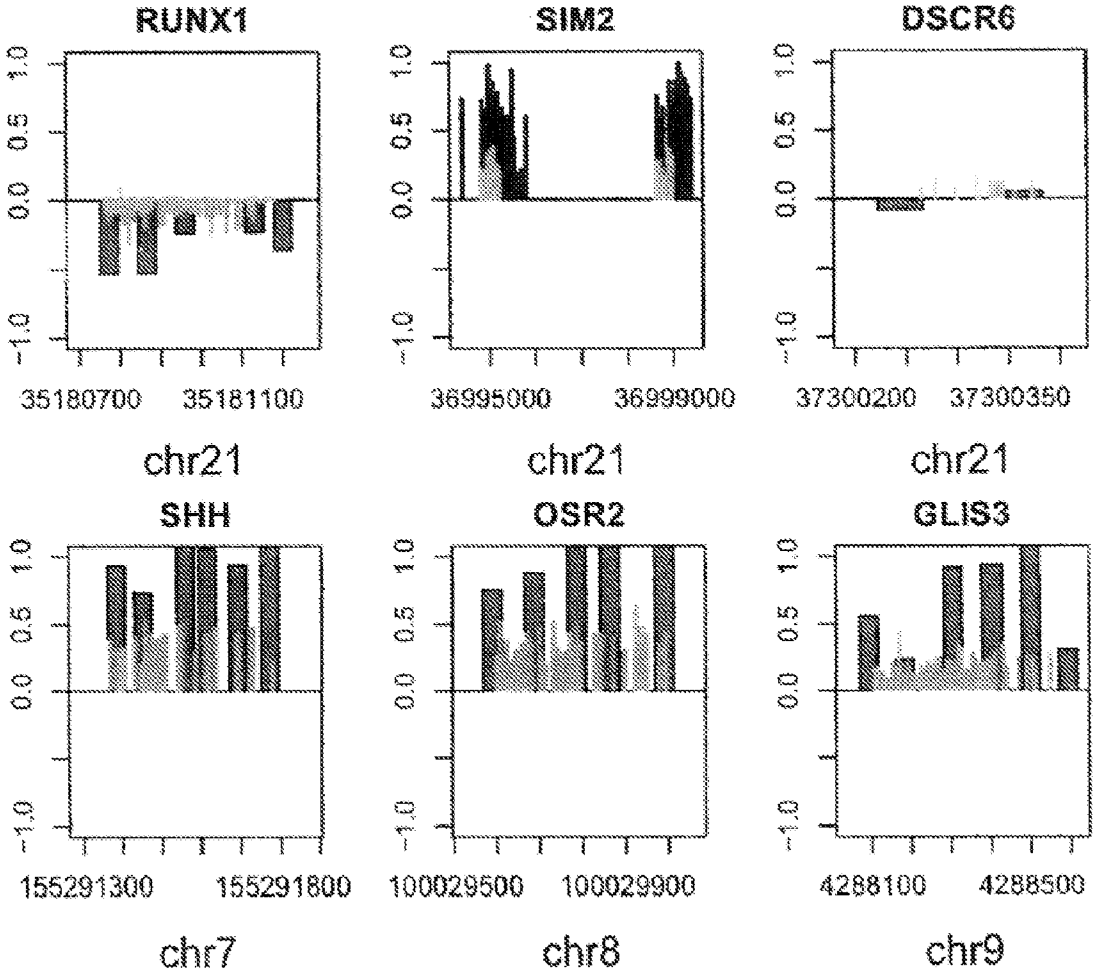

RUNX1
SIM2
DSCR6
1.0
0.5
0.0
-1.0
35180700
35181100
36995000
36999000
37300200
37300350
chr21
chr21
chr21
SHH
OSR2
GLIS3
155291300
155291800
100029500
100029900
4288100
4288500
chr7
chr8
chr9

FIGURE 9L

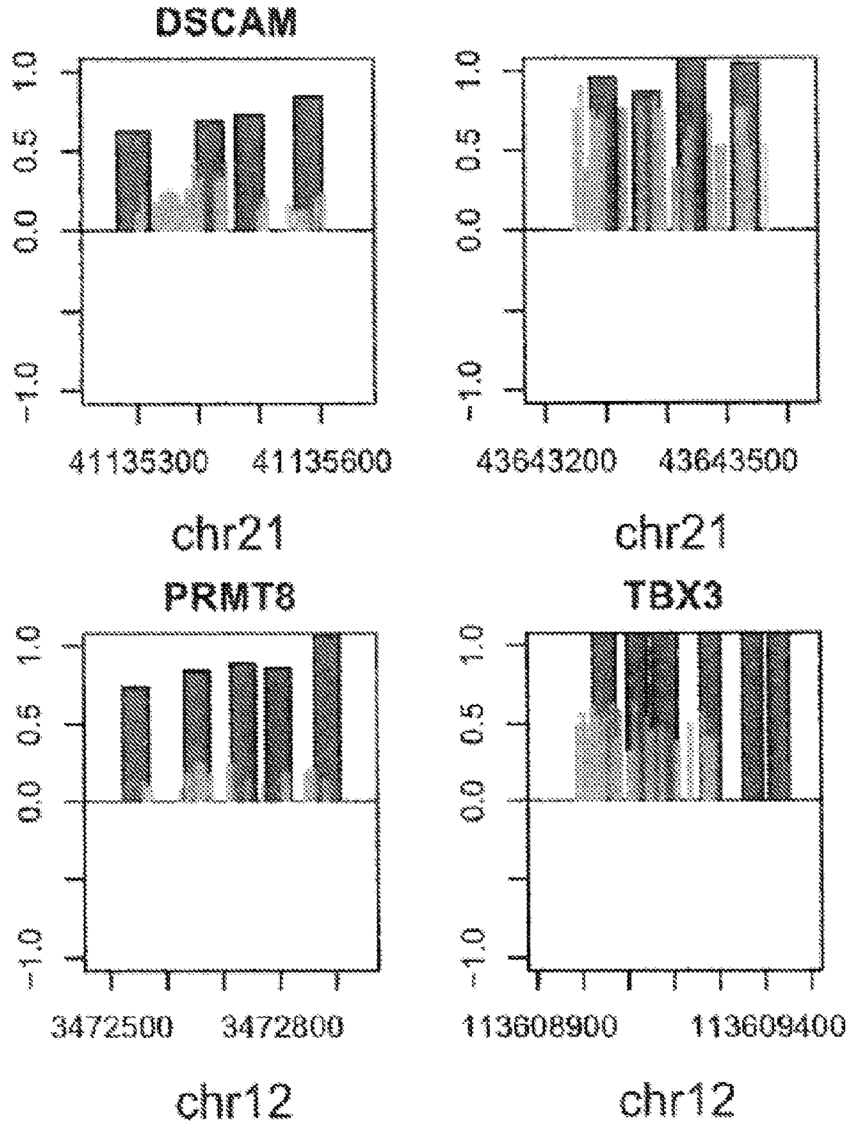

DSCAM
1.0
0.5
0.0
-1.0
41135300
41135600
chr21
1.0
0.5
0.0
-1.0
43643200
43643500
chr21
PRMT8
1.0
0.5
0.0
-1.0
3472500
3472800
chr12
TBX3
1.0
0.5
0.0
-1.0
113608900
113609400
chr12

FIGURE 10

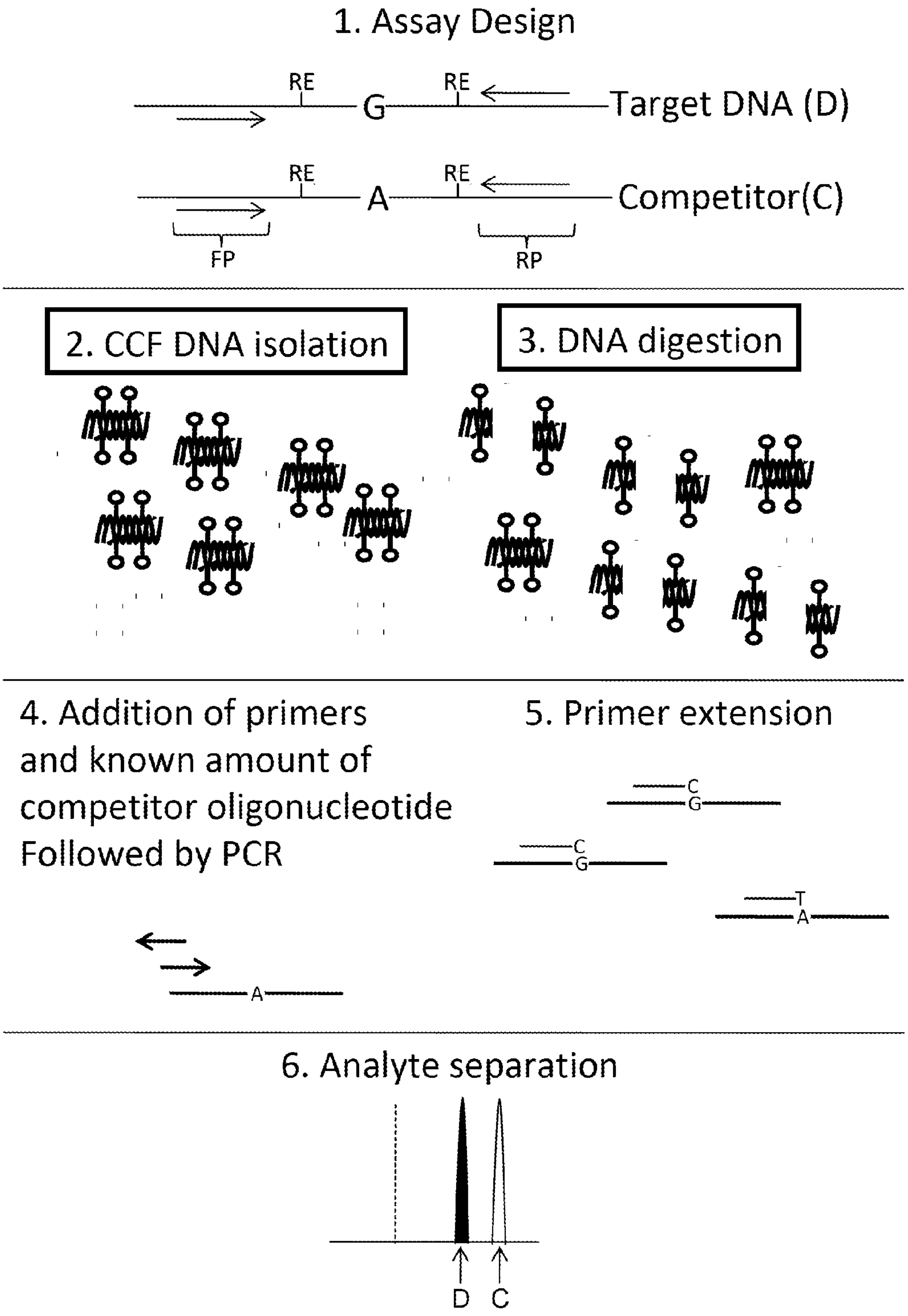

1. Assay Design
RE
RE
G
Target DNA (D)
RE
RE
A
Competitor(C)
FP
RP
2. CCF DNA isolation
3. DNA digestion
4. Addition of primers and known amount of competitor oligonucleotide Followed by PCR
A
5. Primer extension
C
G
C
G
T
A
6. Analyte separation
D
C

FIGURE 11

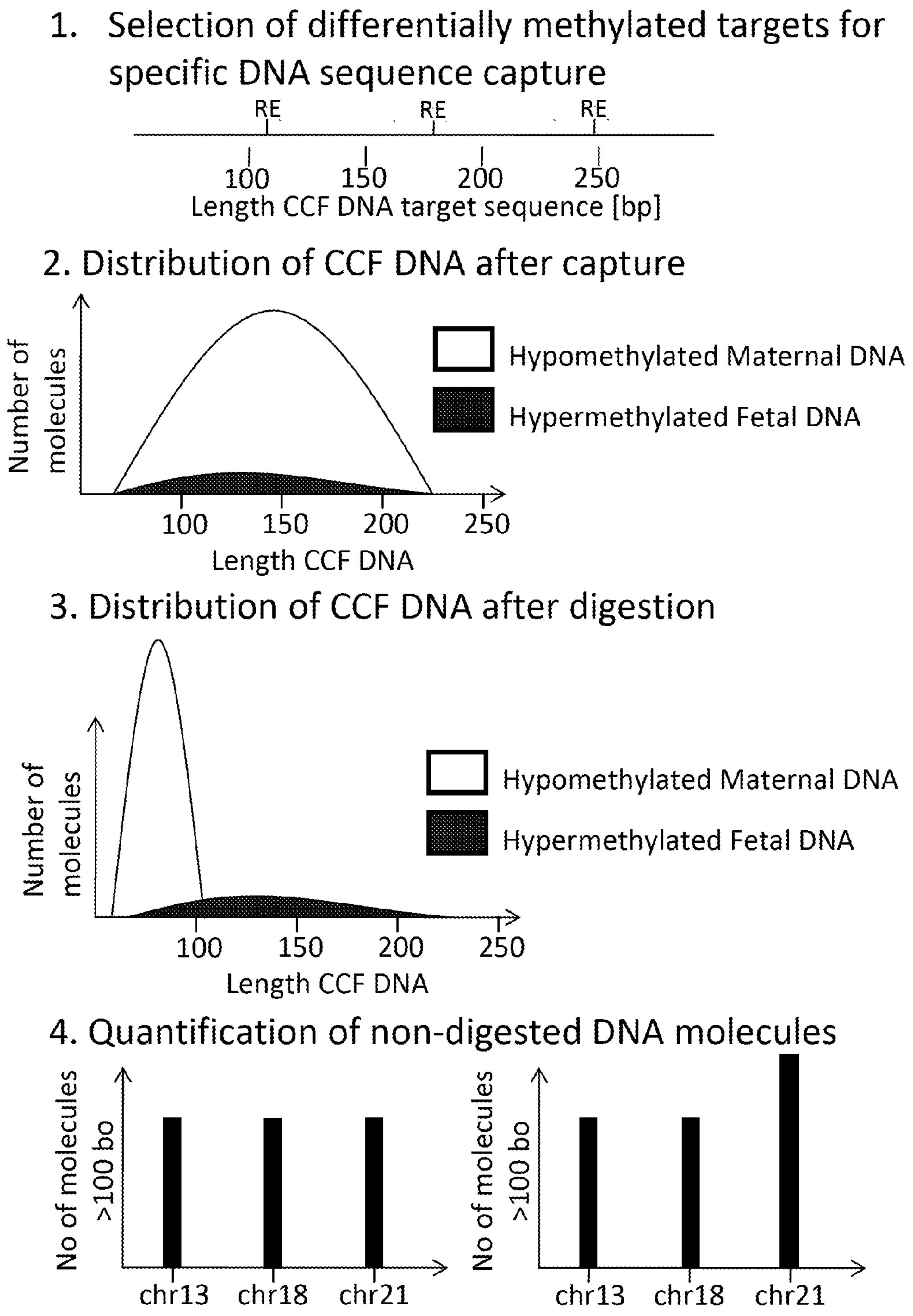

1. Selection of differentially methylated targets for specific DNA sequence capture
RE
RE
RE
100
150
200
250
Length CCF DNA target sequence [bp]
2. Distribution of CCF DNA after capture
Number of molecules
Hypomethylated Maternal DNA
Hypermethylated Fetal DNA
100
150
200
250
Length CCF DNA
3. Distribution of CCF DNA after digestion
Number of molecules
Hypomethylated Maternal DNA
Hypermethylated Fetal DNA
100
150
200
250
Length CCF DNA
4. Quantification of non-digested DNA molecules
No of molecules >100 bo
chr13
chr18
chr21
No of molecules >100 bo
chr13
chr18
chr21

FIGURE 12

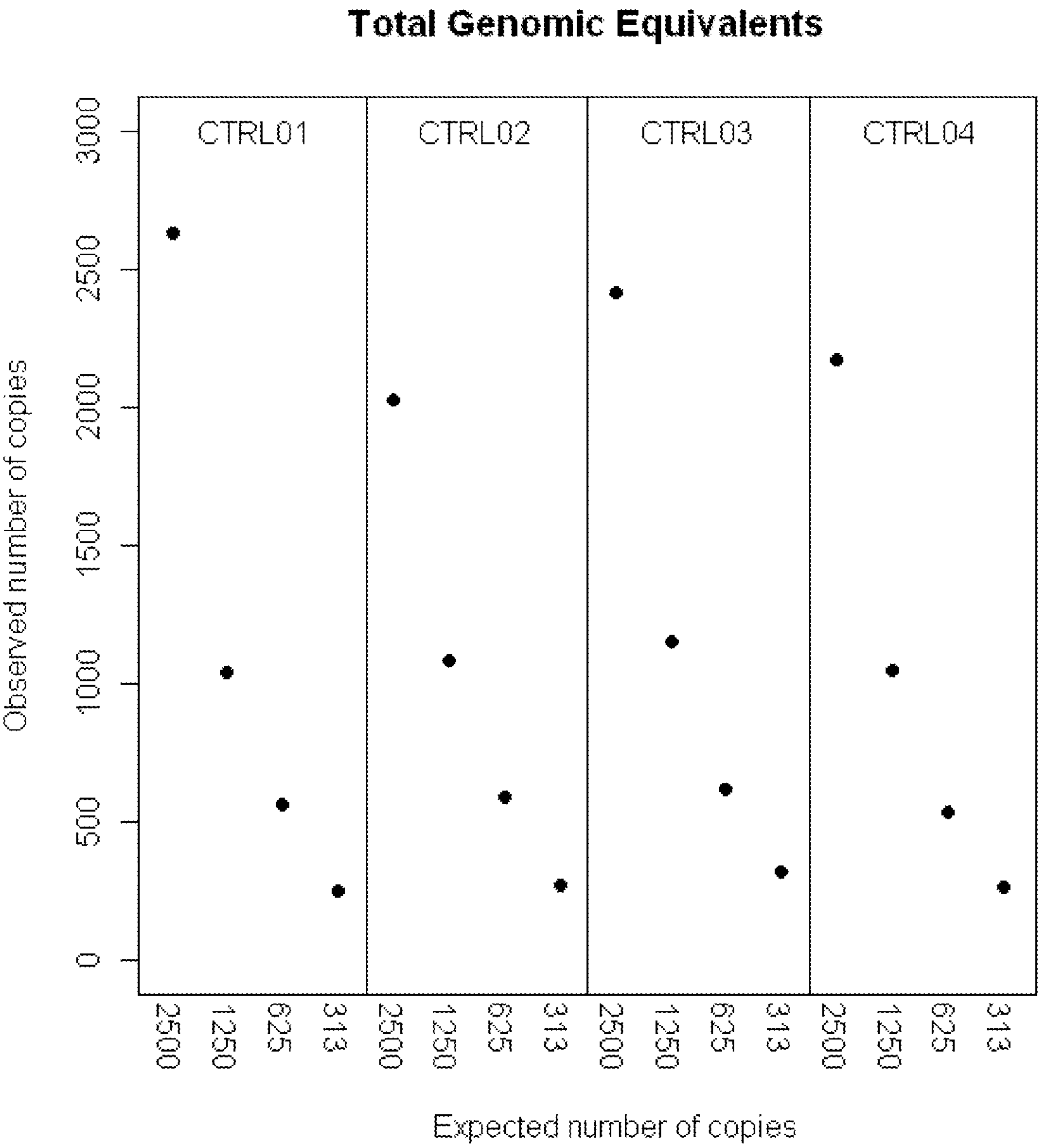

Total Genomic Equivalents
CTRL01
CTRL02
CTRL03
CTRL04
Observed number of copies
0
500
1000
1500
2000
2500
3000
2500
1250
625
313
2500
1250
625
313
2500
1250
625
313
2500
1250
625
313
Expected number of copies

FIGURE 13A

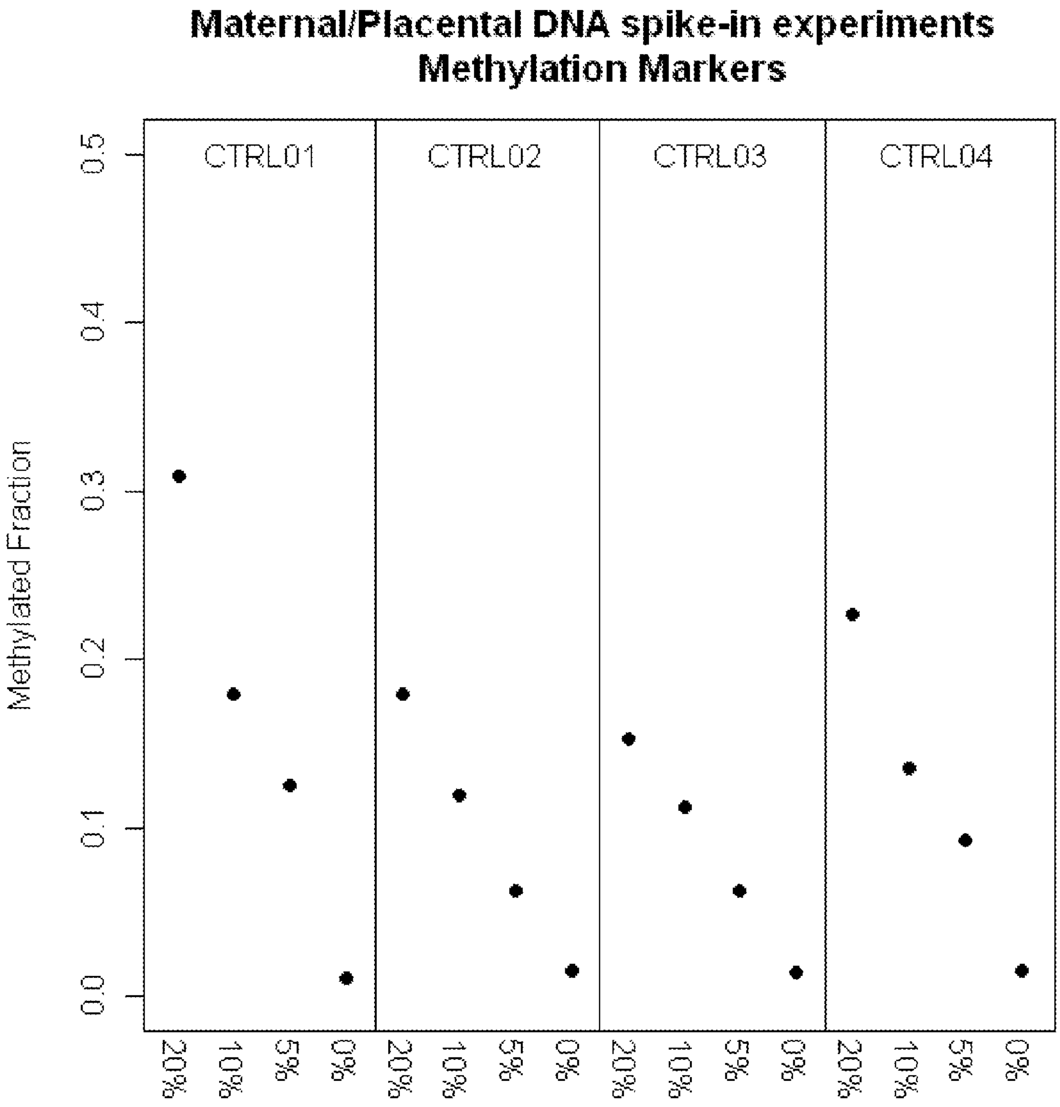

Maternal/Placental DNA spike-in experiments
Methylation Markers
Methylated Fraction
0.0
0.1
0.2
0.3
0.4
0.5
CTRL01
CTRL02
CTRL03
CTRL04
20%
10%
5%
0%
20%
10%
5%
0%
20%
10%
5%
0%
20%
10%
5%
0%

FIGURE 13B

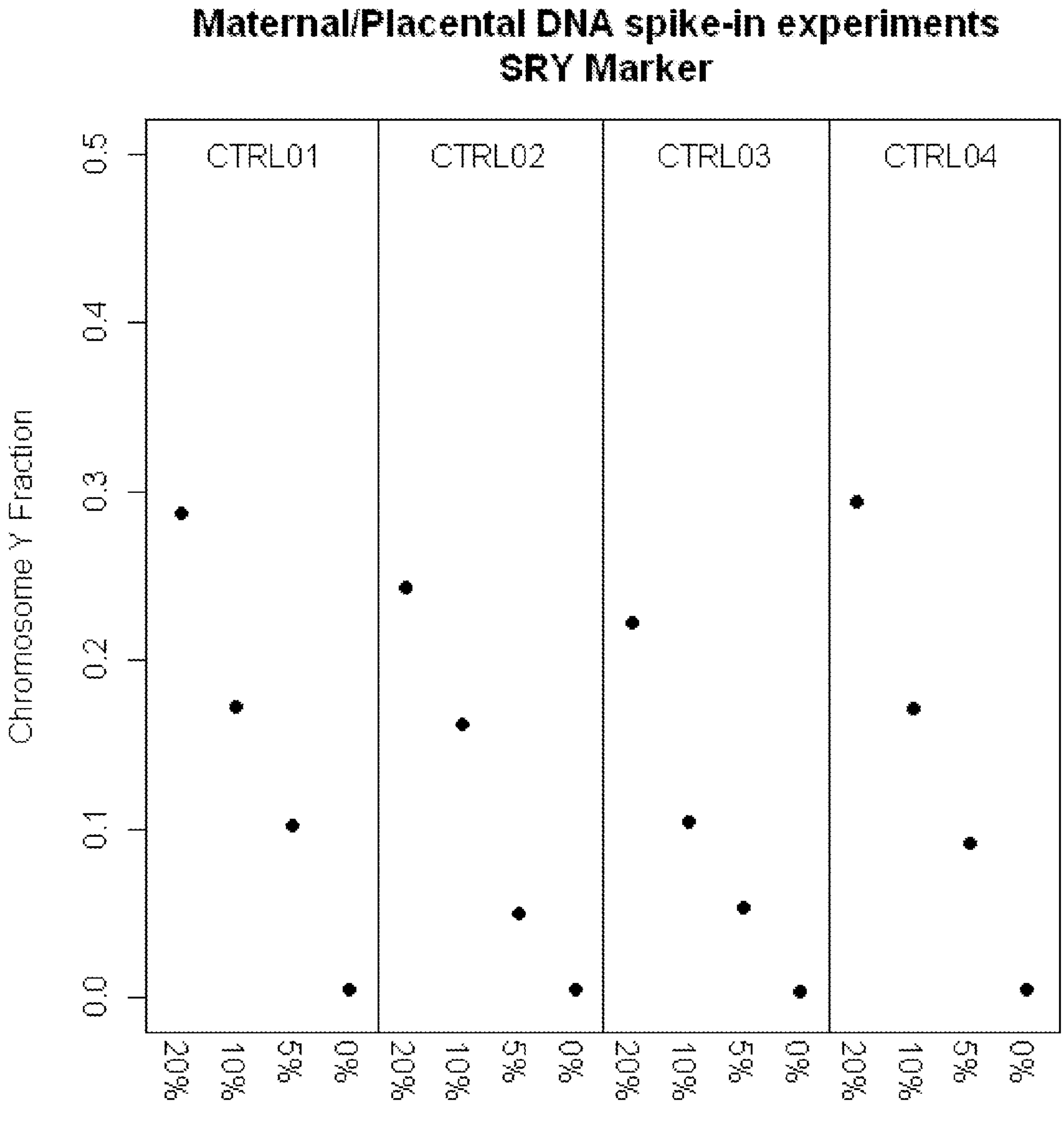

Maternal/Placental DNA spike-in experiments
SRY Marker
Chromosome Y Fraction
0.0
0.1
0.2
0.3
0.4
0.5
CTRL01
CTRL02
CTRL03
CTRL04
20%
10%
5%
0%
20%
10%
5%
0%
20%
10%
5%
0%
20%
10%
5%
0%

FIGURE 14A-B

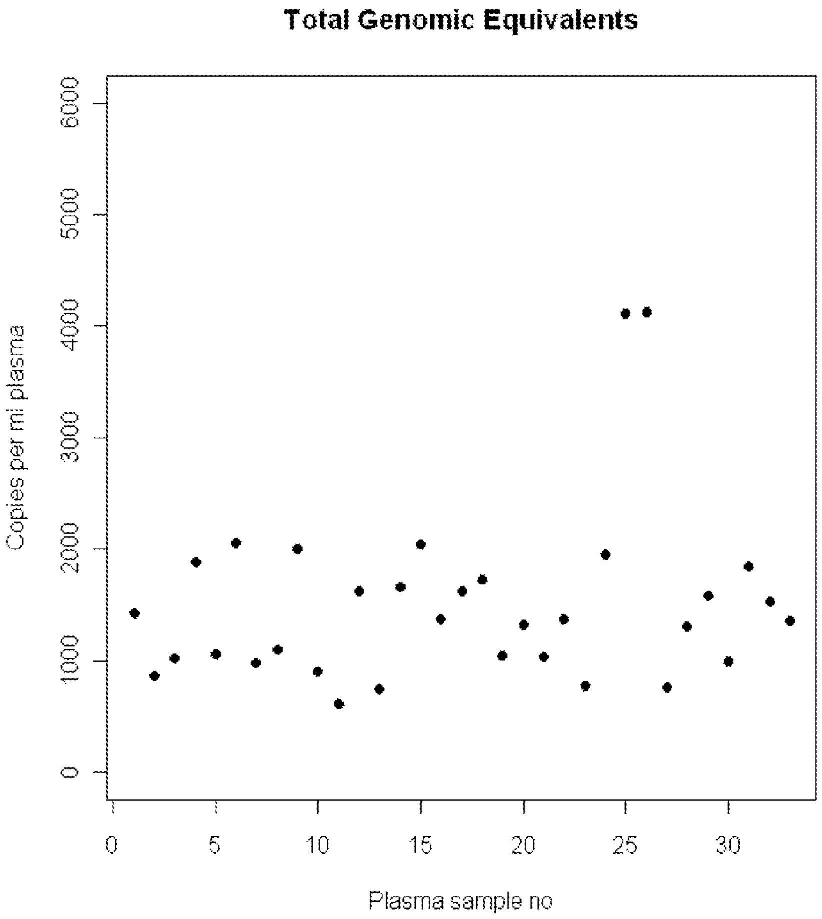


Figure 14A

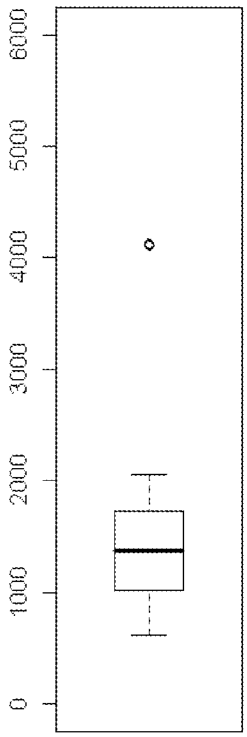


Figure 14B

FIGURE 15A-B

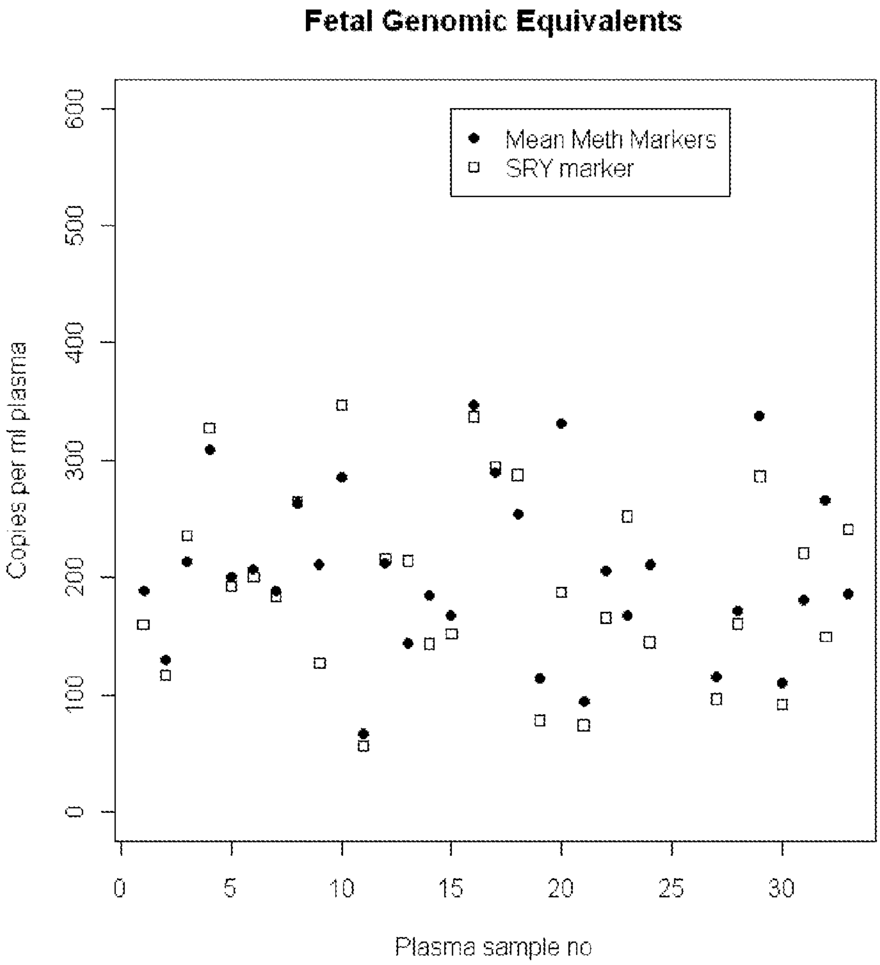


Figure 15A

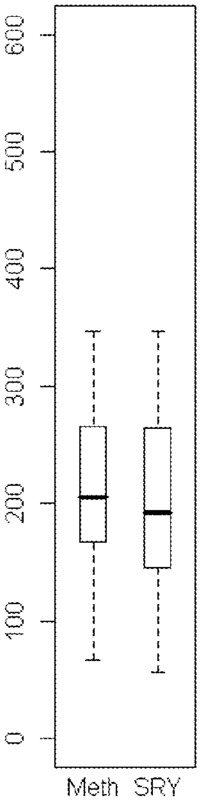


Figure 15B

FIGURE 16

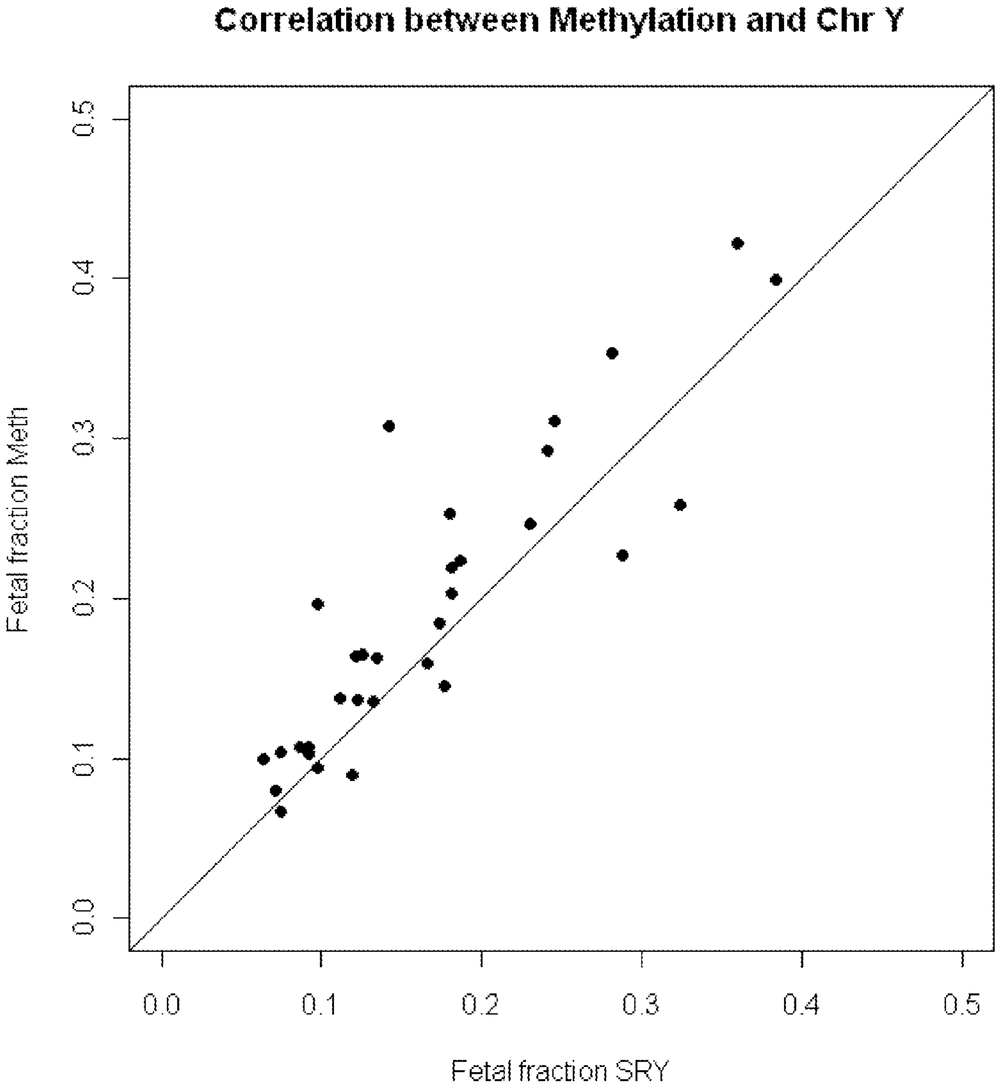

Correlation between Methylation and Chr Y
Fetal fraction Meth
0.0
0.1
0.2
0.3
0.4
0.5
Fetal fraction SRY
0.0
0.1
0.2
0.3
0.4
0.5

FIGURE 17

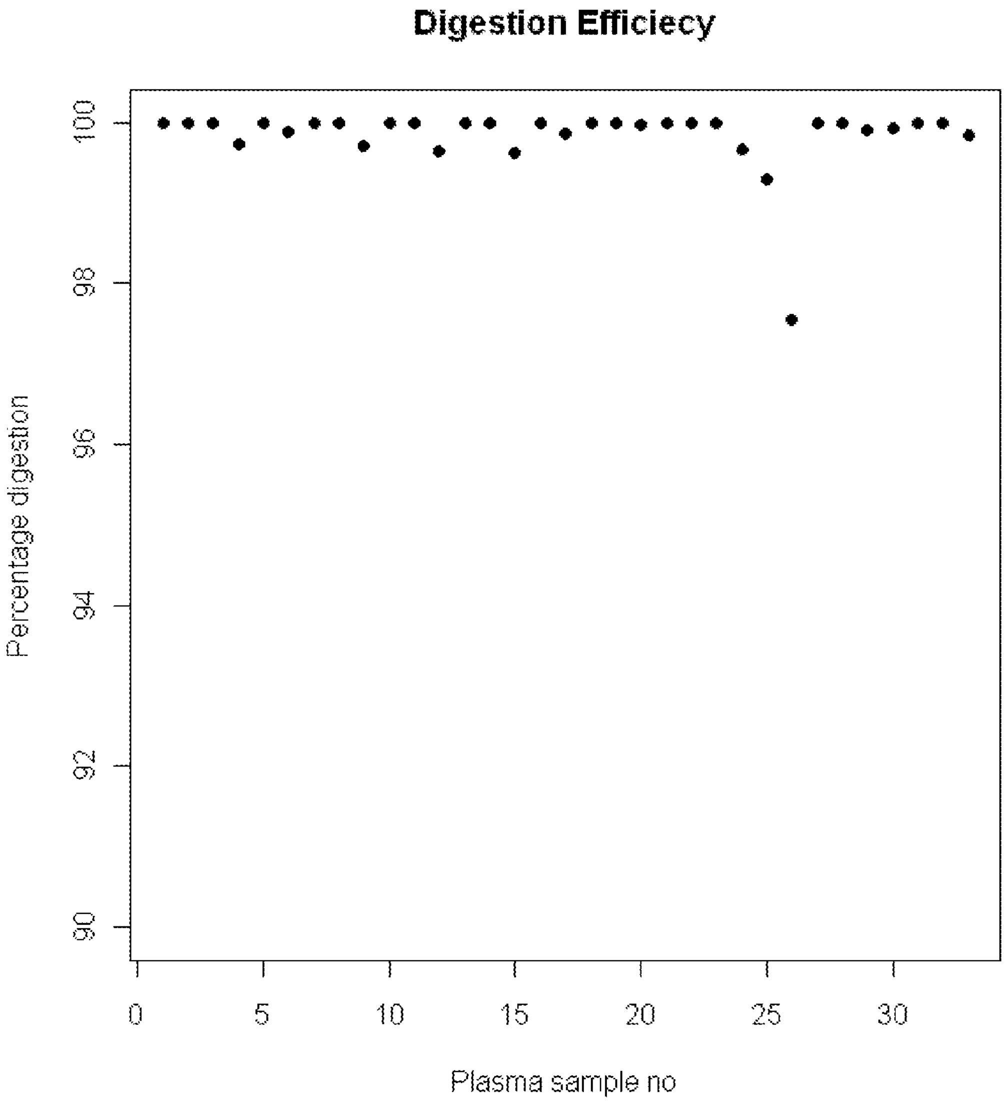
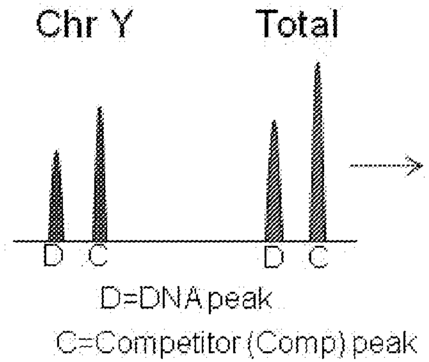

Digestion Efficiecy
Percentage digestion
100
98
96
94
92
90
0
5
10
15
20
25
30
Plasma sample no

FIGURE 18



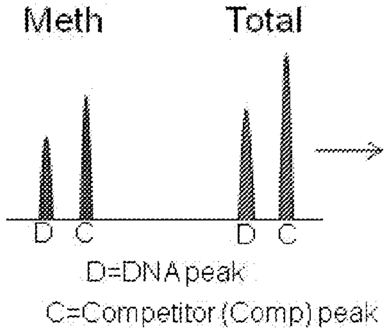
$$\xrightarrow{} \frac{\text{Chr Y(Ratio(D/C)}}{\text{Total(Ratio(D/C)}} \times \frac{\text{\# Comps (Chr Y)}}{\text{\# Comps (Total)}} = \text{Ratio (Chr Y/Total)}$$

Chr Y= 1x fetal genomes G(f)
Total = 2x fetal G(f) and 2x maternal genomes G(m)
G(f) is a fraction of G(m) = k x G(m)

$$\frac{G(f)}{2G(f)+2G(m)} = \frac{k}{2k+2} = R \longrightarrow k=R(2k+2) \longrightarrow k=\frac{2R}{1-2R}$$

Limit: $G(f) < 0.5\ G(m)$

FIGURE 19

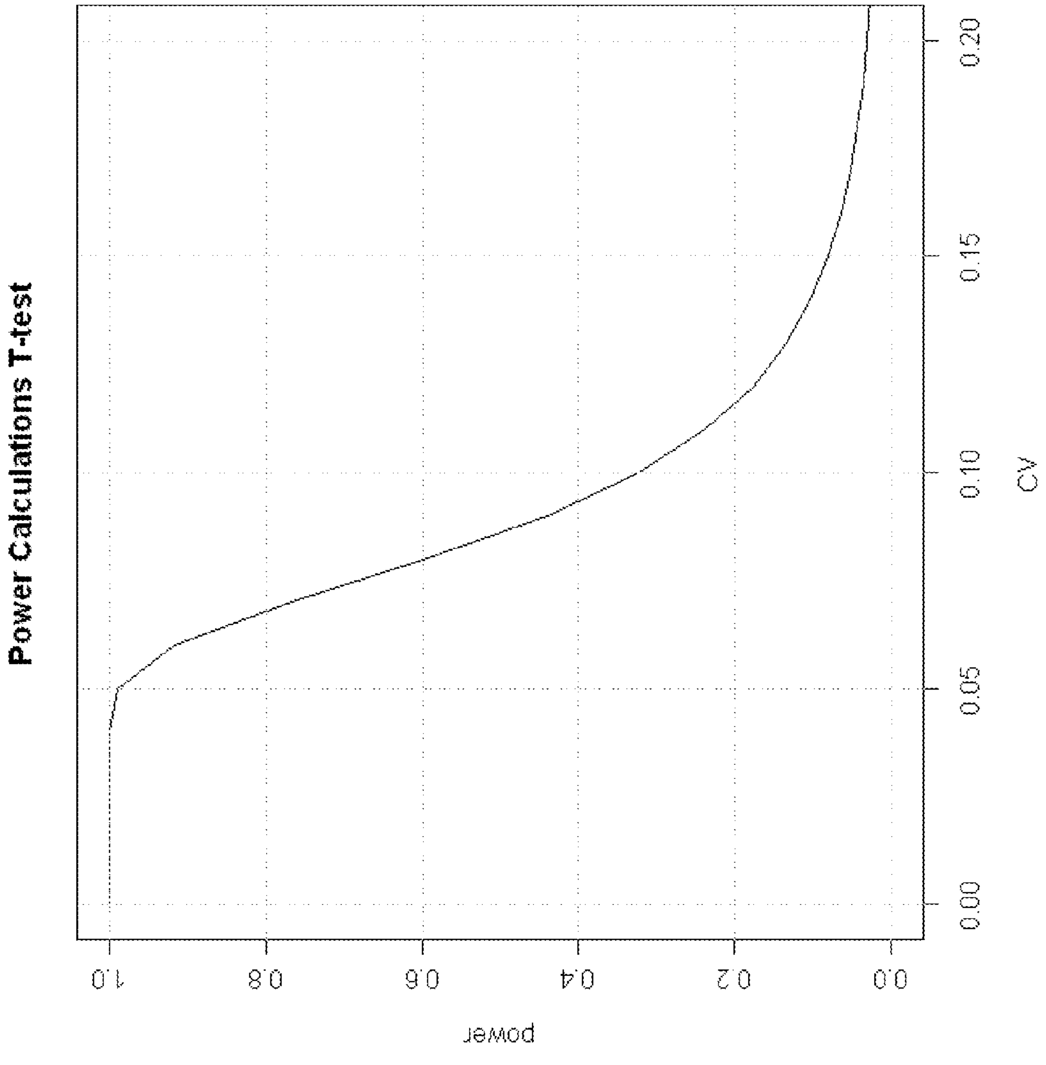


$$\longrightarrow \frac{\text{Meth(Ratio(D/C)}}{\text{Total(Ratio(D/C)}} \times \frac{\text{\# Comps (Meth)}}{\text{\# Comps (Total)}} = \text{Ratio (Meth/Total)}$$

Meth= 2x fetal genomes G(f)
Total = 2x fetal G(f) and 2x maternal genomes G(m)
G(f) is a fraction of G(m) = kG(m)

$$\frac{2\,G(f)}{2G(f)+2G(m)} = \frac{k}{k+1} = R \longrightarrow k=R(k+1) \longrightarrow k=\frac{R}{1-R}$$

FIGURE 20


Power Calculations T-test
power
1.0
0.8
0.6
0.4
0.2
0.0
CV
0.00
0.05
0.10
0.15
0.20