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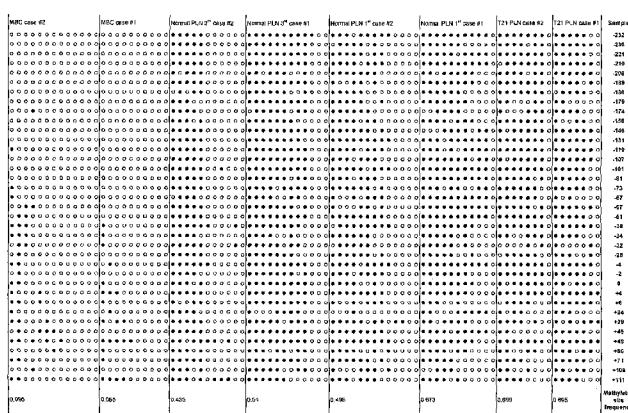
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(54) Title: METHOD FOR DETECTING CHROMOSOMAL ANEUPLOIDY

Figure 1



(57) Abstract: The present invention relates to a new, non-invasive method for detecting chromosomal aneuploidy by analyzing a sample from a pregnant woman. The detection is based on the ratio between the amount of a fetal methylation marker located on a chromosome relevant to the aneuploidy and the amount of a fetal genetic marker located on a reference chromosome, offering improved accuracy.

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METHOD FOR DETECTING CHROMOSOMAL ANEUPLOIDY

RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application No. 61/233,042, filed August 11, 2009, and U.S. Provisional Patent Application No. 61/308,578, filed February 26, 2010, the contents of each are hereby incorporated by reference in the entirety for all purposes.

BACKGROUND OF THE INVENTION

[0002] Various genetic disorders, such as chromosomal aneuploidies, are detectable during pregnancy according to a variety of methods currently in use by hospitals and medical professionals. Most of these methods, however, are invasive and carry a risk of unintended loss of fetus or miscarriage. Noninvasive prenatal diagnosis of chromosomal aneuploidies using fetal DNA in maternal plasma is an actively researched area, and there exists a clear need for new and more reliable methods for early detection. This invention provides a novel method for detecting chromosomal aneuploidies such as trisomy 21 by the combination of fetal-specific epigenetic and genetic markers.

[0003] In particular, the present inventors searched for fetal DNA markers on chromosomes 21, 18, and 13 that were differentially methylated in the placenta and maternal blood cells using methods including combined bisulfite restriction analysis and immunoprecipitation of methylated DNA followed by tiling array hybridization (MeDIP-chip), with confirmation of any target locus with bisulfite sequencing. The resultant markers were analyzed using methylation-sensitive restriction endonuclease digestion followed by real-time polymerase chain reaction (PCR) or microfluidics digital PCR analysis. Chromosome dosage analysis was performed by comparing the dosage of these epigenetic markers to a genetic marker, a DNA sequence that is derived from a fetus and can be distinguished from a maternal DNA sequence by virtue of its distinct polynucleotide sequence. One example of such genetic marker is the *zinc finger protein, Y-linked* (*ZFY*) gene present on chromosome Y.

[0004] For example, it was discovered that the putative promoter of the *holocarboxylase synthetase* (*HLCs*) gene was hypermethylated in the placenta and hypomethylated in maternal blood cells. Chromosome dosage comparison using the hypermethylated *HLCs* and *ZFY* loci can distinguish trisomy 21 and euploid placental DNA samples. The inventors

showed that the epigenetic-genetic chromosome dosage approach is a new method for the noninvasive prenatal detection of chromosomal aneuploidies such as trisomy 21 (T21), trisomy 18 (T18), or trisomy 13 (T13). This approach provides a generally usable technique for noninvasive prenatal diagnosis, utilizing epigenetic markers on any chromosome involved 5 in a chromosomal aneuploidy.

BRIEF SUMMARY OF THE INVENTION

[0005] This invention provides a method for detecting a chromosomal aneuploidy in a fetus carried by a pregnant woman. The method comprises the steps of: (a) determining in a biological sample taken from the pregnant woman the amount of a methylation marker of fetal origin, wherein the methylation marker is located on a chromosome relevant to the chromosomal aneuploidy or within a section of a chromosome relevant to the chromosomal aneuploidy, and wherein the methylation marker of fetal origin is distinguished from its counterpart of maternal origin due to differential DNA methylation; (b) determining the amount of a genetic marker of fetal origin in the sample, wherein the genetic marker is located on a reference chromosome, and wherein the genetic marker of fetal origin is distinguished from its counterpart of maternal origin in the sample due to difference in polynucleotide sequence, or the genetic marker does not exist in the maternal genome; (c) determining the ratio of the amounts from (a) and (b); and (d) comparing the ratio with a standard control, wherein the ratio higher or lower than the standard control indicates the presence of the chromosomal aneuploidy in the fetus. Typically, the standard control value approximates the expected gene or chromosome dosage or ratio in the human genome, although slight variations may exist depending on the specific methodology used in the detection method. For example, there are two copies of chromosome 21 and one copy of chromosome Y in a euploid male genome. Hence, the ratio of dosage between an epigenetic 20 marker on chromosome 21 to a genetic marker on chromosome Y would be approximately 2. 25

[0006] In some cases, the methylation marker of fetal origin is from the placenta. In other cases, the counterpart of maternal origin is from the pregnant woman's blood cells. The methylation marker of fetal origin may be more methylated than its counterpart of maternal origin, or it may be less methylated than its counterpart of maternal origin.

30 [0007] In some cases, the sample is maternal whole blood, serum, plasma, urine, amniotic fluid, genital tract lavage fluid, placental-tissue sample, chorionic villus sample, or a sample containing fetal cells isolated from maternal blood. In other cases, the sample is any sample that contains fetal nucleic acids.

[0008] In some cases, the methylation marker is part of, or in proximity to, the *Holocarboxylase Synthetase (HLCs)* gene. For example, several regions of the *HLCs* gene (including the putative promoter region) as shown in Table 2 may be used as methylation markers. Other methylation markers include *Marker 18A* and *Marker 13A*. Additional 5 methylation markers are defined as follows: a region of about 15 to 450 nucleotides and comprises one cytosine, the region being (1) a genomic locus selected from the group consisting of *MAT.18.0094*, *MAT.13.0023*, *MAT.13.0020*, *MAT.13.0038*, *MAT.18.0071*, *MAT.18.0097*, *MAT.21.0178*, and *TAS.21.1175*; or (2) no more than 10 kb upstream and/or downstream from the locus. Some particular markers on chromosome 21 are defined as 10 follows: a region of about 15 to 450 nucleotides on chromosome 21 and comprises one cytosine, the region being (1) a genomic locus selected from the group consisting of *CGII37*, *phosphodiesterase 9A (PDE9A)*, *Homo sapiens protein phosphatase 1, regulatory (inhibitory) subunit 2 pseudogene 2 (PPP1R2P2)*, and *Similarity to Fem1A (Caenorhabditis elegans)*, or (2) no more than 10 kb upstream and/or downstream from the locus. These 15 methylation markers are intended for use in accordance with the detection method of this invention in combination with any one or more of the genetic markers described herein.

[0009] In some cases, step (a) comprises treating the sample with a reagent that differentially modifies methylated and unmethylated DNA. Such reagent may comprise bisulfite or a protein or chemical that binds to DNA based on methylation status; or the 20 reagent may comprise an enzyme that either preferentially cleaves methylated DNA or preferentially cleaves unmethylated DNA.

[0010] In some cases, the genetic marker does not exist in the maternal genome. In other cases, the genetic marker is located on the Y chromosome. Frequently, the genetic marker of fetal origin is distinguished from the genetic marker of maternal origin based on genetic 25 polymorphism, which includes a single nucleotide polymorphism (SNP), simple tandem repeat polymorphism, and insertion-deletion polymorphism. In one example, the genetic marker is the *Zinc-Finger protein, Y-linked (ZFY)* gene. In another example, the genetic marker is a genomic sequence comprising SNP rs6636 in *TMED8* gene, e.g., SEQ ID NO:1 or SEQ ID NO:2.

30 [0011] In some cases, more than one methylation marker or more than one genetic marker may be used. Frequently, step (a) or step (b) may include the process of amplification of the methylation marker and/or the genetic marker, especially the methylation marker and the genetic marker of the fetal origin. As one example, the amplification is by a polymerase

chain reaction (PCR), such as a methylation-specific PCR; or the amplification may be a nucleic acid sequence-specific amplification.

[0012] In some cases, step (a) or (b) is performed by molecular counting. In other cases, step (a) or (b) comprises digital polymerase chain reaction, real-time quantitative polymerase chain reaction, array capture, a nucleic acid sequence-based detection, massively parallel genomic sequencing, single molecule sequencing, or multiplexed detection of polynucleotide with color-coded probe pairs. In some other cases, step (a) or (b) comprises mass spectrometry or hybridization to a microarray, fluorescence probe, or molecular beacon.

[0013] In some cases, the chromosome relevant to the chromosomal aneuploidy is chromosome 13, 18, 21, or X. For the purpose of data interpretation, in some cases, when the ratio is higher or lower than the standard control by at least 1 standard deviation, it indicates the presence of the chromosomal aneuploidy in the fetus, whereas in other cases, the presence of the chromosomal aneuploidy in the fetus is indicated when the ratio is higher or lower than the standard control by at least 2 or even 3 standard deviations.

[0014] Broadly applied, the above described methods can be used in a non-diagnostic context for assessing any potential change in the amount of a particular chromosome of interest in relation to another chromosome, a reference chromosome that is not suspected to have any change in its relative amount.

[0015] The present invention can also be embodied in a device or a system comprising one or more such devices, which is capable of carrying out all or some of the method steps described herein. For instance, in some cases, the device or system performs the following steps upon receiving a biological sample taken from the pregnant woman: (a) determining in sample the amount of a fetus derived methylation marker relevant to a chromosomal aneuploidy; (b) determining in the sample the amount of a fetus derived genetic marker on a reference chromosome; (c) determining the ratio of the amounts from (a) and (b); and (d) comparing the ratio with a standard control and providing an output indicating whether the chromosomal aneuploidy is present in the fetus. In other cases, the device or system of the invention performs the task of steps (c) and (d), after steps (a) and (b) have been performed and the amounts from (a) and (b) have been entered into the device. Preferably, the device or system is partially or fully automated.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] Figure 1. Cloning and bisulfite sequencing results of *HLCS* region B2.

Individual CpG sites are numbered across the first row, with nucleotide positions defined relative to the transcription start site (position 0). The first CpG site (-232) corresponds to 5 chr21: 37275031 (reverse strand) of the Human March 2006 (hg18) assembly of the UCSC Genome Browser. Each subsequent row depicts the methylation status across the CpG sites in a single DNA molecule studied by cloning. Filled and unfilled circles represent methylated and unmethylated CpG sites, respectively. Clones from trisomy 21, normal first-trimester and normal third-trimester placental tissue samples are labeled with the prefix “T21 PLN,” 10 “Normal PLN 1st,” and “Normal PLN 3rd,” respectively, while those from maternal blood cells are labeled with the prefix “MBC.” Placenta and maternal blood cells from different pregnant individuals are identified by sample numbers following the prefix.

[0017] Figure 2. Digital readout of the gene dosage experiment. The *HLCS* and

RASSF1A assays were run as monoplex, while the *ZFX/Y* assays were run in a duplex

15 fashion. Each chip is divided into 12 panels with each panel being compartmentalized into 765 reaction wells. Reaction wells with the target molecules are shown as red or blue colored dots, while reaction wells with no amplification are shown as grey-colored dots. *HLCS*, *RASSF1A* and *ZFY* are shown in red. *ZFX* is shown in blue. (A) Illustration of the digital PCR results for enzyme-digested euploid placental DNA samples. Four panels were 20 counted for each sample, hence each chip could accommodate three samples. (B) Illustration of the digital PCR results for an enzyme-digested maternal plasma DNA sample. DNA from the sample was evenly distributed in all 12 panels. It is noted that for maternal plasma analysis, the copy number of *ZFY* (fetal DNA) was much less than that of *ZFX* (fetal plus maternal DNA).

25 **[0018] Figure 3. Gene dosage comparison in euploid and trisomy 21 placental tissue DNA samples.** (A) Ratios of *HLCS* to *RASSF1A*. (B) Ratios of *HLCS* to *ZFY*. The normal reference range is depicted by the dotted lines.

[0019] Figure 4. Gene dosage comparison in euploid and trisomy 21 maternal plasma DNA samples. The ratios of *HLCS* to *ZFY* were plotted for each sample. The normal

30 reference range is depicted by the dotted lines.

[0020] Figure 5. Genomic sequences of the four digital PCR assays (*HLCS*, *RASSF1A*, *ZFY* and *ZFX*). The chromosomal location on the March 2006 human reference sequence (NCBI Build 36.1) of the UCSC Genome Browser is indicated. The underlined nucleotides

represent the enzyme recognition sites. The bold nucleotides in capital letters represent the forward and reverse primers. The bold nucleotides in small letters represent the minor groove binding (MGB) probe sequence.

[0021] **Figure 6. Gel electrophoresis of COBRA results for (A) *HLCS* region B2, and**

5 **(B) *C21orf81*.** Two trisomy 21 placentas (T21 PLN), two first-trimester normal placentas (Normal PLN 1st), two third-trimester normal placentas (Normal PLN 3rd), and two first-trimester maternal blood cells (MBC) were analyzed. PCR products were incubated with (+) or without (-) the *Bst*UI enzyme. DNA methylation was detected by the appearance of the smaller size digestion products. One kb ladder (Invitrogen Carlsbad, CA) (M) was used in 10 gel electrophoresis.

[0022] **Figure 7. Quantification of *HLCS* DNA from four placental tissues and eight**

maternal blood cells. The methylation index of a sample was calculated by dividing the copy number of *HLCS* after enzyme digestion by that obtained from mock digestion.

“Normal PLN” represents placental DNA and “MBC” represents maternal blood cell DNA.

15 [0023] **Figure 8. Postpartum clearance in plasma.** Detection of *HLCS* in maternal

plasma samples (A) with (+), or (B) without (-) restriction enzyme digestion. “Pre”

represents pre-delivery plasma samples and “Post” represents post-delivery plasma samples. Paired samples from the same subject are depicted by identical symbols connected by a line.

[0024] **Figure 9. Chromosome dosage comparison in euploid and trisomy 21 placental**

20 tissue DNA samples by determining the ratio of (A) *HLCS* to *TMED8-C* with mock

digestion, and (B) *HLCS* to *TMED8-C* with *Bst*UI digestion. The normal reference range is depicted by dotted lines.

[0025] **Figure 10. Chromosome dosage comparison in euploid and trisomy 21 placental**

tissue DNA samples by determining the ratio of (A) *HLCS* to *TMED8-G* with mock

25 digestion, and (B) *HLCS* to *TMED8-G* with *Bst*UI digestion. The normal reference range is depicted by dotted lines.

[0026] **Figure 11. Chromosome dosage comparison in *Bst*UI-digested, euploid and**

trisomy 21 maternal plasma DNA samples by determining the ratio of (A) hypermethylated *HLCS* to *TMED8-C* allele, and (B) hypermethylated *HLCS* to *TMED8-G* allele. The normal

30 reference range is depicted by dotted lines.

[0027] **Figure 12. DNA methylation levels of *Marker 18A* in 6 pairs of first-trimester**

euploid placentas and maternal blood cells determined by cloned bisulfite sequencing. A

total of 8 clones were scored for each sample. The methylated sites are represented by a filled circle, while the unmethylated sites are represented by an empty circle. The methylation index (MI) at each CpG site was given by the number of methylated clones over the total number of clones at each CpG site. The methylated site frequency was given by the number of cloned methylated CpG sites over the total number of cloned CpG sites for each sample. The positions of the recognition sites of the methylation-sensitive restriction endonucleases are marked next to each genomic location of the CpG site. The genomic locations were defined according to the Human Genome March 2006 Assembly (hg18) of the UCSC Genome Browser (genome.ucsc.edu).

10 [0028] **Figure 13.** DNA methylation levels of *Marker 18A* in euploid (n = 6) and T18 (n = 6) placentas analyzed by the Epityper assay. All T18 and euploid placentas were obtained from the first trimester. The methylation indices (MIs) of the euploid cases were compared with the T18 cases at each CpG unit using Mann Whitney Rank Sum Test.

15 [0029] **Figure 14. Characterization of digestion-resistant *Marker 18A* sequences in maternal plasma** (A) Concentrations of digestion-resistant *Marker 18A* DNA in maternal plasma before and after delivery of the fetus. (B) Correlation between concentrations of digestion-resistant *Marker 18A* DNA against the concentrations of an established fetal genetic marker, *ZFY*, in pregnancies bearing male fetuses.

20 [0030] **Figure 15.** Relative dosage of chromosome 18 (*Marker 18A*) to chromosome Y (ZFY) in euploid (n = 5) and trisomy 18 (T18) (n = 5) placental DNA samples. The normal reference range (mean \pm 1.96SD) is depicted by the dotted lines.

[0031] **Figure 16.** Relative dosage of fetal chromosome 18 (*Marker 18A*) to chromosome Y (ZFY) in euploid (n = 27) and trisomy 18 (T18) (n = 9) maternal plasma samples. The normal reference range (mean \pm 1.96SD) is depicted by the dotted lines.

25 [0032] **Figure 17.** DNA methylation levels of a region on the *beta-actin* gene in 3 pairs of first-trimester euploid placentas and maternal blood cells determined by cloned bisulfite sequencing. For legends of the bisulfite sequencing data, refer to Figure 12. This region was used for the development of a control assay to check the efficiency of enzyme digestion.

30 [0033] **Figure 18.** DNA methylation levels of *Marker 13A* in euploid placentas (n = 6) and maternal blood cells (n = 6) analyzed by cloned bisulfite sequencing. All of the samples were obtained from the first trimester. A total of 8 clones were scored for each sample. The methylated sites are represented by a filled circle, while the unmethylated sites are

represented by an empty circle. The methylation index (MI) at each CpG site was given by the number of methylated clones over the total number of clones at each CpG site. The methylated site frequency was given by the number of cloned methylated CpG sites over the total number of cloned CpG sites for each sample. The positions of the recognition sites of the methylation-sensitive restriction endonucleases are marked next to each genomic location of the CpG site. The genomic locations were mapped according to the Human Genome March 2006 Assembly (hg18) of the UCSC Genome Browser (genome.ucsc.edu).

5 [0034] **Figure 19.** DNA methylation levels of *Marker 13A* in euploid (n = 5) and T13 (n = 5) placentas analyzed by the Epityper assay. Four of the trisomy 13 (T13) placentas were obtained from the first trimester while one was obtained from the second trimester. All of the 10 euploid placentas were obtained from the first trimester. The MIs of the euploid cases were compared with the T13 cases at each CpG unit using Mann Whitney Rank Sum Test.

15 [0035] **Figure 20.** Chromosome dosage comparison in euploid (n = 10) and trisomy 13 (T13) (n = 5) placental DNA samples by determining the ratio of digestion-resistant *Marker 13A* to *ZFY*. The normal reference range is depicted by dotted lines.

DEFINITIONS

20 [0036] The term "**pregnancy-associated disorder**," as used in this application, refers to any condition or disease that may affect a pregnant woman, the fetus the woman is carrying, 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 25 span of the fetus following its birth. A "pregnancy-associated disorder" is necessarily accompanied by pregnancy and is not merely a condition that is incidentally occurring to a pregnant woman. In other words, the disorder is not one that can occur to a non-pregnant woman. Some examples of a pregnancy-associated disorder include ectopic pregnancy, preeclampsia, preterm labor, and fetal chromosomal abnormalities such as trisomy 13, 18, or 21.

30 [0037] The term "**chromosomal aneuploidy**," as used herein, encompasses any genetic defect exhibiting an abnormal number of chromosomes, including having more or fewer than normal number of any one chromosome, as well as having an extra portion of any one chromosome in addition to the normal pair, or missing a portion of any one chromosome in the normal pair. In some cases, the abnormality can involve more than one chromosome, or more than one portion of one or more chromosomes. The most common chromosome aneuploidy is trisomy, e.g., trisomy 21, where the genome of an afflicted patient has three

rather than the normal two (*i.e.*, a pair) chromosome 21. In rarer cases, the patient may have an extra piece of chromosome 21 (less than full length) in addition to the normal pair. In yet other cases, a portion of chromosome 21 may be translocated to another chromosome, *e.g.* chromosome 14. In this example, chromosome 21 is referred as the "**chromosome relevant**

5 **to the chromosomal aneuploidy**" and a second, irrelevant chromosome, *i.e.*, one that is present in the normal pair in the patient's genome, for example chromosome 1, is a "**reference chromosome.**" There are also cases where the number of a relevant chromosome is less than the normal number of 2. Turner syndrome is one example of a chromosomal aneuploidy where the number of X chromosome in a female subject has been reduced from

10 two to one.

[0038] A "**genetic marker,**" as used in the context of comparison with a "**methylation marker**" to determine their relative amount or concentration, *i.e.*, a ratio, refers to a polynucleotide sequence present in the genomic sequence of a reference chromosome that permits different alleles (*e.g.*, alleles from two different individuals, such as alleles from a 15 fetus *v.* alleles from the pregnant woman) to be distinguished from each other based on difference in the polynucleotide sequence (*e.g.*, polymorphism), or presence or absence of the sequence at all (*e.g.*, a sequence present on the Y chromosome from a male fetus but not present in the pregnant woman's genome). In this context, a "**methylation marker**" located on a chromosome relevant to the chromosomal aneuploidy refers to a genomic polynucleotide 20 sequence on a chromosome having an abnormal number; or in the case where there is an extra piece of the chromosome or a portion of the chromosome is missing, the "**methylation marker**" is located within the piece or portion of the relevant chromosome. Difference in methylation profiles of the methylation marker allows distinction of the corresponding methylation marker from two different individuals, *e.g.*, a fetus and the pregnant woman.

25 **[0039]** The term "**epigenetic state**" or "**epigenetic status**" as used herein refers to any structural feature at the 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, *e.g.*, histones and the modifications of such 30 proteins, *e.g.*, acetylation, deacetylation, and methylation.

[0040] The term "**methylation profile**" or "**methylation status,**" when used in this application to describe the state of methylation of a genomic sequence, refers to the characteristics of a DNA fragment at a particular genetic 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" may also refer to the relative or absolute concentration of methylated C or unmethylated C at any particular stretch of residues in a biological sample.

5 [0041] The term "**single nucleotide polymorphism**" or "SNP" as used herein refers to the polynucleotide sequence variation present at a single nucleotide residue among different alleles of the same gene, which may be the same gene located on the two copies of the same 10 chromosome from the same individual (*e.g.*, two alleles from a fetus) or may be the same gene from two different individuals (*e.g.*, fetus and pregnant woman). This variation may occur within the coding region or non-coding region (*e.g.*, the promoter region or its proximity, or the intron) of a gene, or in the intergenic region. Detection of one or more SNP allows differentiation of different alleles of a single gene.

15 [0042] The term "**simple tandem repeat polymorphism**" as used herein refers to the polynucleotide sequence variation demonstrated in the varying number of tandem repeats of a nucleotide sequence (*e.g.*, a tandem repeat of 1 or more nucleotides) among different alleles of the same gene, which may be the same gene located on two copies of the same chromosome from the same individual (*e.g.*, fetus) or may be the same gene from two 20 different individuals (*e.g.*, fetus and pregnant woman). This variation often occurs within the non-coding region (*e.g.*, the promoter region or its proximity, or intron) of a gene, or in the intergenic region. Detection of difference in tandem repeat numbers allows differentiation of different alleles of a single gene.

25 [0043] The term "**insertion-deletion polymorphism**" as used herein refers to the polynucleotide sequence variation demonstrated in the presence or absence of a short nucleotide sequence (*e.g.*, 1-3 nucleotides) among different alleles of the same gene, which may be the same gene located on two copies of the same chromosome from the same individual (*e.g.*, fetus) or may be the same gene from two different individuals (*e.g.*, fetus and pregnant woman). This variation can occur within both the coding region and the non- 30 coding region (*e.g.*, the promoter region or its proximity, or intron) of a gene, or in the intergenic region. Detection of whether a short nucleotide sequence is present allows differentiation of different alleles of a single gene.

[0044] 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.

[0045] The term "bisulfite" as used herein encompasses all 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.

[0046] As used herein, a reagent that "differentially modifies" methylated or non-methylated DNA encompasses any reagent that reacts differentially with methylated and unmethylated DNA in a process through which distinguishable products or quantitatively distinguishable results (*e.g.* degree of binding or precipitation) are generated 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 an unmethylated C → U conversion by bisulfite), enzymatic treatment (such as cleavage by a methylation-dependent endonuclease), binding, and precipitation. Thus, an enzyme that preferentially cleaves methylated DNA is one capable of cleaving a DNA molecule at a much higher efficiency when the DNA is methylated, whereas an enzyme that preferentially cleaves unmethylated DNA exhibits a significantly higher efficiency when the DNA is not methylated. In the context of the present invention, a reagent that "differentially modifies" methylated and unmethylated DNA also refers to any reagent that exhibit differential ability in its binding to DNA sequences or precipitation of DNA sequences depending on their methylation status. One class of such reagents consists of methylated DNA binding proteins.

[0047] The term "nucleic acid" or "polynucleotide" refers to deoxyribonucleic acids (DNA) or ribonucleic acids (RNA) and polymers thereof in either single- or double-stranded form. 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 gene, cDNA, and mRNA encoded by a gene.

[0048] 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).

[0049] The term “**locus**” means the segment of DNA defined by a start nucleotide position

10 to an end nucleotide position on a chromosome (*i.e.*, a genomic location, or a chromosomal location) of a reference genome assembly (*e.g.*, the Human Genome March 2006 assembly (hg18) on the UCSC Genome Browser). A locus may or may not overlap with the genomic location of a gene, a CpG island, or any product of transcription/translation. In this application, a locus usually refers to a continuous segment of DNA identified by 15 experimental data (*e.g.*, a MeDIP-chip dataset) and the subsequent data analysis (*e.g.*, MAT, TAS) to contain different DNA methylation levels. A locus may contain one or more CpG sites. A locus may be sub-divided into shorter segments (*e.g.*, CpG-containing genomic sequences, fragments or regions) that are amenable to analysis (*e.g.*, Epityper assay, bisulfite sequencing, polynucleotide amplification and determination). A locus may be developed into 20 one or more fetal epigenetic markers. In some context of this application, a locus also refers to a continuous segment of DNA identified by certain bioinformatics criteria.

[0050] 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 25 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.

[0051] The term “**amino acid**” refers to naturally occurring and synthetic amino acids, as

30 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, γ -carboxyglutamate, and O-phosphoserine.

[0052] 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.

5 [0053] As used in this application, an "increase" or a "decrease" refers to a detectable positive or negative change in quantity from an established standard control. An increase is a positive change by at least 10% or 20% or by at least 50%, 80%, or 100% of the standard control value; in some case, an increase can be at least about 1.5-fold, at least about 2-fold, or at least about 3-fold of the control value. Similarly, a decrease is a negative change by at 10 least 1/10, 1/6, 1/5, or by at least 1/3 or even 1/2 of the control value. Other terms indicating quantitative changes or differences from a comparative basis, such as "more," "less," "higher," and "lower," are used in this application in the same fashion as described above.

15 [0054] A "polynucleotide hybridization method" as used herein refers to a method for detecting the presence and/or quantity of a polynucleotide based on its ability to form Watson-Crick base-pairing, under appropriate hybridization conditions, with a polynucleotide probe of a known sequence. Examples of such hybridization methods include Southern blotting and Northern blotting.

20 [0055] "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 gene of interest, *e.g.*, the *HLCS* gene in various methylation states. At least one of the PCR primers for amplification of a polynucleotide sequence is sequence-specific for the sequence.

25 [0056] The term "digital polymerase chain reaction" as used herein refers to a refined version of conventional polymerase chain reaction (PCR) methods that can be used to directly quantify and clonally amplify nucleic acids including DNA, cDNA or RNA, such that the amount of target nucleic acid can be directly quantitatively measured. Digital PCR achieves this direct quantitative measurement by capturing or isolating each individual nucleic acid molecule present in a sample within many separate reaction chambers that are able to localize and concentrate the amplification product to detectable levels. After PCR 30 amplification, a count of chambers containing the PCR end-product is a direct measure of the absolute nucleic acid quantity. The capture or isolation of individual nucleic acid molecules, typically by way of dilution, may be effected in capillaries, microemulsions, arrays of miniaturized chambers, or on nucleic acid binding surfaces. The basic methodology of

digital PCR is described in, *e.g.*, Sykes *et al.*, *Biotechniques* 13 (3): 444-449, 1992; and Vogelstein and Kinzler, *Proc Natl Acad Sci U S A* 1999;96:9236-41.

[0057] The term "**molecular counting**" as used herein refers to any method that allows quantitative measurement of the number of a molecule or molecular complex, often the relative number in the context of other co-existing molecules or complexes of distinct characteristics. Various methods of molecular counting are described in, *e.g.*, Leaner *et al.*, *Analytical Chemistry* 69:2115-2121, 1997; Hirano and Fukami, *Nucleic Acids Symposium Series* No. 44:157-158, 2000; Chiu *et al.*, *Trends in Genetics* 25:324-331, 2009; and U.S. Patent No. 7,537,897.

[0058] "**Standard control**" as used herein refers to a value reflective of the ratio, or the amount or concentration of a fetal genomic sequence located on a chromosome relevant to a particular chromosomal aneuploidy (*e.g.*, trisomy 13, 18, or 21) over the amount or concentration of a fetal genetic marker located on a reference chromosome, as the amounts or concentrations are found in a biological sample (*e.g.*, blood, plasma, or serum) from an average, healthy pregnant woman carrying a chromosomally normal fetus. A "standard control" may be determined differently and represent different value depending on the context in which it is used. For instance, when used in an epigenetic-genetic dosage method where an epigenetic marker is measured against a genetic marker, the "standard control" is a value reflective of the ratio, or the amount or concentration of a fetal genomic sequence located on a chromosome relevant to a particular chromosomal aneuploidy (*e.g.*, trisomy 13, 18, or 21) over the amount or concentration of a fetal genetic marker located on a reference chromosome, as the amounts or concentrations are found in a biological sample (*e.g.*, blood, plasma, or serum) from an average, healthy pregnant woman carrying a chromosomally normal fetus. In some case, a standard control is determined based on an average healthy pregnant woman at a certain gestational age, whereas in other cases, no distinction is made with regard to the gestational age.

[0059] The term "**average**," as used in the context of describing a pregnant woman, refers to certain relevant characteristics, such as the methylation profile of a particular gene or genomic sequence of both maternal and fetal origins found in the woman's blood, that are representative of a randomly selected group of healthy women who are pregnant with chromosomally normal fetuses and not suffering from any pregnancy-related diseases or conditions at the time of sample collection. This selected group should comprise a sufficient number of women such that the average amount or methylation profile of the gene of interest

among these women reflects, with reasonable accuracy, the corresponding profile in the general population of healthy pregnant women with healthy fetuses. In addition, the selected group of women generally have a similar gestational age to that of a woman whose blood is tested for indication of a potential pregnancy-associated disorder. The preferred gestational 5 age for practicing the present invention may vary depending on the disorder that is being screened for. For example, fetal chromosomal aneuploidy is preferably screened for and diagnosed as early as possible. Moreover, the preferred gestational age for testing may also depend on the gene of interest in testing.

10 [0060] The term "**amount**" as used in this application refers to the quantity of a polynucleotide sequence of interest, *e.g.*, a genetic marker or an epigenetic/methylation marker of fetal origin, present in a sample. Such quantity may be expressed in the absolute terms, *i.e.*, the total quantity (*e.g.* in mass, or in number of molecules) of the polynucleotide sequence in the sample, or in the relative terms (*e.g.* a ratio, or relative to other markers), or as the concentration (*e.g.* mass per unit volume, or number of molecules per unit volume) of 15 the polynucleotide sequence in the sample.

DETAILED DESCRIPTION OF THE INVENTION

I. Introduction

20 [0061] The screening for trisomies such as trisomy 21 (T21) constitutes an important component in modern obstetrics care in many countries. The common screening methods that are routinely available in prenatal clinics target the epiphrenomena associated with the chromosomal aneuploidy, rather than directly targeting the actual chromosome dosage in the fetus (Wapner *et al.*, *N Engl J Med* 2003;349:1405-13; Malone *et al.*, *N Engl J Med* 2005;353:2001-11). For a definitive test, invasive procedures, such as chorionic villus sampling and amniocentesis, are needed in conventional screening programs. These 25 methods, however, can lead to procedure-related fetal loss (Tabor *et al.*, *Lancet* 1986;1:1287-93).

30 [0062] The discovery of cell-free fetal DNA in maternal plasma in 1997 has offered new possibilities for noninvasive prenatal diagnosis (Lo *et al.*, *Lancet* 1997;350:485-7; Lo and Chiu, *Nat Rev Genet* 2007;8:71-7). Detection of the fetal-derived, paternally-inherited genetic materials in maternal plasma samples has allowed the prenatal determination of fetal Rhesus D blood group (Lo *et al.*, *N Engl J Med* 1998;339:1734-8; Finning *et al.*, *BMJ* 2008;336:816-8) and fetal sex determination for sex-linked disorders (Costa *et al.*, *N Engl J*

Med 2002;346:1502). The development of noninvasive prenatal diagnostic tests for fetal chromosomal aneuploidies, however, has been more challenging.

[0063] Two main groups of approaches have been developed for the direct detection of T21 from maternal plasma nucleic acid analysis. The first group involves allelic ratio analysis of 5 single nucleotide polymorphisms (SNPs) present in a fetal-specific nucleic acid marker (Lo and Chiu, *Nat Rev Genet* 2007;8:71-7). Examples of the latter include circulating placental mRNA (the so-called RNA-SNP approach) (Lo *et al.*, *Nat Med* 2007;13:218-23) and DNA methylation markers (the so-called epigenetic allelic ratio approach) (Tong *et al.*, *Clin Chem* 2006;52:2194-202). The main disadvantage of this approach is that these methods are only 10 applicable to fetuses that are heterozygous for the analyzed SNP. Furthermore, there is a finite number of SNPs with sufficiently high heterozygosity rates within a particular locus so population coverage is a major challenge for this approach. The second group of approaches involves the use of single molecule counting approaches such as digital PCR (Lo *et al.*, *Proc Natl Acad Sci U S A* 2007;104:13116-21) and massively parallel genomic sequencing (Chiu 15 *et al.*, *Proc Natl Acad Sci U S A* 2008;105:20458-63; Fan *et al.*, *Proc Natl Acad Sci U S A* 2008;105:16266-71). In these approaches, individual plasma DNA molecules are counted. The precision of such methods allows the slight increase in chromosome 21-derived DNA molecules that is present in the plasma of a pregnant woman carrying a T21 fetus to be 20 detected. One disadvantage of these approaches is that if used on markers with no fetal-specificity (e.g., random sequences from chromosome 21), then an extremely large number of molecules would need to be counted, as exemplified in recent experiments using massively parallel genomic sequencing when millions of molecules would need to be analyzed per case (Chiu *et al.*, *Proc Natl Acad Sci U S A* 2008;105:20458-63; Fan *et al.*, *Proc Natl Acad Sci U S A* 2008;105:16266-71). This latter requirement is associated with the use of expensive 25 equipment and reagents, and relatively complex bioinformatics.

[0064] This application describes a new approach for the noninvasive prenatal detection of chromosomal aneuploidies (e.g., trisomy 21) from maternal plasma that is based on the determination of the ratio of the concentrations of a fetal-specific DNA methylation marker on chromosome potentially involved in the aneuploidy (e.g., chromosome 21) and a fetal-specific DNA marker on a reference chromosome. This is called the epigenetic-genetic (EGG) chromosome dosage approach. Compared with the epigenetic allelic ratio described above, the EGG approach does not require the fetal-specific DNA methylation marker and the SNP to be present within a short stretch of DNA and thus population coverage would be much easier to be achieved. The EGG approach is more precise than an approach based 30

purely on epigenetic markers, the latter being typically performed with one on chromosome 21 and one on a reference chromosome.

[0065] For the EGG approach to work for trisomy detection, it is crucial to have a good fetal-specific DNA methylation marker on the relevant chromosome, such as chromosome 21, 18, or 13. One preferred type of markers would be one that is hypermethylated in the fetus and hypomethylated in maternal blood cells, so that one can use methylation-sensitive restriction enzyme to digest away the maternal sequences. Indeed, previous work has shown that the promoter of the *RASSF1A* gene is one such marker, except that it is on chromosome 3 (Chan *et al.*, *Clin Chem* 2006;52:2211-8; Chiu *et al.*, *Am J Pathol* 2007;170:941-50). A number fetal DNA methylation markers on chromosome 21 has been reported (Chim *et al.*, *Clin Chem* 2008;54:500-11; Old *et al.*, *Reprod Biomed Online* 2007;15:227-35; Papageorgiou *et al.*, *Am J Pathol* 2009;174:1609-18). The present inventors searched for additional markers using combined bisulfite restriction analysis (COBRA) (Xiong and Laird, *Nucleic Acids Res* 1997;25:2532-4) targeting 35 gene promoter regions on chromosome 21. This has led to the discovery of differential methylation of the putative promoter region of the *Holocarboxylase Synthetase* (biotin-(proprionyl-Coenzyme A-carboxylase (ATP-hydrolysing)) ligase) (*HLCS*) gene as a new hypermethylated fetal DNA marker. The EGG concept was then implemented using *HLCS*. The discovery of fetal-specific methylation markers on chromosomes 18 and 13 further permits the use of similar strategies for detection of trisomy 18 and 13.

II. General Methodology

[0066] Practicing this invention utilizes routine techniques in the field of molecular biology. Basic texts disclosing the general methods of use in this invention 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)).

[0067] 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.

[0068] Oligonucleotides that are not commercially available can be chemically synthesized, *e.g.*, according to the solid phase phosphoramidite triester method first described by Beaucage and 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).

5 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 and Reanier, *J. Chrom.* **255**: 137-149 (1983).

[0069] The sequence of a genetic marker or genomic sequence used in this invention, *e.g.*, the polynucleotide sequence of *HLCS* or *ZFY* gene, and synthetic oligonucleotides (*e.g.*, primers) can be verified using, *e.g.*, the chain termination method for sequencing double-stranded templates of Wallace *et al.*, *Gene* **16**: 21-26 (1981).

III. Acquisition of Blood Samples and Extraction of DNA

[0070] The present invention relates to analyzing the epigenetic-genetic chromosome dosage of appropriate fetal chromosomal 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 this invention are to obtain a blood sample from a pregnant woman and extract DNA from the sample.

A. Acquisition of Blood Samples

[0071] A blood sample is obtained from a pregnant woman at a gestational age suitable for testing using a method of the present invention. 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 maybe stored according to standard procedure prior to further preparation.

25 B. Preparation of Blood Samples

[0072] The analysis of fetal DNA found in maternal blood according to the present invention may be performed using, *e.g.*, 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, NJ) to prevent blood clotting, and plasma can then be obtained from whole blood through centrifugation. As alternatives to EDTA, heparin or citrate can be used as anticoagulants. 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 x g. Plasma or serum may be subjected to additional centrifugation steps before being transferred to a fresh tube for DNA extraction. Instead or in 5 additional to such centrifugation steps, it is also possible to use one or more filtration steps to remove particulate matters from plasma or serum.

[0073] 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. The 10 cellular fraction can also be previously enriched for fetal nucleated cells circulating in maternal blood. Such enrichment procedures may include a sorting or selection procedure involving one or more antibodies against fetal cells or a procedure targeting physical, chemical, biochemical or other characteristics which can differentiate fetal from maternal cells. The sorting procedure can involve technologies such as the fluorescence activated cell 15 sorter, magnetic activated cell sorting or microfluidics. The selection procedure can involve micromanipulation, or laser-captured microdissection, manipulation with optical tweezers or any other single cell manipulation procedure. Fetal cell types that can be targeted include nucleated red blood cells, lymphocytes, mononuclear cells, trophoblasts, or cellular remnants, *e.g.*, apoptotic fetal cells.

20 **C. Extraction of DNA**

[0074] 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 the QIAamp DNA Mini Kit or 25 QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), GenomicPrep™ Blood DNA Isolation Kit (Promega, Madison, WI), and GFX™ Genomic Blood DNA Purification Kit (Amersham, Piscataway, NJ), 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.

IV. Methylation-Specific Modification of DNA

30 [0075] To properly identify the origin of an epigenetic marker or a genomic sequence (*e.g.*, the *HLCs* gene) that is differentially methylated between the fetal and maternal DNA present in a sample, DNA isolated from the last step must be analyzed for their methylation status so as to distinguish the fetal and maternal DNA. Upon being extracted from a blood sample of a

pregnant woman, the DNA is treated with a reagent capable of chemically modifying DNA in a methylation differential manner, *i.e.*, different and distinguishable chemical structures will result from a methylated cytosine (C) residue and an unmethylated C residue following the treatment. Typically, such a reagent reacts with the unmethylated C residue(s) in a DNA 5 molecule and converts each unmethylated C residue to a uracil (U) residue, whereas the methylated C residues remain unchanged. This C → U conversion allows detection and comparison of methylation status based on changes in the primary sequence of the nucleic acid. An exemplary reagent suitable for this purpose is bisulfite, such as sodium bisulfite. Methods for using bisulfite for chemical modification of DNA are well known in the art (see, 10 *e.g.*, Herman *et al.*, *Proc. Natl. Acad. Sci. USA* 93:9821-9826, 1996) and will not be discussed in detail here.

[0076] As a skilled artisan will recognize, any other reagents that are unnamed here but have the same property of chemically (or through any other mechanism) modifying methylated and unmethylated DNA differentially can be used for practicing the present 15 invention. For instance, methylation-specific modification of DNA may also be accomplished by methylation-sensitive restriction enzymes, some of which typically cleave an unmethylated DNA fragment but not a methylated DNA fragment, while others (*e.g.*, methylation-dependent endonuclease McrBC) cleave DNA containing methylated cytosines but not unmethylated DNA. In addition, a combination of chemical modification and 20 restriction enzyme treatment, *e.g.*, combined bisulfite restriction analysis (COBRA), may be used for practicing the present invention.

V. Polynucleotide Sequence Amplification and Determination

[0077] Following the modification of DNA in a methylation-differential manner, the treated DNA is then subjected to sequence-based analysis, such that the genomic sequence 25 acting as the epigenetic marker (*e.g.*, the *HLCS* gene) derived from the fetal DNA of a chromosome relevant to an aneuploidy may be distinguished from the same gene from the maternal DNA, and that the amount or concentration of the fetal gene in the sample may be quantitatively determined and compared to the amount or concentration of a genetic marker from a reference chromosome of fetal origin, and the ratio of the two is then compared to a 30 standard control.

A. Amplification of Nucleotide Sequences

[0078] An amplification reaction is optional prior to the epigenetic marker's sequence analysis after methylation specific modification. In some embodiments of this invention, the

amplification is performed to preferentially amplify a portion of the marker that has a particular methylation pattern, such that only the marker from one particular source, *e.g.*, from the placenta or other tissues of the fetus, is detected and analyzed for its quantity or concentration. If desired, the amplification of a genetic marker on a reference chromosome 5 that allows determination of fetal or maternal origin based on differences in polynucleotide sequence is carried out using known amplification methods that selectively amplify the fetal version of the marker sequence. Typically, preferential amplification is achieved by careful primer design.

[0079] A variety of polynucleotide amplification methods are well established and 10 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 15 available from commercial vendors, such as Roche Molecular Systems.

[0080] 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. Improved and 20 more sensitive PCR methods such as real-time PCR and digital PCR are also useful in certain embodiments of the present invention.

[0081] Although PCR amplification of a target polynucleotide sequence (*e.g.*, a portion of the epigenetic marker where the fetal and maternal sequence is differentially methylated) is typically used in practicing the present invention, one of skill in the art will recognize that 25 amplification of the *HLCS* gene sequence found in a maternal blood sample may be accomplished by any known method, such as the 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. Branched-DNA technology may also be used to qualitatively demonstrate the presence of a particular 30 marker sequence (which represents a particular methylation pattern or a particular polynucleotide sequence), or to quantitatively determine the amount or concentration of a particular marker 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.

B. Determination of Polynucleotide Sequences

[0082] 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 invention. Additional means suitable for detecting changes (e.g., C → U) in a polynucleotide sequence for practicing the methods of the present invention include but are not limited to mass spectrometry, primer extension, polynucleotide hybridization, real-time PCR, melting curve analysis, high resolution melting analysis, heteroduplex analysis, pyrosequencing, and electrophoresis.

15 VI. Establishing a Standard Control

[0083] In order to establish a standard control for practicing the method of this invention, a group of healthy pregnant women carrying healthy fetuses is first selected. These women are within the appropriate time period of pregnancy for the purpose of screening for pregnancy-associated conditions such as fetal chromosomal aneuploidy and others using the methods of the present invention. Optionally, the women are of similar gestational age, e.g., within the same trimester of pregnancy, such as in the first or second trimester.

[0084] The healthy status of the selected pregnant women and the fetuses they are carrying are confirmed by well established, routinely employed methods including but not limited to general physical examination of the women, genetic analysis of the women, and fetal genetic analysis using CVS and amniocentesis.

[0085] Furthermore, the selected number of healthy pregnant women carrying healthy fetuses must be of a reasonable size, such that the average amount/concentration of fetal genetic and epigenetic markers in the maternal blood, the amount/concentration ratio, and the methylation profile of one or more of the epigenetic markers in the maternal blood obtained from the group can be reasonably regarded as representative of the normal or average level or methylation profile among the general population of healthy women carrying healthy fetuses. Preferably, the selected group comprises at least 10 women.

[0086] A fetal epigenetic marker methylation profile may reflect multiple different and separable aspects of the methylation status of this gene. For example, one aspect of a methylation profile is whether the C residue is methylated or not; another aspect is the number of methylated C bases within a particular region of the marker; a further aspect of the 5 profile is the percentage(s) of methylated C at any given locations in a number of analyzed DNA molecules. Additional aspects of a methylation profile may include, but are not limited to, the allelic difference in methylation, the ratio of differentially methylated alleles, and the like. Fetal epigenetic marker methylation profile may also vary depending on the tissue type, e.g., placental or other fetal tissue. Thus, separate standard controls may be established for 10 different fetal tissues used in testing.

[0087] Once an average value for the fetal epigenetic-genetic marker ratio is established for the particular set of fetal markers present in the maternal sample based on the individual values found in each woman of the selected healthy control group, this average or median or representative value or profile is considered a standard control. The samples taken from 15 these healthy control women should ideally be taken prior to an invasive procedure. A standard deviation is also determined during the same process. In some cases, separate standard controls may be established for different aspects of the methylation profile of the epigenetic marker, such as based on different regions of the epigenetic marker sequence.

EXAMPLES

20 [0088] 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.

EXAMPLE 1

I. Materials and Methods

25 STUDY PARTICIPANTS

[0089] Women with euploid and trisomy 21 pregnancies who attended the Department of 30 Obstetrics and Gynaecology, Prince of Wales Hospital, Hong Kong, were recruited. Informed consent was obtained from individuals who joined the study, and ethics approval was obtained from the Joint Chinese University of Hong Kong – New Territories East Cluster Clinical Research Ethical Committee.

SCREENING OF DIFFERENTIALLY-METHYLATED REGIONS ON CHROMOSOME 21 BY COBRA

[0090] COBRA (Xiong and Laird, *Nucleic Acids Res* 1997;25:2532-4) was used to assess the methylation status of genomic sequences on chromosome 21 in DNA samples from

5 placentas and maternal blood cells.

CLONING AND BISULFITE SEQUENCING ANALYSIS OF THE DIFFERENTIALLY-METHYLATED LOCUS IDENTIFIED BY COBRA

[0091] PCR products from the COBRA analysis were used for cloning and bisulfite sequencing. To analyze methylation status at the resolution of a single molecule, the PCR

10 product was TA-cloned into a plasmid vector using the pGEM-T Easy Vector System (Promega, Madison, WI). The inserts from the positive recombinant clones were PCR amplified using vector primers T7 and SP6, and were then analyzed by cycle sequencing using the BigDye Terminator Cycle Sequencing v1.1 kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. After ethanol precipitation, the samples
15 were resuspended in 10 µL of Hi-Di formamide and run on a 3100 DNA Analyzer (Applied Biosystems). The sequencing data were analyzed using the SeqScape software (Applied Biosystems). The completeness of bisulfite conversion was confirmed before scoring. The methylated site frequency was calculated by dividing the total number of methylated CpG sites over all cloned CpG sites (Chiu *et al.*, *Am J Pathol* 2007;170:941-50).

20 CONVENTIONAL REAL-TIME QUANTITATIVE PCR ANALYSIS OF THE *HLCS* LOCUS

[0092] Based on the COBRA screening and bisulfite sequencing results, we developed a methylation-sensitive restriction endonuclease (MSRE) digestion assay followed by real-time quantitative PCR analysis targeting the *HLCS* locus region B2, which is hypermethylated in
25 the placenta and unmethylated in maternal blood cells. The use of enzyme digestion allowed the analysis of the methylation pattern of the *HLCS* locus in different genomic DNA samples. In addition, the background maternal DNA molecules in maternal plasma samples could be eliminated. The sequences for primers and probe for the real-time PCR assay are listed in Table 1.

30 GENE DOSAGE ANALYSIS BY THE MICROFLUIDICS DIGITAL PCR PLATFORM

[0093] Gene dosage comparison of the chromosome 21 and the reference markers was analyzed by the polymerase chain reaction on a microfluidics digital PCR platform (Ottesen *et al.*, *Science* 2006;314:1464-7; Warren *et al.*, *Proc Natl Acad Sci U S A* 2006;103:17807-

12). The loci for an epigenetic-epigenetic comparison were *HLC5* on chromosome 21 and *RASSF1A* on chromosome 3 (Chiu *et al.*, *Am J Pathol* 2007;170:941-50). Both were hypermethylated fetal DNA markers. The loci for an epigenetic-genetic comparison were *HLC5* on chromosome 21 and *ZFY* on chromosome Y from pregnancies with a male fetus.

5 The sequences of primers and probes with the PCR thermal cycle conditions for all the assays are listed in Table 1.

STATISTICAL ANALYSIS

[0094] Statistical analyses were performed using the SigmaStat 3.5 software (SPSS).

COLLECTION OF BLOOD AND TISSUE SAMPLES

10 [0095] Chorionic villus samples (CVS) were collected during conventional prenatal diagnosis sessions in the first trimester of pregnancy. Placental tissue samples were collected from euploid third-trimester pregnancies after delivery and from euploid and trisomy 21 pregnancies after termination of pregnancy. The fetal chromosomal status was confirmed by full karyotyping. Maternal peripheral blood samples (12 - 20 mL EDTA) were collected from all subjects. An additional 12 mL of blood was collected into EDTA tubes from the third trimester pregnancies after delivery. Gestational ages of the first-, second- and third-trimester samples were 12 – 14 weeks, 17 – 21 weeks and 38 – 40 weeks, respectively.

PROCESSING OF BLOOD AND TISSUE SAMPLES

20 [0096] Maternal peripheral blood samples were processed by a double centrifugation protocol as previously described (Chiu *et al.*, *Clin Chem* 2001;47:1607-13). The blood cell portion was re-centrifuged at 2,500 g, and any residual plasma was removed. DNA from the peripheral blood cells and that from maternal plasma was extracted with the blood and body fluid protocol of the QIAamp DNA Blood Mini Kit and the QIAamp DSP DNA Blood Mini Kit, respectively (Qiagen, Hilden, Germany). DNA from the CVS and placentas was extracted with the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's tissue protocol.

SCREENING OF DIFFERENTIALLY-METHYLATED REGIONS ON CHROMOSOME 21 BY COBRA

25 [0097] The COBRA procedure is described as follows. Bisulfite conversion was performed on one microgram of each DNA sample using the EZ DNA Methylation Kit (Zymo Research, Orange, CA) according to manufacturer's instructions. Forty nanograms of bisulfite-converted DNA (calculation based on the original DNA input) were then subjected

to PCR amplification with reagents supplied in the HotStar *Taq* DNA Polymerase Kit (Qiagen, Hilden, Germany). Reagent compositions for each PCR are listed in Table 2. Typically, PCR was performed in a 20 μ L reaction with 1x PCR Buffer, MgCl₂, 50 μ M of each dNTP, forward and reverse primers, HotStar *Taq* polymerase, and with or without 2X 5 PCRx Enhancer (Invitrogen, Carlsbad, CA). The thermal profile was 95 °C for 15 min, followed by 45-55 cycles of 95 °C for 20 s, appropriate annealing temperature for 30 s, 72 °C for 1.5 min, and a final extension of 72 °C for 3 min. PCR products were then subjected to restriction enzyme digestion. The restriction enzyme to be used for each respective locus was chosen for its ability to distinguish between the methylated and unmethylated sequences 10 after bisulfite conversion. In essence, restriction sites were only present in either the methylated or unmethylated sequence but not both, so that one of the sequences would be digested while the other would remain intact. Restriction enzyme digestions were performed in 20 μ L reactions with 5 μ L PCR products, 1x appropriate buffer, and 10 U restriction 15 enzyme (or none for mock digestion) under the manufacturer's recommended temperatures for 2 h. All enzymes were purchased from New England Biolabs (Ipswich, MA). Digested products were then analyzed by agarose gel electrophoresis.

CONVENTIONAL REAL-TIME QUANTITATIVE PCR ANALYSIS OF THE HLCS LOCUS

[0098] **MSRE digestion.** For each placental and maternal blood cell DNA sample, 100 ng 20 DNA was subjected to MSRE digestion. Restriction enzyme digestion was performed in a 50 μ L reaction with 1x appropriate buffer, 25 U of *Hpa*II and 50 U of *Bst*UI (or none for mock digestion) (New England Biolabs) under the manufacturer's recommended temperatures for at least 16 h. For each maternal plasma sample, 1.6 mL plasma was used for DNA extraction, and was eluted in 50 μ L of deionized water, 21 μ L of which was subjected to 25 restriction enzyme digestion. Enzyme digestion was performed in a 30 μ L reaction with 1x appropriate buffer, 20 U of *Hpa*II and 30 U of *Bst*UI (or none for mock digestion) under the manufacturer's recommended temperatures for at least 16 h. Digested products were then analyzed by real-time quantitative PCR. The selected restriction enzymes only digested the unmethylated DNA but not the methylated DNA. Since the data from the COBRA analysis 30 have shown that *HLCS* is hypermethylated in placental tissues and unmethylated in maternal blood cells, it was expected that a proportion of DNA from placental tissues would remain detectable while most DNA from maternal blood cells would be digested, thus becoming undetectable after restriction enzyme treatment.

[0099] **Real-time quantitative PCR analysis.** Real-time PCR assay was developed for quantitative analysis of *HLCS* genomic DNA with and without restriction enzyme digestion. Four microliters of restriction enzyme treated DNA or mock digestion sample were used in the real time PCR assay. Each reaction contained 1x TaqMan Universal PCR Master Mix (Applied Biosystems), 300 nM of each of the forward and reverse primers (Integrated DNA Technologies, Coralville, IA), and 100 nM of the TaqMan probe (Applied Biosystems). The sequences of the primers and probes are listed in Table 1. The thermal profile was 50 °C for 2 min, 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s, and 60 °C for 1 min. All reactions were run in duplicates, and the mean quantity was taken. Serially-diluted human genomic DNA originally quantified by optical density measurement was used as the quantitative standard for the assay and the detection limit for the assay was 1 copy per reaction. As the detectable *HLCS* molecules after restriction enzyme digestion represented the methylated fraction, the real-time quantitative PCR was expressed as a methylation index. The methylation index of a sample was calculated by dividing the copy number of *HLCS* after enzyme digestion by that obtained with mock digestion. Maternal plasma DNA analysis was performed to show the postpartum clearance of the hypermethylated *HLCS* molecules.

GENE DOSAGE ANALYSIS BY THE MICROFLUIDICS DIGITAL PCR PLATFORM

[0100] **MSRE digestion.** The MSRE, *Bst*UI (New England Biolabs), was used to digest the hypomethylated DNA. Extracted DNA was digested with the *Bst*UI enzyme at 60 °C for 16 h. For CVS, placental tissues and maternal blood cells, 40 U of *Bst*UI enzyme was used to digest 200 ng of DNA for the microfluidics digital PCR assays. A mock-digested aliquot was included as the digestion control. For mock-digestion, an equal amount of DNA was subjected to the same digestion condition without the addition of enzyme. For the plasma samples, 20 U of the *Bst*UI enzyme was used to digest the DNA from 3.4 – 4.8 mL plasma in the third-trimester samples. For the first- and second-trimester plasma samples, 40 U or 60 U of the *Bst*UI enzyme was used to digest the DNA extracted from 4.4 – 9.1 mL plasma. The target sequences with the *Bst*UI restriction sites underlined are illustrated in Figure 5.

[0101] **Microfluidics digital PCR analysis.** Microfluidics digital PCR assays were designed for the *HLCS*, *RASSF1A* and *ZFY* loci (Figure 5), representing the dosage of chromosome 21, chromosome 3 and chromosome Y, respectively. The *ZFX/Y* assay and the basis of the digital PCR analysis have been described previously (Lun *et al.*, *Clin Chem* 2008;54:1664-72). The sequences of the primers and probes are listed in Table 1. The digital experiments were carried out on the BioMark System (Fluidigm, South San Francisco,

CA) using the 12.765 Digital Arrays (Fluidigm). The Digital Array consists of 12 panels, and each panel is further partitioned into 765 reaction wells. With each panel, the reaction was set up as a 10 μ L mixture at a final concentration of 1x TaqMan Universal PCR Master Mix (Applied Biosystems), 125 nM TaqMan probe (Applied Biosystems), and 900 nM forward and reverse primers (Integrated DNA Technologies) diluted with the assay loading buffer and sample loading buffer according to the manufacturer's protocol. The input DNA volume was 3.5 μ L for each of the 10 μ L reaction mixture. The thermal profile was 50 °C for 2 min, 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 or 30 s, and 57 °C or 60 °C for 1 min. The thermal cycle condition for each assay is specified in Table 1. The *HLCS* and *RASSF1A* assays were performed as a monoplex assay. The *ZFX/Y* assays were performed as a duplex reaction.

[0102] **Assay specificity on the *Bst*UI-digested genomic DNA samples.** DNA samples from the CVS, placenta, and maternal blood cells were subjected to the *HLCS*, *RASSF1A*, and *ZFX/Y* digital PCR analysis after *Bst*UI digestion. After enzyme digestion, the DNA was diluted to a concentration of 1 - 2 ng/ μ L for loading into the reaction mixture for digital PCR analysis. DNA samples from the CVS and placenta constituted enzyme digestion-resistant *HLCS* and *RASSF1A* molecules, thus signals should be detected after restriction enzyme digestion. On the other hand, we would expect no or low level of detection in the blood cells as they are hypomethylated at these two loci.

[0103] The *ZFX/Y* loci did not contain any *Bst*UI enzyme digestion sites, thus restriction enzyme treatment should not confer any effects on the DNA molecules. The *ZFY* molecules constituted the fetal-derived sequences from a male fetus for the ratio comparison.

[0104] **Assay specificity on the *Bst*UI-digested plasma DNA samples.** After *Bst*UI-digestion on the maternal plasma DNA samples, a four-fold dilution with deionized water was performed. The samples were then mixed with the reaction mixture for loading onto the microfluidics chips for analysis.

[0105] **Gene dosage comparison by the epigenetic-epigenetic approach and the epigenetic-genetic approaches.** Digital PCR analyses of the *HLCS*, *RASSF1A* and *ZFX/Y* loci were performed on euploid and T21 placental DNA samples after *Bst*UI restriction enzyme digestion. For maternal plasma analysis, we compared the ratio of *HLCS* to *ZFY* in *Bst*UI-digested DNA samples from euploid and T21 pregnancies.

II. Results

SCREENING OF DIFFERENTIALLY-METHYLATED REGIONS ON CHROMOSOME 21 BY COBRA

[0106] Thirty-five promoter regions on chromosome 21 were selected for differential methylation screening using the COBRA method, thirty of which were not associated with CpG islands (CGIs) while the other five were associated with CGIs. The criteria to define a CGI were as follows: length > 400 bp, GC content > 50%, and observed/expected CpG ratio > 0.6 (Yamada *et al.*, *Genome Res* 2004;14:247-66). DNA sequences proximal to the transcription start sites (-1 kb to +500 bp; transcription start site being 0) of the 35 promoters were obtained from the March 2006 human reference sequence of the UCSC Genome Bioinformatics Database (website: www.genome.ucsc.edu/) (NCBI Build 36.1), and designed 51 COBRA assays to compare the methylation patterns between placental tissues and maternal blood cells (Table 2).

[0107] Among the 51 assays, the methylation profiles of the promoter regions between 15 placental tissues collected from the first- and third-trimesters and maternal blood cells were compared. At least one placental tissue and one maternal blood cell sample from normal pregnancies were used for the COBRA screening (Table 2). Among the screened regions, the putative promoter regions of *HLCS* and *C21orf81* (GenBank accession AF326257) were identified by COBRA to be differentially-methylated between placental tissues and maternal 20 blood cells. Representative COBRA results are illustrated in Figure 6A for *HLCS* region B2, in which the placenta was hypermethylated when compared to maternal blood cells; and 6B for *C21orf81*, in which the maternal blood cells were slightly methylated when compared to placental tissues.

CLONING AND BISULFITE SEQUENCING ANALYSIS OF THE *HLCS* LOCUS

[0108] Cloning and bisulfite sequencing experiments were performed on the *HLCS* region for further analysis of the methylation status at the resolution of a single molecule, and the results are shown in Figure 1. Sequencing results for the *HLCS* region confirmed that the hypermethylation of *HLCS* is placenta-specific with a methylated site frequency from 0.435 to 0.699. Although low methylation level was detected at the 3'-end (*i.e.*, position -57 to 30 +111, transcription start site being 0) of the target sequence in maternal blood cell samples (methylated site frequency <0.100), almost no methylation was observed for CpG sites from -232 to -67.

REAL-TIME QUANTITATIVE PCR FOR MSRE-DIGESTED PLACENTAL,
MATERNAL BLOOD CELL AND PLASMA DNA SAMPLES

[0109] Based on the COBRA and bisulfite sequencing data of the *HLCS* region B2, an MSRE digestion assay was developed followed by real-time quantitative PCR analysis to

5 analyze the differential methylation of genomic DNA extracted from placentas and maternal blood cells.

[0110] The methylation profiles of the putative promoter region of *HLCS* from eight maternal blood cell samples were compared with those from two first-trimester and two third-trimester placental tissue samples collected from euploid pregnancies. Restriction

10 enzyme digestion followed by real-time quantitative PCR analysis was performed and the results are shown in Figure 7. DNA from all maternal blood cell samples were mostly digested by restriction enzymes, resulting in methylation indices approaching 0 (Median: 0.0178, interquartile range (IQR): 0.0121 – 0.0281). Placental tissue DNA samples were partially digested, resulting in methylation indices ranging from 0.567 to 0.966.

15 [0111] Previous data suggested that the placenta was the predominant source of fetal DNA (Bianchi, *Placenta* 2004;25 Suppl A:S93-S101), while maternal blood cells were the main contributor of the background maternal DNA (Lui *et al.*, *Clin Chem* 2002;48:421-7)

detectable in maternal plasma. It was therefore hypothesized that the placental-specific fraction of the *HLCS* DNA molecules, namely the methylated or non-digestible fraction,

20 could be detected in maternal plasma. 25 pairs of pre- and post-delivery third-trimester maternal plasma samples were recruited, and restriction enzyme digestion followed by real-time quantitative PCR analysis were performed. The results are illustrated in Figure 8.

Positive *HLCS* signals were obtained in all of the enzyme-digested pre-delivery plasma samples. The clearance pattern from the enzyme-treated pre- and post-delivery plasma

25 samples suggested that the digestion-resistant *HLCS* molecules were pregnancy-specific ($P = <0.001$, Wilcoxon signed-rank test). The median *HLCS* concentration in the post-delivery plasma samples was only 8.1% of that in the pre-delivery samples (20.1 vs. 247.8 copies/mL, respectively (Fig. 8A)). For samples which underwent mock-digestions, the median *HLCS* concentration in the post-delivery plasma samples was 83.8% of that in the pre-delivery

30 samples (1208.5 vs. 1442.4 copies/mL, respectively) (Fig. 8B). With mock-digestion, both the fetal and maternal DNA were detected in the pre-delivery plasma samples while only maternal DNA was counted in the post-delivery samples. It is noted that the difference in the *HLCS* concentration between pre- and post-delivery samples with mock digestion is statistically significant ($P = 0.003$, Wilcoxon signed-rank test). Data from the *Bst*UI

digestion aliquot show that there were five maternal plasma samples having a relatively high level of fetal DNA, and these five samples were identical to the ones demonstrating a major drop in *HLCS* concentration in post-delivery sample even without enzyme digestion. This may contribute to the apparent “clearance” pattern even without enzyme digestion. The

5 reduction of *HLCS* concentration observed in the post-delivery plasma samples might be due to some samples having unusually high levels of fetal DNA (P = 0.003, Wilcoxon signed-rank test). It is noted that the five pre-delivery samples with extraordinarily high concentration of *HLCS* matched with the top five samples in the enzyme-digestion comparison.

10 GENE DOSAGE ANALYSIS ON THE MICROFLUIDICS DIGITAL PCR PLATFORM

[0112] The gene dosage comparison of the chromosome 21 and reference chromosome markers was performed on a microfluidics digital PCR platform. The chromosome 21 marker was the hypermethylated *HLCS*, and the reference markers on chromosome 3 and chromosome Y were the hypermethylated *RASSF1A* and male-specific *ZFY*, respectively.

15 [0113] Two pairs of placental tissues and maternal blood cells samples were used to check the performance of the *HLCS* and *RASSF1A* assays with and without enzyme digestion. The *ZFX/Y* assay was also performed (GenBank accession of *ZFX*: NM_003410 and *ZFY*: NM_003411). Since there is no enzyme digestion site on these loci, it was expected the copy number would not be affected by the enzyme digestion. Two post-delivery maternal plasma 20 samples were used to test the postpartum clearance of the digestion-resistant *HLCS* and *RASSF1A* molecules.

[0114] One panel for each assay was performed on each of the genomic DNA samples. For the post-delivery maternal plasma, enzyme-digested DNA was distributed into two panels for the scoring of any wells with positive signals. Results for the genomic DNA and 25 plasma DNA samples are summarized in Tables 3A and 3B, respectively. The number of wells that were positive for each target locus were counted. The actual number of molecules distributed into the panel followed the Poisson distribution, and was corrected by the following equation:

$$\text{Target} = -\ln [E/N] \times N,$$

30 where Target is the Poisson-corrected counts of the target molecules, ln is the natural logarithm, E is the number of negative (empty) wells, and N is the total number of digital PCR wells in the reaction.

[0115] Results from the *Bst*UI-digested placental DNA samples showed that about 60% – 70% of the *HLCS* and *RASSF1A* molecules remained detectable when compared to the mock-digested samples. In the maternal blood DNA, the hypomethylated *RASSF1A* molecules were completely digested by *Bst*UI enzyme treatment, while a few *HLCS* molecules

5 remained detectable in the samples. For the *ZFY* and *ZFX* assays, there was no change in the copy number counted from the mock- or *Bst*UI-digested DNA samples (Table 3A).

[0116] Post-delivery maternal plasma analysis showed that extremely low levels of both the *HLCS* and *RASSF1A* molecules were detected after *Bst*UI enzyme digestion (Table 3B).

GENE DOSAGE COMPARISON IN PLACENTAL DNA SAMPLES BY MSRE

DIGESTION FOLLOWED BY DIGITAL PCR ANALYSIS

[0117] The *HLCS* and *RASSF1A* assays were applied to ten euploid and 12 trisomy 21 placental DNA samples (Table 4A). Four panels were counted for each assay (Figure 2A).

The ratios of the number of positive *HLCS* and *RASSF1A* amplifications in the euploid and T21 samples were statistically significantly different ($P = 0.005$, Mann-Whitney Rank Sum

15 Test). However, there was a large overlap between the two groups (Figure 3A). A normal reference range, defined as the mean *HLCS* to *RASSF1A* ratio $\pm 1.96\text{SD}$, was calculated from the euploid samples as 0.86 – 1.63. More than half of the T21 cases fell within the reference range. The heterogeneity in the methylation density of the two loci across different samples may contribute to the large inter-individual variation in the *HLCS* to *RASSF1A* ratio. To

20 solve this precision problem, a more stable baseline would be required for the dosage comparison between the euploid and T21 DNA samples.

[0118] A fetal-specific locus which is independent of its methylation status, namely the *ZFY* locus, was then explored to serve as a baseline for the gene dosage comparison in pregnancy involving a male fetus. This is an important feature of the epigenetic-genetic

25 (EGG) approach.

[0119] The same restriction enzyme-digested placental DNA samples used for the *HLCS* and *RASSF1A* comparison were subjected to the *ZFY* digital PCR analysis (Figure 2A). The total copy number from four panels was used as the reference baseline for the total copy number of the *HLCS* molecules obtained above. A normal reference range, defined as the

30 mean *HLCS* to *ZFY* ratio $\pm 1.96\text{SD}$, was calculated from the euploid samples as 1.08 – 1.62. All the T21 samples showed a higher ratio than the normal reference range (Figure 3B).

GENE DOSAGE COMPARISON IN MATERNAL PLASMA DNA SAMPLES BY MSRE DIGESTION FOLLOWED BY DIGITAL PCR ANALYSIS

[0120] The EGG technique was then applied to maternal plasma DNA samples from euploid and T21 pregnancies to determine whether the EGG approach can be applied for noninvasive prenatal detection of fetal trisomy 21. It was reasoned that while the maternal *HLCS* molecules in the maternal plasma was restriction digested by the *Bst*UI enzyme, leaving the fetal-derived hypermethylated *HLCS* molecules intact for analysis, the fetal-derived *ZFY* locus would not be affected as there was no *Bst*UI enzyme recognition site located within the PCR amplicon, thus providing a stable baseline for gene dosage comparison.

[0121] Eight maternal plasma samples were obtained from each of the third-, second-, and first-trimester pregnancies with a euploid fetus. After *Bst*UI digestion, each DNA sample was analyzed in at least six panels on the microfluidics chips for the *HLCS* and *ZFY* assays (Figure 2B). The total number of positive wells was used to calculate the ratio of *HLCS* to *ZFY* in each sample (Table 4B).

[0122] A normal reference range of 1.49 – 2.88 was calculated from the 24 maternal plasma samples with euploid pregnancies. The ratio of one euploid DNA sample fell out of the normal reference range. This sample dried up during Speedvac concentration, and was reconstituted with water during sample preparation, which might explain the inaccuracy. All five maternal plasma samples from T21 pregnancies had a higher ratio of *HLCS* to *ZFY* than the reference range (Figure 4). The data showed that with the EGG approach, fetal trisomy 21 can be detected noninvasively in maternal plasma samples.

III. Discussion

[0123] In the first part of this study, the present inventors have successfully validated a hypermethylated fetal DNA marker on chromosome 21, namely, the promoter region of the *HLCS* gene (US Patent Application Publication No. 2007/0275402). The hypermethylated nature of this marker allows it to be detected relatively easily in maternal plasma through the use of methylation-sensitive restriction enzyme digestion and PCR amplification.

[0124] *HLCS* was then used to demonstrate that the novel EGG approach is a feasible method for fetal chromosome dosage analysis. The main advantage of the EGG method over the previously epigenetic allelic ratio analysis (Tong *et al.*, *Clin Chem* 2006;52:2194-202) is that using this method one has bypassed the requirement of the latter method for the epigenetic target and the genetic target (*i.e.*, the SNP) to be present in the same locus and

within a short distance from one another. In this study, the *ZFY* gene on the Y chromosome was used as a model for the fetal-specific genetic target. In practice, however, any fetal-specific genetic target, *e.g.*, an SNP allele that the fetus has inherited from the father but absent in the pregnant mother, can be used. A panel of such markers would be relatively easily developed to ensure a broad population coverage of this approach.

5 [0125] It was also demonstrated that the EGG approach has a higher discrimination power for T21 than an approach based purely on epigenetic markers, using the ratio of the hypermethylated *HLCS* to hypermethylated *RASSF1A* as an example. One possible explanation of the suboptimal performance of the latter approach is because of the fact that 10 there is a variability in the level of DNA methylation of individual fetal-derived *HLCS* and *RASSF1A* molecules, as can be seen in Figure 1 and in previously published bisulfite sequencing results (Chiu *et al.*, *Am J Pathol* 2007;170:941-50). Thus, one would expect that a ratio determined from these two potentially varying parameters would have a wider 15 reference interval than if one of the parameter is a relatively stable genetic marker (as in the case for the EGG approach).

[0126] In this study, microfluidics digital PCR was used as the detection and measurement platform because previous results have shown it to be a highly precise analytical method (Lun *et al.*, *Clin Chem* 2008;54:1664-72; Lun *et al.*, *Proc Natl Acad Sci USA* 2008;105:19920-5). The EGG approach can also be implemented in other variation of this 20 molecular counting theme, *e.g.*, by targeted sequencing using a 'next-generation' sequencer.

[0127] The development of fetal epigenetic markers on the other chromosomes important for prenatal screening, *e.g.*, chromosomes 18 and 13, would further broaden the usefulness of the EGG approach. One example of a fetal epigenetic marker is the *SERPINB5* gene, coding for maspin, on chromosome 18 (Chim *et al.*, *Proc Natl Acad Sci USA* 2005;102:14753-8).

25 Other examples of fetal epigenetic markers on chromosome 18 and 13 are described by Papageorgiou *et al.* (Papageorgiou *et al.*, *Am J Pathol* 2009;174:1609-18).

EXAMPLE 2

[0128] A novel epigenetic-genetic chromosome dosage approach is recently developed for fetal trisomy 21 detection using a fetal epigenetic marker, the putative promoter of 30 *holocarboxylase synthetase* (*HLCS*) on chromosome 21, and a fetal genetic marker, the *zinc finger protein, Y-linked* (*ZFY*) gene present on chromosome Y (Tong *et al.*, *Clin Chem* 2010;56:90-8). To demonstrate that this method can be used for the detection of both male and female fetuses, the inventors have explored the use of a paternally-inherited fetal single

nucleotide polymorphism (SNP) allele on a reference chromosome to serve as the baseline for epigenetic-genetic chromosome 21 dosage determination in place of the chromosome Y marker.

I. MATERIALS AND METHODS

5 Study Participants

[0129] Women with euploid and trisomy 21 (T21) pregnancies who attended the Department of Obstetrics and Gynaecology, Prince of Wales Hospital were recruited between October 2009 and March 2010. Informed consent was obtained from individuals who joined the study, and ethics approval was obtained from the Joint Chinese University of Hong Kong

10 – New Territories East Cluster Clinical Research Ethical Committee.

[0130] Chorionic villus samples (CVS) were collected during conventional prenatal diagnosis sessions in the first trimester of pregnancy. Placental tissue samples were collected from euploid third-trimester pregnancies after delivery and from T21 pregnancies after termination of pregnancy (TOP). The chromosome status of each T21 case was confirmed by

15 full karyotyping. Maternal peripheral blood samples (12 mL in EDTA tubes) were collected from all subjects.

Processing of Blood and Tissue Samples

[0131] Peripheral blood samples were processed by a double centrifugation protocol as previously described (Chiu *et al. Clin Chem* 2001;47:1607-13). The blood cell portion was 20 re-centrifuged at 2,500 g, and any residual plasma was removed. DNA from the peripheral blood cells and that from maternal plasma was extracted with the blood and body fluid protocol of the QIAamp DNA Blood Mini Kit and the QIAamp DSP DNA Blood Mini Kit, respectively (Qiagen).

[0132] DNA from the CVS and placentas was extracted with the QIAamp DNA Mini Kit

25 (Qiagen) according to the manufacturer's tissue protocol.

Chromosome Dosage Analysis

[0133] Chromosome dosage comparison of the chromosome 21 and the reference marker in 30 placental tissue and maternal plasma DNA samples was analyzed by real-time quantitative polymerase chain reaction (qPCR) and digital PCR (Vogelstein and Kinzler *Proc Natl Acad Sci USA* 1999;96:9236-41), respectively. Chromosome dosage analysis was performed by comparing the amount of hypermethylated *HLCS* to that of a SNP allele (rs6636, a C/G SNP) that the fetus has inherited from the father but absent in the pregnant mother. SNP rs6636 is

located within the *transmembrane emp24 protein transport domain containing 8 (TMED8)* gene on chromosome 14 with an average heterozygosity of 0.451 +/- 0.149 (dbSNP build 130). rs6636 is one of the SNPs that have been previously described (Chow *et al. Clin Chem* 2007;53:141-2). The rs6636 SNP assays are denoted as *TMED8*-C/G SNP assays in this

5 document.

Methylation-sensitive restriction endonuclease (MSRE) digestion

[0134] A methylation-sensitive restriction endonuclease, *Bst*UI (New England Biolabs), was used to digest the hypomethylated DNA. Extracted DNA was digested with the *Bst*UI enzyme at 60 °C for 16 hours. For CVS, placental tissues and maternal and normal control blood cells, 40 U of *Bst*UI enzyme was used to digest 100 ng of DNA for the PCR assays. A mock-digested aliquot was included as the digestion control. For mock-digestion, an equal amount of DNA was subjected to the same digestion condition without the addition of enzyme. For the plasma samples, 20 U to 40 U of the *Bst*UI enzyme was used to digest the DNA from 1.6 – 5.2 mL plasma.

15 Assay design and reaction conditions for conventional real-time qPCR analysis

[0135] Real-time PCR analysis was performed for the *HLCS* and *TMED8* loci. The sequences of the primers and probes are listed in Table 5. The *HLCS*, *TMED8*-C allele and *TMED8*-G allele assays were all performed as a monoplex reaction. Within the PCR amplicon of the *HLCS* locus, there were two *Bst*UI enzyme recognition sites. In contrast, the *TMED8* SNP assays did not contain any *Bst*UI enzyme recognition sites.

[0136] Each reaction was set up as a 25 µL mixture at a final concentration of 1x TaqMan® Universal PCR Master Mix (Applied Biosystems), 100 nM TaqMan® probe (Applied Biosystems), and 300 nM of each of the forward and reverse primers (Integrated DNA Technologies) with 25 ng DNA input. The reaction was initiated at 50°C for 2 min and continued at 95°C for 10 min and followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The experiments were carried out on the 7300 Real-time PCR System (Applied Biosystems) and the fluorescence data were collected and analyzed by the SDS v1.3.0 software (Applied Biosystems). All reactions were run in duplicates with the mean quantity taken. A calibration curve was constructed by serially-diluted genomic DNA extracted from adult male blood cells with concentration ranging from 10000 genome equivalents (GE) to 3 GE per reaction.

Assay design and reaction conditions for the digital PCR analysis

[0137] The same oligonucleotide sequences for real-time PCR analysis were used for the *HLCs* and *TMED8*-C/G SNP digital PCR analysis (Table 5). Here, the *HLCs* assay was performed as a duplex reaction with each of the *TMED8*-C/G SNP assays. The fluorescent

5 probes were labeled as *HLCs*(VIC) duplex with *TMED8*-C(FAM) and *HLCs*(FAM) duplex with *TMED8*-G(VIC).

[0138] The basis of the digital PCR analysis have been described previously (Lo *et al.* *Proc Natl Acad Sci U S A* 2007;104:13116-21; Lun *et al.* *Clin Chem* 2008;54:1664-72). The total reaction volume was 5 µL per well in a 384-well plate at a final concentration of 1x

10 TaqMan® Universal PCR Master Mix (Applied Biosystems), 100 nM TaqMan® probe (Applied Biosystems), and 300 nM of each of the forward and reverse primers (Integrated DNA Technologies). The reaction was initiated at 50°C for 2 min and continued at 95°C for 10 min and followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. The experiments were carried out on the 7900HT Sequence Detection System (Applied Biosystems) in a 384-well format, and the fluorescence data were collected by the "Absolute Quantification" 15 application of SDS 2.3 software (Applied Biosystems).

Specificity of the *TMED8*-C/G SNP assay

[0139] Genomic DNA extracted from placental tissues with known *TMED8* genotypes were subjected to the *HLCs* and *TMED8* duplex assays. Samples that were homozygous for 20 one allele were tested with the *TMED8*-C/G SNP assay for the other allele. A sample homozygous for the C allele should not show any signals for the *TMED8* assay detecting the G allele, and *vice versa*.

***Beta-actin* assay as a digestion control**

[0140] A previously-described *beta-actin* region that is unmethylated in both the placenta and maternal blood cells was used to determine the efficiency of *Bst*UI digestion (Chan *et al.* *Clin Chem* 2006;52:2211-8). The *beta-actin* assay was modified to contain two *Bst*UI enzyme recognition sites to match the number of *Bst*UI enzyme recognition sites of the current *HLCs* assay. The sequences of the primers and probes are listed in Table 5. The same sequences were used for both the real-time qPCR and digital PCR analyses.

30 **Statistical analysis**

[0141] Statistical analyses were performed using the SigmaStat 3.5 software (SPSS).

II. RESULTS AND DISCUSSION

Chromosome dosage analysis by conventional real-time qPCR

[0142] The *HLCS* and *TMED8*-C/G SNP assays were applied to a total of 20 euploid and nine T21 placental tissue samples with the heterozygous C/G genotype. Six euploid and four

5 T21 samples were analyzed by using the *HLCS* to *TMED8*-C ratio, and the remaining 14 euploid and five T21 samples were analyzed by the *HLCS* to *TMED8*-G ratio. A calibration curve constructed by serially-diluted genomic DNA extracted from adult male blood cells with concentration ranging from 10000 GE to 3 GE per reaction was used to determine the absolute copy number of the two loci in the tested samples.

10 [0143] All three assays were optimized to have similar efficiencies, as indicated by the slopes and y-intercepts of the calibration curves. The slopes of the calibration curves of the *HLCS*, *TMED8*-C and *TMED8*-G assays were -3.71, -3.70 and -3.62, respectively, whereas the y-intercepts showed threshold cycle values of 39.62, 39.79 and 42.04, respectively.

HLCS to *TMED8*-C ratio in placental DNA samples

15 [0144] The *HLCS* to *TMED8*-C ratio was first determined in mock-digested placental DNA samples. A normal reference range, defined as the mean *HLCS* to *TMED8*-C ratio \pm 1.96SD, was calculated from the six euploid samples as 1.78 – 2.66. All four T21 samples showed a higher ratio than the normal reference range (Table 6A) (Figure 9A).

20 [0145] The same samples were then subjected to *Bst*UI restriction enzyme digestion before real-time PCR analysis. While the unmethylated *HLCS* molecules would be digested by *Bst*UI enzyme treatment, leaving only the digestion-resistant, hypermethylated *HLCS* molecules for PCR detection, the *TMED8* molecules would remain intact as there was no *Bst*UI enzyme recognition site within the *TMED8* PCR amplicon. A normal reference range was calculated as 1.24 – 2.26. All samples were correctly classified (Table 6B) (Figure 9B).

25 [0146] In both the mock and *Bst*UI digestion experiments, a placental DNA sample with the homozygous GG genotype for the *TMED8* SNP was included for testing the assay specificity. There was no signal when the *TMED8* assay for detecting the C allele was applied (Table 6A and 6B).

HLCS to *TMED8*-G ratio in placental DNA samples

30 [0147] The *HLCS* to *TMED8*-G ratio was first determined in mock-digested placental DNA samples. A normal reference range was calculated from the 14 euploid samples as 1.36 –

3.03. One sample from each of the euploid and T21 groups was misclassified (Table 7A) (Figure 10A).

[0148] The same samples were then subjected to *Bst*UI restriction enzyme digestion before real-time PCR analysis. A normal reference range was calculated as 0.90 – 1.99. One

5 sample from each of the euploid and T21 groups was misclassified (Table 7B) (Figure 10B). These are not the same samples as the misclassified ones in the mock digestion experiment.

[0149] In both mock and *Bst*UI digestion experiments, a placental DNA sample with the homozygous CC genotype for the *TMED8* SNP was included for testing the assay specificity.

There was no signal when the *TMED8* assay for detecting the G allele was applied (Table 7A

10 and 7B).

Beta-actin as a digestion control

[0150] The *Bst*UI enzyme digestion efficiency was evaluated by applying the beta-actin assay to the same mock- and *Bst*UI-digested placental DNA samples as those used for *HLCS*

15 to *TMED8* chromosome dosage analysis. The same calibration standard was used to quantify

the amount of beta-actin sequences in the samples. The slope of the calibration curve was -

3.41, whereas the y-intercept showed threshold cycle value of 37.97. Results showed that

over 96% of the DNA was digested in all tested samples (Table 8A and 8B).

Chromosome dosage analysis by digital PCR

Specificity of the *TMED8*-C/G SNP assay

[0151] Placental DNA samples of the heterozygous C/G, homozygous C/C and homozygous G/G genotypes were tested with the *HLCS* and *TMED8* duplex assay to ascertain the specificity for detecting either one of the SNP alleles. Two samples were used for each of the three groups. A sample homozygous for the C allele should not show any signals for the *TMED8* assay detecting the G allele, and *vice versa*.

[0152] The number of wells that were positive for each of the target loci was counted. The total number of molecules distributed into the 384-well plate followed the Poisson distribution, and was corrected as previously described (Tong *et al. Clin Chem* 2010;56:90-8).

[0153] By applying the *HLCS* and *TMED8*-C duplex assay, placental DNA samples of the heterozygous C/G and homozygous C/C genotypes showed positive wells for both loci, whereas samples of the homozygous G/G genotypes were only positive for the *HLCS* locus, and no detectable signal was observed for the *TMED8*-C allele (Table 9A).

[0154] By applying the *HLCS* and *TMED8*-G duplex assay, placental DNA samples of the heterozygous C/G and homozygous G/G genotypes showed positive wells for both loci, whereas samples of homozygous C/C were only positive for the *HLCS* locus, and no detectable signal was observed for the *TMED8*-G allele (Table 9B).

5 [0155] The results showed that the *TMED8*-C/G SNP assays were specific in detecting the individual SNP allele. The specificity was conferred by the allele-specific probes labeled with difference fluorescent dyes.

HLCS to *TMED8*-C ratio in maternal plasma DNA samples

10 [0156] Maternal plasma DNA analysis was performed by comparing the ratio of digestion-resistant *HLCS* to fetal-specific *TMED8* SNP allele in informative samples. An informative sample was defined as one in which the fetus was heterozygous and the mother was homozygous, being the *TMED8*-C/G SNP in the current example. The extra allele that the fetus had inherited from the father was used as the reference baseline for chromosome 21 dosage determination.

15 [0157] The *HLCS* and *TMED8*-C duplex assay was applied to a total of 16 euploid and two T21 pregnancies. In these samples, the fetal and maternal genotypes for the *TMED8* SNP were C/G and G/G, respectively. The fetal-specific SNP allele was the C allele. Maternal plasma DNA samples were analyzed by digital PCR after *Bst*UI enzyme digestion. Each sample was analyzed in at least one 384-well plate. The total number of positive wells was 20 used to calculate the ratio of *HLCS* to *TMED8*-C allele in each sample.

25 [0158] A normal reference range of 2.12 – 3.65 was calculated from the euploid maternal plasma samples. Eight samples were collected from the third-trimester, and four samples were collected from each of the second- and first-trimester pregnancies. Among these 16 euploid samples, ten were from pregnancies carrying a female fetus, and six from those carrying a male fetus. The *HLCS* to *TMED8*-C allele ratios of all euploid samples fell within the reference range. The T21 samples collected from the second- and first-trimester pregnancies showed a higher ratio than the reference range (Figure 7A). Both T21 cases carried a female fetus.

HLCS to *TMED8*-G ratio in maternal plasma DNA samples

30 [0159] In the other group of informative samples, the fetal and maternal genotypes for the *TMED8* SNP were C/G and C/C, respectively. The fetal-specific SNP allele was the G allele. The *HLCS* and *TMED8*-G duplex digital PCR assay was applied to *Bst*UI-digested maternal

plasma DNA samples from nine euploid pregnancies and one T21 pregnancy. Each sample was analyzed in at least one 384-well plate. The total number of positive wells was used to calculate the ratio of *HLCS* to *TMED8*-G allele in each sample.

[0160] A normal reference range of 2.06 – 3.68 was calculated from euploid maternal plasma samples. Among the nine euploid samples, five, three and one cases were collected from the third-, second- and first-trimester pregnancies, respectively. They included four pregnancies with a female fetus, and five with a male fetus. The *HLCS* to *TMED8*-G allele ratios of all euploid samples fell within the reference range. The first-trimester T21 pregnancy with a female fetus showed a higher *HLCS* to *TMED8*-G ratio than the reference range (Figure 7B).

Beta-actin as a digestion control

[0161] The *Bst*UI enzyme digestion efficiency was evaluated by applying the *beta-actin* digital PCR assay to the same *Bst*UI-digested maternal plasma DNA samples as those used for *HLCS* to *TMED8* chromosome dosage analysis. An aliquot of the digested DNA samples (1/50 of the total digestion mixture) was confirmed to show no more than one positive well for the *beta-actin* assay before subjected to chromosome dosage analysis.

III. CONCLUSION

[0162] In an earlier example, the inventors have demonstrated in principle that the EGG approach was a feasible method for fetal chromosome dosage analysis in maternal plasma DNA samples. The hypermethylated *HLCS* locus was used as the epigenetic component, which represented a class of fetal-specific molecules, and the *ZFY* locus was a genetic marker which was specific to a male fetus. By comparing the ratio between the fetal-specific epigenetic marker on chromosome 21 and the fetal-specific genetic marker on a reference chromosome, the chromosome 21 dosage could be deduced.

[0163] In this example, we further demonstrated that the epigenetic-genetic chromosome dosage approach can be applied to the prenatal diagnosis of trisomy 21 using a fetal-specific SNP allele as a genetic reference baseline in place of a chromosome Y marker derived from a male fetus.

[0164] Here, SNP rs6636 located within the *TMED8* locus on chromosome 14 was used as an example. By comparing the ratio between the hypermethylated *HLCS* and the fetal-specific *TMED8* SNP allele, the chromosome 21 dosage could be deduced. It was demonstrated that both SNP alleles could serve as such a genetic reference baseline.

[0165] By using an informative SNP allele as the genetic reference baseline, the EGG chromosome dosage approach can be applied to the prenatal diagnosis of trisomy 21 for both male and female fetuses. Furthermore, development of a panel of such markers will ensure a broad population coverage of this approach. As previously described (Chow *et al. Clin Chem* 2007;53:141-2), one could first test a maternal buffy coat sample, which comprises of mostly maternal DNA, to ascertain the maternal genotypes for the panel of SNPs. For SNPs where the mother was shown to be homozygous, one could then aim to detect the allele that is not represented in the maternal genotype in maternal plasma. If the plasma sample is positive for the non-maternal allele, it suggests that the fetus has inherited that allele from the father. The quantification of that paternally inherited fetal-specific allele in maternal plasma could then be used as the reference for gene dosage assessment using the epigenetic-genetic approach. Finally, the use of epigenetic markers for the other chromosomes involved in other aneuploidies important for prenatal testing, *e.g.*, chromosomes 18 and 13, will further expand the clinical utility of the EGG approach.

15 EXAMPLE 3: Epigenetic-genetic chromosome dosage approach for the detection of fetal trisomy 18

[0166] This example demonstrates the application of epigenetic-genetic (EGG) chromosome dosage approach for the detection of fetal trisomy 18 (T18). This example of the EGG approach involved a fetal epigenetic marker located on chromosome 18 and a fetal genetic marker located on a reference chromosome, respectively, in maternal plasma. The fetal epigenetic marker is, preferably, *Marker 18A* [genomic location chr18:10022533-10022724, defined according to the Human Genome March 2006 Assembly (hg18)], which is a 146-bp intergenic region located 75 kb downstream of the gene *VAPA* (*vesicle-associated membrane protein*)-associated protein A and 421 kb upstream of the gene *APCDD1* (*adenomatosis polyposis coli* down-regulated 1), as identified by an methylated DNA immunoprecipitation and tiling array analysis (described in USSN 61/308,578). However, this fetal epigenetic marker can also be any cytosine-containing DNA genomic region located within 100 kb upstream or downstream of the above locus, *i.e.*, chr18:10022533-10022724. Furthermore, this fetal epigenetic marker can also be any cytosine-containing DNA genomic region with different epigenetic signature (DNA methylation levels) that distinguishes the fetal from the maternal chromosome 18 in maternal plasma.

[0167] The fetal genetic marker in maternal plasma is, preferably, a region located in the *zinc-finger Y-linked* (*ZFY*) gene on chromosome Y. However, this fetal genetic marker can

also be any genetic differences between the fetus and its mother, including single-nucleotide polymorphism (SNP) and insertion/deletion (indel) polymorphism.

5 [0168] In this example, the inventors adopted the EGG approach to compare the concentrations of *Marker 18A* on chromosome 18 with those of a fetal genetic marker, the *zinc finger protein, Y-linked (ZFY)* gene on chromosome Y for the noninvasive prenatal detection of T18.

I. MATERIALS AND METHODS

Sample collection

10 [0169] Samples were obtained from women attending the Department of Obstetrics and Gynaecology at the Prince of Wales Hospital, Hong Kong, or the Prenatal Diagnostic and Counselling Department at the Tsan Yuk Hospital, Hong Kong, or the Harris Birthright Research Centre for Fetal Medicine, at the King's College Hospital, London, UK. All of the pregnancies were recruited with informed consent. The study was approved by the respective institutional review boards.

15 [0170] Chorionic villus samples (CVS) were collected during conventional prenatal diagnosis sessions in the first and second trimesters of pregnancy. The chromosome status of each euploid and T18 case was confirmed by full karyotyping. Maternal peripheral blood samples (12 mL in EDTA tubes) were collected from all subjects. Plasma from the UK was harvested, kept frozen and sent to Hong Kong in batches on dry ice.

20 **Sample processing**

[0171] Peripheral blood samples were processed by a double centrifugation protocol as previously described (Chiu *et al. Clin Chem* 2001; 47:1607-13). The blood cell portion was re-centrifuged at 2,500 g, and any residual plasma was removed.

25 [0172] DNA from the peripheral blood cells and that from maternal plasma was extracted with the blood and body fluid protocol of the QIAamp DNA Blood Mini Kit and the QIAamp DSP DNA Blood Mini Kit, respectively (Qiagen). DNA from the CVS and placentas was extracted with the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's tissue protocol.

Analysis of *Marker 18A* methylation status in placenta and maternal blood cells

30 DNA methylation analysis by cloning and bisulfite sequencing

[0173] The methylation status of *Marker 18A* were analyzed in six pairs of placental tissues and maternal blood cells by cloning and bisulfite sequencing. Briefly, extracted DNA was

bisulfite-converted using the EZ DNA Methylation Kit (ZymoResearch) according to the manufacturer's instructions. Bisulfite-converted DNA was subjected to PCR amplification by primers targeting the *Marker 18A* region. The primer sequences for the PCR are listed in Table 10. The PCR conditions are summarized in Table 11A. The PCR product was subsequently TA-cloned into a plasmid vector with the pGEM T-Easy Cloning Kit (Promega) according to manufacturer's instructions. Cloning was done with *Escherichia coli* strain JM109 (Promega). The inserts from the clones were then amplified using T7 and SP6 promoter primers (Promega) according to the manufacturer's instructions (Reaction conditions are summarized under the sub-title colony PCR assays in Table 11A). The PCR product was then subjected to sequencing reaction with the BigDye Terminator Cycle Sequencing v1.1 kit (Applied Biosystems) according to the manufacturer's instructions (Reaction conditions are summarized under the sub-title sequencing reaction in Table 11A). DNA was then precipitated with ethanol and resuspended in 10 µL of Hi-Di formamide and sequenced on a 3100 DNA Analyzer (Applied Biosystems). The sequencing data were analyzed using the SeqScape v2.5 software (Applied Biosystems). The inventors ensured > 99% bisulfite conversion of each clone by examining the conversion rate at non-CpG cytosine residues.

[0174] The MI at each CpG site was calculated by the number of methylated clones divided by the total number of clones for each sample. The average MI of the two biological replicates was calculated for each CpG site. The methylated site frequency was given by the number of methylated clones across each sample over the total number of clones scored.

Analysis of *Marker 18A* methylation status in T18 and euploid placentas

DNA methylation analysis by the Epityper assay

[0175] The Epityper assay was performed with the standard MassCLEAVE protocol (Sequenom) (Ehrlich *et al. Proc Natl Acad Sci U S A* 2005; 102:15785-90) (Details of the conditions for individual reactions involved in the protocol are summarized in Table 11B). Briefly, the bisulfite-converted DNA was subjected to PCR amplification with the Epityper assay targeting *Marker 18A*. The primer sequences were listed in Table 10. A T7 promoter tag was added to the 3' end of the reverse primer, and a 10-base tag was added to the 5' end of the primer to equalize the melting temperature between the forward and reverse primers. Extracted DNA was subjected to bisulfite conversion with an EZ DNA Methylation Kit (ZymoResearch) according to the manufacturer's instruction and amplified with PCR. The PCR products were then in vitro transcribed as RNA using the T7 DNA polymerase and T7

RNA polymerase, and cleaved specifically at bases with A- or G-residues by RNase A. The cleavage reaction generated CpG-containing fragments, or CpG unit, whose sizes would be dependent on the methylation status of the CpG sites (*i.e.*, CpG or TpG for methylated and unmethylated CpGs, respectively, after bisulfite conversion). The products were then cleaned up, and resolved with a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrophotometer (MassARRAY Analyzer Compact). The methylation index (MI) of each CpG unit were calculated by dividing the peak height of the methylated product by the sum of peak heights of both the methylated and unmethylated products.

10 **Detection of methylated *Marker 18A* sequences in pre- and post-delivery maternal plasma**

[0176] Quantitative real-time PCR (qPCR) assays were designed to target *Marker 18A*. The primer and probes were strategically positioned such that the unmethylated CpG sites in maternal DNA, but not the methylated CpG sites in the fetal DNA, would be specifically cleaved by the relevant restriction endonucleases.

Methylation-sensitive restriction endonucleases (MSRE) digestion

[0177] Two types of MSRE, namely *Hpa*II and *Hin*P1I (New England Biolabs), were used to digest the plasma DNA. For each plasma sample, DNA was extracted from 0.8 mL – 3.2 mL plasma and eluted in 50 µL water. 35 µL of the eluted DNA was then incubated with 20 U each of *Hpa*II and *Hin*P1I (New England Biolabs) in 1X NEBuffer 1 at 37 °C for 2 hours.

Conventional real-time quantitative PCR analysis

*I) Concentrations of digestion-resistant *Marker 18A* before and after delivery of the fetus*

[0178] After digestion by MSRE, hypermethylated DNA sequences of *Marker 18A* were amplified by conventional qPCR on the ABI 7300 HT sequence detection system (Applied Biosystems). The reaction volume of each qPCR assay was 50 µL, which contained 1X TaqMan® Universal PCR Master Mix (Applied Biosystems), 1500 nmol/L of each forward and reverse PCR primers (Integrated DNA Technologies), and 250 nmol/L TaqMan® probe (Integrated DNA Technologies). The probe was labeled with FAM on its 5' end as reporter. The thermal profile was 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 minute. The primer and probe sequences are listed in Table 12.

2) Relationship between the concentrations of digestion-resistant Marker 18A and those of ZFY in pre-delivery maternal plasma obtained from pregnancies involving male fetuses

[0179] The concentrations of ZFY were determined by qPCR on the ABI7300 HT sequence detection system (Applied Biosystems) with a previously established assay (Lun *et al. Clin Chem* 2008; 54:1664-72). The reaction volume of each qPCR assay was 50 µL, which contained 1X TaqMan® Universal PCR Master Mix (Applied Biosystems), 300 nmol/L of each forward and reverse PCR primers, and 100 nmol/L TaqMan® probe (Applied Biosystems). The probe was labeled with VIC on its 5' end as reporter. The thermal profile was 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 minute.

5 The primer and probe sequences are listed in Table 12.

[0180] For quantification by qPCR analysis, a calibration curve was derived by serial dilutions of known concentrations of genomic DNA extracted from a male peripheral blood cell sample, which was quantified by NanoDrop ND-1000 (Thermo Fisher Scientific Waltham) using the DNA-50 setting. It was then used to construct the quantitative standards with a linear dynamic range from 3 copies per reaction to 10,000 copies per reaction. The limit of detection (LOD) was 3 copies per reaction. Twenty duplicated reactions (40 replicates) were performed at the LOD concentration and 100% of the wells were positive with a median quantification cycle of 39.1 (range: 38.2 – 40.8). Any signals detected beyond that limit were considered undetectable. All reactions were duplicated and the mean quantity was taken. Multiple water blanks (no template control) were included in every PCR run.

EGG Chromosome Dosage Analysis

[0181] The inventors adopted the EGG approach to compare the relative dosage of the fetal epigenetic marker, *Marker 18A*, on chromosome 18, and that of a fetal genetic marker, *ZFY*, on chromosome Y, in placental tissues (CVS) and maternal plasma samples obtained from 25 euploid and T18 pregnancies.

Methylation-sensitive restriction endonucleases (MSRE) digestion

[0182] The digestion protocol for the EGG analysis involving *Marker 18A* was the same as that used for qPCR analysis. For analyzing placental samples, 50 ng of DNA was subjected to digestion. All of the placental tissues were obtained from the first trimester. For analyzing 30 plasma samples, DNA was extracted from 1.6 mL – 3.2 mL plasma obtained from first, second and third trimester of euploid or T18 pregnancies. Extracted DNA from each case was eluted into 50 µL water, of which 35 µL was subjected to digestion.

Assay design and reaction conditions for the digital PCR analysis

[0183] A duplex digital PCR assay was developed to amplify digestion-resistant *Marker 18A* and *ZFY* sequences. The basis of the digital PCR analysis have been described previously (Lo *et al.* *Proc Natl Acad Sci U S A* 2007; 104:13116-21; Tong *et al.* *Clin Chem* 2010; 56: 90-8).

[0184] The reaction volume was 5 μ L per well at a final concentration of 1x TaqMan® Universal PCR Master Mix (Applied Biosystems) with the respective primers and probes concentrations for each target. For *Marker 18A*, the reaction involved 250 nM TaqMan® probe (Integrated DNA Technologies) and 1500 nM of each of the forward and reverse primers (Integrated DNA Technologies). For *ZFY*, the reaction involved 100 nM TaqMan® probe (Applied Biosystems), and 300 nM of each of the forward and reverse primers (Integrated DNA Technologies). The probe for *Marker 18A* was labeled with the reporter FAM while that for *ZFY* was labeled with the reported VIC (Table 12). The total number of digital PCR reactions for each target was 384. The primer and probe sequences are the same as those for qPCR.

[0185] To evaluate the completeness of digestion, another digital PCR assay was developed to target a region on the *beta-actin* gene, which (i) is completely unmethylated in both placentas and maternal blood cells from the first trimester (Figure 17); and (ii) contains similar number of recognition sites and type of MSRE as *Marker 18A*. The methylation status of this particular region was confirmed by cloned bisulfite sequencing (Figure 17). The primer sequences for PCR amplification involved in bisulfite sequencing analysis were listed in Table 10. The reaction conditions were the same as that described for *Marker 18A*. No *beta-actin* sequences were expected to be detectable after a complete digestion. The reaction volume was 5 μ L per well at a final concentration of 1x TaqMan® Universal PCR Master Mix (Applied Biosystems) with 250 nM TaqMan® probe (Applied Biosystems) and 900 nM of each of the forward and reverse primers (Integrated DNA Technologies). The probe was labeled with the reporter VIC at its 5' end (Table 10). The assay was carried out on the 7900HT Sequence Detection System (Applied Biosystems). The reaction was initiated at 50°C for 2 min and continued at 95°C for 10 min, followed by 55 cycles of 95°C for 15 s, and 60°C for 1 min. The total number of digital PCR reactions for each target was 96.

[0186] The fluorescence data of all the digital PCR assays were collected by the "Absolute Quantification" application of SDS 2.3 software (Applied Biosystems).

Statistical analysis

[0187] Statistical analyses were performed with the SigmaStat 3.0 software (SPSS).

II. RESULTS

Marker 18A is a potential fetal epigenetic marker

5 [0188] Previous studies have shown that a genomic region that is differentially methylated between placenta and maternal blood cells has the potential to be developed into fetal epigenetic marker in maternal plasma (Chim *et al.* *Clin Chem* 2008; 54:500-11; Tong *et al.* *Clin Chem* 2010; 56:90-8). Cloning and bisulfite sequencing results revealed that *Marker 18A* is predominantly methylated in euploid placentas (n = 6) and essentially unmethylated in 10 maternal blood cells (n = 6) (Figure 12). The average methylation indices in the placentas and maternal blood cells are 0.68 and 0.01, respectively.

15 [0189] The inventors have also studied the methylation levels of *Marker 18A* in euploid (n = 6) and T18 (n = 6) placentas by the Epityper. Mann Whitney Rank Sum Test was performed to compare the MIs of the euploid placentas with those of the T18 placentas at each CpG unit (Figure 13). It was reasoned that if the methylation level of *Marker 18A* does not change significantly between euploid and T18 placentas, it can be used to compare the dosage of fetal chromosome 18 in euploid and T18 maternal plasma. It was found that within the target region of *Marker 18A*, all of the CpG units had no significant differential methylation between euploid and T18 placentas (Figure 13).

20 **Detection of *Marker 18A* in maternal plasma after enzyme digestion**

25 [0190] The inventors analyzed and compared the concentrations of digestion-resistant *Marker 18A* in maternal plasma DNA obtained before delivery and at 24 hour post-delivery. Unmethylated *beta-actin* sequences were not detectable in every post-digestion sample, indicating digestion was completed. Before delivery of the fetus, the median concentrations of digestion-resistant *Marker 18A* sequences were 806 copies/mL in third-trimester pregnancies (n = 10). After delivery, the plasma concentrations dropped below the detection limit of the assay, which was estimated to be 3 copies per reaction. The plasma concentrations of digestion-resistant *Marker 18A* sequences before and after delivery of the fetuses were statistically significantly different (Wilcoxon signed-rank test, P-value = 0.002) 30 (Figure 14A).

35 [0191] It has been further demonstrated that the plasma concentrations of digestion-resistant *Marker 18A* sequences were significantly correlated with those of the *ZFY* gene, an established fetal genetic marker, in pre-delivery (n = 13, r = 0.91; P-value < 0.00001;

Spearman correlation) (Figure 14B). These results suggested that the digestion-resistant *Marker 18A* sequences in maternal plasma were derived from the fetus.

EGG Chromosome dosage analysis

Marker 18A to ZFY ratio in placental DNA samples

5 [0192] First-trimester placental DNA samples (5 for T18 and 5 for euploid) from pregnancies with male fetuses were analyzed with the duplex digital PCR assays for *Marker 18A* and *ZFY*. The ratios of *Marker 18A* to *ZFY* in euploid and T18 samples were significantly different (Mann Whitney Rank Sum Test, P-value = 0.023). The normal reference range, defined as the mean ratio of *Marker 18A* to *ZFY* \pm 1.96SD, for the euploid 10 samples was 1.20 – 1.66. All of the T18 cases had a ratio greater than the upper reference limit (Figure 15).

Marker 18A to ZFY ratio in maternal plasma samples

15 [0193] To demonstrate that this approach is feasible to detect T18 noninvasively in maternal plasma, the inventors analyzed 27 maternal plasma samples obtained from pregnancies with euploid male fetuses and 9 from pregnancies with T18 male fetuses. Among the 27 euploid cases, 18, 4 and 9 cases were obtained from the first, second and third trimesters, respectively. All T18 cases were obtained from the first trimester. The median gestational age of the euploid and T18 groups were 14.1 and 13.3, respectively. The normal reference range was 0.34 – 3.04 (Figure 16). Based on this reference range, 26 out of 27 20 euploid cases and 8 out of 9 T18 were correctly classified, giving a sensitivity of 88.9% and a specificity of 96.3%.

Beta-actin as a digestion control

25 [0194] The enzyme digestion efficiency was evaluated by analyzing the enzyme-digested maternal plasma samples with the *beta-actin* assay that targeted at a region completely unmethylated in placentas and maternal blood cells while containing similar MSRE restriction sites as the *Marker 18A* amplicon (Figure 17). *Beta-actin* sequences were not detectable in all the digested plasma samples.

III. CONCLUSION

30 [0195] This example illustrated that, using hypermethylated *Marker 18A* as the fetal epigenetic marker on chromosome 18 and *ZFY* as the fetal genetic marker on chromosome Y, the EGG approach can be applied to infer the relative dosage of fetal chromosome 18 from maternal plasma. It has also been demonstrated that, by comparing such relative

chromosome dosage in euploid and T18 maternal plasma samples by the EGG approach, noninvasive prenatal detection of trisomy 18 is feasible. The noninvasive prenatal detection of trisomy 18 using this EGG approach could be extended to cover more fetuses in the general population by replacing the Y-specific fetal genetic marker, which was used in this 5 example as an illustration, with other fetal genetic marker, such as paternally-inherited SNP on a reference chromosome, to specifically measure the dosage of the fetal reference chromosome in maternal plasma.

EXAMPLE 4: Epigenetic-genetic chromosome dosage approach for the detection of fetal trisomy 13

10 [0196] This example demonstrates the application of epigenetic-genetic (EGG) chromosome dosage approach for the detection of fetal trisomy 13 (T13). This EGG approach involved a fetal epigenetic marker located on chromosome 13 and a fetal genetic marker located on a reference chromosome, respectively. The fetal epigenetic marker is, preferably, *Marker 13A* [genomic location chr13:105941431-105941818, defined according 15 to the Human Genome March 2006 Assembly (hg18)]. It is a 188-bp region located at the 3'end of the gene *EFNB2* (*ephrin-B2*), as identified by an methylated DNA immunoprecipitation and tiling array analysis (described in USSN 61/308,578). However, this fetal epigenetic marker can also be any cytosine-containing DNA genomic region located within 100 kb upstream or downstream of the above locus, *i.e.*, chr13:105941431-

20 Furthermore, this fetal epigenetic marker can also be any cytosine-containing DNA genomic region with different epigenetic signature (DNA methylation levels) that distinguishes the fetal from the maternal chromosome 13 in maternal plasma.

25 [0197] The fetal genetic marker in maternal plasma is, preferably, a region located in the *zinc-finger Y-linked (ZFY)* gene on chromosome Y. However, this fetal genetic marker can also be any genetic differences between the fetus and its mother, including single-nucleotide polymorphism (SNP) and insertion/deletion (indel) polymorphism.

MATERIALS AND METHODS

Sample collection

30 [0198] Samples were obtained from women attending the Department of Obstetrics and Gynaecology at the Prince of Wales Hospital, Hong Kong, or the Harris Birthright Research Centre for Fetal Medicine, at the King's College Hospital, London, UK. All of the pregnancies were recruited with informed consent. The study was approved by the respective institutional review boards.

[0199] Chorionic villus samples (CVS) were collected during conventional prenatal diagnosis sessions in the first and second trimesters of pregnancy. The chromosome status of each euploid and T13 case was confirmed by full karyotyping. Maternal peripheral blood samples (12 mL in EDTA tubes) were collected from all subjects. CVS from the UK were harvested, kept frozen and sent to Hong Kong in batches on dry ice.

5 [0200] In this study, 5 T13 cases (4 from the first trimester and 1 from the second trimester) and 10 euploid cases (all from the first trimester) were recruited.

Sample processing

[0201] Peripheral blood samples were processed by a double centrifugation protocol as previously described (Chiu *et al. Clin Chem* 2001; 47:1607-13). The blood cell portion was 10 re-centrifuged at 2,500 g, and any residual plasma was removed. DNA from the peripheral blood cells was extracted with the blood and body fluid protocol of the QIAamp DNA Blood Mini Kit (Qiagen).

[0202] DNA from the CVS and placentas was extracted with the QIAamp DNA Mini Kit 15 (Qiagen) according to the manufacturer's tissue protocol.

Analysis of Marker 13A methylation status in placentas and maternal blood cells

DNA methylation analysis by cloning and bisulfite sequencing

[0203] The methylation status of *Marker 13A* were analyzed in six pairs of placental tissues and maternal blood cells by cloning and bisulfite sequencing. Extracted DNA was bisulfite 20 converted using the EZ DNA Methylation Kit (ZymoResearch) according to the manufacturer's instructions. Bisulfite-converted DNA was subjected to PCR amplification with the forward primer '5 - aggaagagagGGAGGATAGGAGGGAGGTATAGT - 3' and reverse primer '5 -

cagtaatacgactcaactatagggagaaggctAAAATTACACACACAATACACACACCAAAAAAAT - 3'

25 targeting Marker 13A region. This set of primers were designed for analysis by the Epityper assay, but was also applied in the PCR amplification of bisulfite-converted DNA for bisulfite sequencing analysis. The PCR conditions are summarized in Table 13A. The PCR product was subsequently TA-cloned into a plasmid vector with the pGEM T-Easy Cloning Kit (Promega) according to manufacturer's instructions. Cloning was done with *Escherichia coli* 30 strain JM109 (Promega). The inserts from the clones were then amplified using T7 and SP6 promoter primers (Promega) according to the manufacturer's instructions (Reaction conditions are summarized under the sub-title colony PCR assays in Table 13A). The PCR product was then subjected to sequencing reaction with the BigDye Terminator Cycle

Sequencing v1.1 kit (Applied Biosystems) according to the manufacturer's instructions (Reaction conditions are summarized under the sub-title sequencing reaction in Table 13A). DNA was then precipitated with ethanol and resuspended in 10 μ L of Hi-Di formamide and sequenced on a 3100 DNA Analyzer (Applied Biosystems). The sequencing data were 5 analyzed using the SeqScape v2.5 software (Applied Biosystems). The inventors ensured > 99% bisulfite conversion of each clone by examining the conversion rate at non-CpG cytosine residues.

10 [0204] The MI at each CpG site was calculated by the number of methylated clones divided by the total number of clones for each sample, which was 8 in our experiments. The average MI of the two biological replicates was calculated for each CpG site. The methylated site frequency was given by the number of methylated clones across each sample over the total number of clones scored.

Analysis of Marker 13A methylation status in T13 and euploid placentas

DNA methylation analysis by the Epityper assay

15 [0205] The Epityper assay was performed with the standard MassCLEAVE protocol (Sequenom) (Ehrlich *et al. Proc Natl Acad Sci U S A* 2005; 102:15785-90) (Details of the conditions for individual reactions involved in the protocol are summarized in Table 13B). Briefly, the bisulfite-converted DNA was subjected to PCR amplification with the forward primer '5' - aggaagagagGGAGGATAGGAGGGAGGTATAGT - 3' and reverse primer '5' 20 - cagtaatacgactcaactatagggagaaggctAAAATTACACACAATACACACACCAAAAAAT - 3' targeting Marker 13A region. In this set of primer, a T7 promoter tag was added to the 3' end of the reverse primer, and a 10-base tag was added to the 5' end of the primer to equalize the melting temperature between the forward and reverse primers. Extracted DNA was subjected to bisulfite conversion with an EZ DNA Methylation Kit (ZymoResearch) 25 according to the manufacturer's instruction and amplified with PCR. The PCR products were then in vitro transcribed as RNA using the T7 DNA polymerase and T7 RNA polymerase, and cleaved specifically at bases with A- or G-residues by RNase A. The cleavage reaction generated CpG-containing fragments, or CpG unit, whose sizes would be dependent on the methylation status of the CpG sites (*i.e.*, CpG or TpG for methylated and unmethylated 30 CpGs, respectively, after bisulfite conversion). The products were then cleaned up, and resolved with a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrophotometer (MassARRAY Analyzer Compact). The methylation index (MI) of

each CpG unit were calculated by dividing the peak height of the methylated product by the sum of peak heights of both the methylated and unmethylated products.

EGG Chromosome Dosage Analysis

[0206] The inventors adopted the EGG approach to compare the relative dosage of the fetal epigenetic marker, *Marker 13A* on chromosome 13, and that of a fetal genetic marker, *ZFY* on chromosome Y, in placental tissues (CVS) obtained from euploid and T13 pregnancies.

Methylation-sensitive restriction endonucleases (MSRE) digestion

[0207] Three types of MSRE, namely AccI, NlaIV and HpaII (FastDigest® enzymes, Fermentas Life Sciences), were used to digest the placental DNA. For each sample, 50 ng of placental DNA were incubated with 2 FDU each of the above enzymes, in 1X FastDigest® Buffer at 37 °C for 2 hours, followed by enzyme inactivation at 65 °C for 20 minutes.

Assay design and reaction conditions for the digital PCR analysis

[0208] Two monoplex digital PCR assays were developed to amplify digestion-resistant *Marker 13A* and *ZFY*, respectively. The basis of the digital PCR analysis have been described previously (Lo *et al.* *Proc Natl Acad Sci U S A* 2007; 104:13116-21; Tong *et al.* *Clin Chem* 2010; 56:90-8).

[0209] The reaction volume was 5 µL per well at a final concentration of 1x TaqMan® Universal PCR Master Mix (Applied Biosystems) with the respective primers and probes concentrations for each target. For *Marker 13A*, the reaction involved 250 nM TaqMan® probe (Integrated DNA Technologies) and 900 nM of each of the forward and reverse primers (Integrated DNA Technologies). For *ZFY*, the reaction involved 100 nM TaqMan® probe, and 300 nM of each of the forward and reverse primers (Integrated DNA Technologies). The probe for *Marker 13A* was labeled with the reporter FAM while that for *ZFY* was labeled with the reporter VIC (Table 14). The total number of digital PCR reactions for each target was 192.

[0210] The experiments were carried out on the 7900HT Sequence Detection System (Applied Biosystems). The reaction was initiated at 50°C for 2 min and continued at 95°C for 10 min, followed by 55 and 45 cycles of 95°C for 15 s for *Marker 13A* and *ZFY*, respectively, and 60°C for 1 min. The fluorescence data were collected by the "Absolute Quantification" application of SDS 2.3 software (Applied Biosystems).

Statistical analysis

[0211] Statistical analyses were performed with the SigmaStat 3.0 software (SPSS).

II. RESULTS

Marker 13A is a potential fetal epigenetic marker

5 [0212] Previous studies have shown that genomic region that is differentially methylated between placenta and maternal blood cells has the potential to be developed into fetal epigenetic markers in maternal plasma (Chim *et al. Clin Chem* 2008; 54:500-11; Tong *et al. Clin Chem* 2010; 56:90-8). Cloning and bisulfite sequencing results revealed that *Marker 13A* is predominantly methylated in euploid placentas (n = 6) and essentially unmethylated in 10 maternal blood cells (n = 6) (Figure 18). The average methylation indices in placentas and maternal blood cells are 0.78 and 0.00, respectively.

15 [0213] The inventors have also studied the methylation levels of *Marker 13A* in euploid (n = 5) and T13 (n = 5) placentas by the Epityper. Mann Whitney Rank Sum Test was performed to compare the MIs of the 5 euploid placentas with those of the 5 T13 placentas at each CpG unit (Figure 19). It was reasoned that if the methylation level of *Marker 13A* does not change significantly between euploid and T18 placentas, it can be used to compare the dosage of fetal chromosome 13 in euploid and T18 fetal tissues or maternal plasma. It was found that within the target region of *Marker 13A*, 3 CpG units had no significant differential methylation between euploid and T13 placentas (Figure 19). The inventors therefore design 20 their subsequent digital PCR assays by targeting at these 3 CpG units.

EGG Chromosome-dosage analysis using placental DNA samples

25 [0214] Placental DNA samples (5 for T13 and 10 for euploid) from pregnancies with male fetuses were analyzed with the respective monoplex digital PCR assays for *Marker 13A* and *ZFY*. The ratios of *Marker 13A* to *ZFY* in euploid and T13 samples were significantly different (Mann Whitney Rank Sum Test, P-value = 0.023). A normal reference range, defined as the mean ratio of *Marker 13A* to *ZFY* \pm 1.96SD, of the euploid samples was 1.40 – 2.10. All except one of the T13 cases had a ratio greater than the upper reference limit (Figure 20).

III. CONCLUSION

30 [0215] This example illustrated that using hypermethylated *Marker 13A* as the fetal epigenetic marker on chromosome 13 and *ZFY* as the fetal genetic marker on chromosome Y, the EGG approach can be applied to infer the dosage of fetal chromosome 13 in placental

tissues obtained during the first and second trimesters. It is feasible to detect trisomy 13 by comparing such relative chromosome dosage in euploid and T13 placental tissues. This approach has the potential to be applied to infer the relative chromosome dosage of fetal chromosome 13 in maternal plasma and to detect trisomy 13 in an noninvasive manner.

5 [0216] All patents, patent applications, and other publications, including GenBank Accession Numbers, cited in this application are incorporated by reference in the entirety for all purposes.

Sequence Listing

10 **SEQ ID NO:1** dbSNP for rs6636 (taken from website ncbi.nlm.nih.gov/snp)

GAAGATTTTC CAAACTACTT TATGTCATT AGCTTCTATT TTCTGAAGGG CTTTCTTGG
TGCCATGTAC TCAGATCAGT CAGTTGACTG AAAGATGATC ATGTTTCTT CGTAAAGATT
TAAGCAATTG GCAACTACAA AGACATTATT TTCTTACTGT TCTATATCAT GTACTGTTGC
15 TGACATTACA AAAAGGGTCT GGAAGGGAAA CCGTGTCACT GTTTTATCTT TTTTCTTAA
AATACAAAAG TATCCCAACT AATCATTAT TATGGTCAGC TTGTTTACA TGTCCCTAT
[C/G]
ATGAGAAATG CTATCAACAT CTGTGATTTC TAAGAGTCTT ACCAAATTGT TACTTTAATT
CTTGTGTCCT GCTGAGTGGT TTTTCTTTA AAATACCATT TTTATCACCC TGTGGCACTG
20 GGTGTGTTAC TCGGATTACA CTGATGATTC TGAGCTGTGC TTCTCAAGT AGCTCAGTTC
TTGCGTTTA TATTAGGTAA CAGTTTGTG ATGCTTTGT GCATTCTTG TCATCTCTTC
TGAGTTTCG AATCTGTCAT AAATAAAACTT TTTCACTATG CACCTGGTAA CATCTGAGTT

25 **SEQ ID NO:2** DNA sequence including 100bp upstream and downstream of SNP rs6636 (taken from website genome.ucsc.edu/cgi-bin/hgGateway)

GGAAGGGAAACCGTGTCACTGTTTATCTTTCTTAAACAAAAG
TATCCCAACTAATCATTATTATGGTCAGCTGTTACATGTCCCTAT
30 [C/G]
ATGAGAAATGCTATCAACATCTGTGATTCTAAGAGTCTACCAAATTGT
TACTTTAATTCTGTGTCCTGCTGAGTGGTTTCTTTAAACACCATT

Table 1. Oligonucleotide sequences and specific PCR conditions for the conventional real-time PCR and digital PCR assays.

Assay	Oligonucleotide Sequence (5' to 3')	Specific Thermal Cycling Condition
Conventional		
<i>HLCS</i>	Forward Primer CCGTGTGGCCAGAGGTG	As described in specification
	Reverse Primer TGGGAGCCGGAACCTTAC	
	TaqMan Probe FAM- TCCCGACCTGGCCCTTGCC-TAMRA ^a	
Digital and Conventional		
<i>HLCS</i>	Forward Primer CCGTGTGGCCAGAGGTG	50 cycles of 95 °C for 30 seconds and 60 °C for 1 minute
	Reverse Primer AAAGGGCCAGGTCGGGA	
	TaqMan Probe FAM-AGGATTGGGGCTGCGC(MGB) ^b	
<i>RASSF1A</i>	Forward Primer AGCTGGCACCCGCTGG	50 cycles of 95 °C for 30 seconds and 60 °C for 1 minute
	Reverse Primer GTGTGGGTTGCACGCG	
	TaqMan Probe VIC-ACCCGGCTGGAGCGT(MGB) ^c	
<i>ZFX/Y</i>	Forward Primer CAAGTGCTGGACTCAGATGTAAGT	50 cycles of 95 °C for 15 seconds and 57 °C for 1 minute
	Reverse Primer TGAAGTAATGTCAGAAGCTAAACATCA	
	TaqMan Probe <i>ZFY</i> FAM-TCTTTACCACTGCAC(MGB)	
	TaqMan Probe <i>ZFX</i> VIC-TCTTTAGCACATTGCA(MGB)	

^a FAM = 6-carboxyfluorescein, TAMRA = 6-carboxytetramethylrhodamine. ^b MGB = minor-

5 groove binding. ^c VIC = Applied Biosystems proprietary dye.

Table 2

The list of information regarding the 51 COBRA assays, including the names and locations of the loci, the primer sequences, reaction conditions and results.

Gene Name	Common Name	Genebank	# of COBRA assay regions	Sequence name	(hg18) UCSC Mar 2006 (5' - 3')	F-primer (5' - 3')	R-primer (5' - 3')	Conclusion
212940_at	COL6A1	BE350145	1	COL6A1	chr21:46,245,62 7-46,246,005	GTGTGGATAGTT TAGAGAGTATGG GTTGGT	AAACCCACACCCCA ACACTCAAA	both placenta and buffy coat are methylated
233741_at	FTCD	AL109817	1	FTCD region A	chr21:46,399,97 2-46,400,232	AGGGTTATTYGT AGTGGTATAGGG AGGT	CACCRACCTCTCA TTAACCAACAAAA	both placenta and buffy coat are methylated
			2	FTCD region B	chr21:46,384,24 5-46,384,408	GTAATTITTAGAT GGAATAGAAAAAT TGTATTTTTT	TAATCTCCCTAAA CAAATCTTAACCA AAAA	both placenta and buffy coat are methylated
				FTCD region C	chr21:46,382,73 7-46,383,058	TGGGGTAGTGTG TGTATTGTTTG TT	AAATCTCCCTTAC TAACTCAACCCAA	both placenta and buffy coat are methylated
202245_at	LSS	AW084510	1	LSS	chr21:46,438,73 6-46,439,010	TTTGTTAGGATGTT TGTGTAGAGTT TTT	TCCCTCTACATTC ACTCAACCCAA	both placenta and buffy coat are methylated
241233_X_at	C21orf81	AI978581	2	C21orf81 region A	chr21:14,275,13 6-14,275,436	GGTTAGGGGTAG TATTGTTGGTAT ATTGT	CCRCCACAAACAA TACAACAACTAAT AA	both placenta and buffy coat are methylated
				C21orf81 region B	chr21:14,274,79 1-14,275,078	GGAGTTGTYGG GGTTAA GTTGGTT	TCCCCAACCTAC CCCTTA	both placenta and buffy coat are methylated
			2	C21orf81 region C	chr21:14,274,28 5-14,274,628	GGTTTTAGTATA TTAGTGGTTATA AAGAAGGAGTT	RATCCACCAACAA CTTCAACAA	placenta unmethylated and BC methylated
				C21orf81 region D	chr21:14,273,72 5-14,274,165	AGGTGAAAAGGT GATAGGGAGTT TT	CTCCACCAACTAC TCCAACCAAA	both placenta and buffy coat are methylated
232536_at	C21orf30	AL117578	2	C21orf30 region A	chr21:44,703,68 6-44,703,950	GGATGTTAGGT GAATGATTGGTG	AAAACACTAAC ACCCACAAACAC	both placenta and buffy coat are methylated

Gene Name	Common Name	Genebank	# of COBRA assay regions	Sequence name	(hg18) UCSC Mar 2006	F-primer (5' - 3')	R-primer (5' - 3')	Conclusion
239602_at	C21orf87	AI942340	1	C21orf87	chr21:44,704,10 6-44,704,401	TTATT GGGTGTTAGTTAGT TTAGGGTTGGTT TGATT	AAA CAAACACCCACRA TAACAACTCCAAT A	both placenta and buffy coat are methylated
219482_at	C21orf18	NM_017438	1	C21orf18	chr21:39,608,36 6-39,608,641	AAAATTGTTTGT GGGTAGTATAAA GGTAGTATTAGG AATAGGGGTT GGTTT	CCACAAATTATAA CTACTCAAAATC AAACTAATAA ACTCATCCACCTT ATCTCAAAACAAC AATAA	both placenta and buffy coat are unmethylated
238428_at	KCNJ15	BG542347	1	KCNJ15	chr21:38,549,76 2-38,550,032	GTATGTTTTGG ATTAGTGTAGAG GAGGTAATAGAG TT	CAAAAATTCCATCC TAAAAAAAAACTT CTAATAA	both placenta and buffy coat are methylated
230470_at	DSCR9	AW510868	2	DSCR9	chr21:37,514,41 8-37,514,645	GTGAYGTAATAA TITAAATTATGAA GAGGTAATAGAG TT	CCCCACCRTTCCA TCCCCAAA	both placenta and buffy coat are methylated
236631_at	C21orf12	AA522688	2	DSCR9	chr21:37,515,19 7-37,515,378	GYGTAGGTTAT GTTGGGGTTGGTAT TATT	ACAAACCRAAAAAC ACCACCTAA	both placenta and buffy coat are unmethylated
5			5	C21orf12	chr21:43,693,74 2-43,694,068	TTAATAGTATCAA GGGTGGGTGGGT TT	CTACCTAACATAA AAATATTAAATTCT TCCCTATCTA	both placenta and buffy coat are methylated
237268_at	DSCAM	BE503065	2	DSCAM	chr21:43,694,82 4-43,695,108	GTATGGTTAGGT AGGAGGGGGGT T	CAATACCAAATA AAATAAAATCCCCA AAA	both placenta and buffy coat are methylated
				DSCAM	chr21:40,647,21 9-40,647,485	GGTTGGGTAGYG TGTGGAGTTGGT	RATAAAAACCAAC ACCACCTTACAA A	both placenta and buffy coat are methylated
233818_at	ZNF294	AK023499	2	ZNF294	chr21:29,286,99 3-29,287,264	GGGTAAATGTTT GGTTTTATGG	AACTACAAAAAA ACATAAAAATTTA CTTATAACAAAAA	both placenta and buffy coat are methylated
							CCCATAAATCRCA TTACAACTATACT	both placenta and buffy coat are unmethylated

Gene Name	Common Name	Genebank	# of COBRA assay regions	Sequence name	(hg18) UCSC Mar 2006	F-primer (5' - 3')	R-primer (5' - 3')	Conclusion
				ZNF294 region B	chr21:29,286,764-29,287,046	TT	CTAAA CACRATTCCCCAA TCTACTCAAAA	both placenta and buffy coat are unmethylated
242913_at	CLIC6	AW816405	1	CLIC6	chr21:34,963,094-34,963,335	AYGTGTTAATYG GGGTTTGAGTGT T	AACTCCCTAAACT CTTCCTCCACTAA AA	both placenta and buffy coat are methylated
236910_at	MRPL39	AI809483	2	MRPL39 region A	chr21:25,901,919-25,902,230	GAGGGAGGGAGGG GAAGATAAATT	CCCCACCAACRTA AACTCTACAAA	both placenta and buffy coat are unmethylated
241726_at	HLCS	AI682088	3	HLCS region A	chr21:37,275,090-37,275,427	GGGGAYGTGT YGAGGTTTTATT	TCCCCATAAATT ATCCTCCCAA ATA	both placenta and buffy coat are unmethylated
				HLCS region B1	chr21:37,274,765-37,275,074	GGAGGTAGAYG TTTAGTTYGTGT	CRCTACCCCTCTCC ACTAACTACTCAA A	placenta methylated and buffy coat unmethylated
				HLCS region B2	chr21:37,274,651-37,275,063	GGTT GT	CTAAACACCCRAA TCCCCAAA	placenta methylated and buffy coat unmethylated
231696_x_at	C21orf4	AV648424	1	C21orf4 region A	chr21:33,744,397-33,744,764	GTTTTTTTTTT GAGATTAGAAT TTGATTATT	CTAAAAATAAAAA AACAAAATCCAAA ACAAA	placenta methylated and buffy coat unmethylated
			2	C21orf4 region B	chr21:33,764,164-33,764,488	TTAGTAGAGAYG GGATTTTATTATG TTGGTT	AAAAAAATTTCAC TCTCCCCAAA ATAACTATAAAA TA	both placenta and buffy coat are methylated
				C21orf4 region C	chr21:33,763,170-33,763,602	TTAGGGTGGAGT GTAGTGGTATAA TTTGTAT	CCTTTCTTAAACT TTACACACAACTT TACTTTAA	both placenta and buffy coat are methylated
232755_at	UBE2G2	AL355686	2	UBE2G2 region A	chr21:45,020,136-45,020,445	GGAAATGTYGTT TGTAAATAGATT	CTCTTACCTATCA ATTATATTTTAA	both placenta and buffy coat are methylated

Gene Name	Common Genebank	Sequence name	(hg18) UCSC Mar 2006	F-primer (5' – 3')	R-primer (5' – 3')	Conclusion		
	# of COBRA assay regions			GTTGTT	CTTCTTATATAAA			
		UBE2G2 region B	chr21:45,019,358-45,019,633	AGAGGTGGTGTGGATT	CCACCTATCCTTA CACTATACCCAAA	both placenta and buffy coat are methylated		
236061_at	PRDM15	BF058757	2	GTATTATAATT TGATTAGTTTT	CAAATAACACTCAC TCCTACAAAACC	both placenta and buffy coat are methylated		
		PRDM15 region A	chr21:42,109,470-42,109,789	ATGTTAGGGTT	TAA			
		PRDM15 region B	chr21:42,109,068-42,109,280	ATTYGTGTGTGT TTGAAGGTATGA AGAT	ACCCACCTAACCT CCCTACACAAATA	both placenta and buffy coat are methylated		
		1	PRDM15 region C	GTATTAGGGTAGT GTGTTGGTTGTTT	CTTCACACACATA AAATCCACACATA CACTATA	both placenta and buffy coat are methylated		
		1	PRDM15 region D	chr21:42,093,090-42,093,290	AAGTAGAGAGGT TAGTTTATTAAATA GAAGTATTAAATT GTT	AAACITCTCCCCA CACCTCTCAATA	both placenta and buffy coat are methylated	
242784_at	ETS2	AV646177	1	chr21:39,101,959-39,102,202	GTAGGTATGTGG ATTTTGGGTAGAT TGTTT	CACATTCACTCAA AATCTCCACTTAA TAA	both placenta and buffy coat are methylated	
231245_s_at	C21orf80	AW294061	1	C21orf80	chr21:45,532,732-45,533,105	TTYGGGGAAATAG YGGGAGTTGT	AATACTCTCRCCC CTCTAAAACTAAA	both placenta and buffy coat a little methylated
234315_at	BACH1	AF317902	1	BACH1	chr21:29,644,841-29,645,100	GAAGAATGGTTA TGTAGGGTAGAG GATAATT	AATTCRTAACTTTC ATAAAAATCTACT CCTCTCTAA	both placenta and buffy coat are methylated
239968_at	C21orf84	AW206007	1	C21orf84	chr21:43,722,837-43,723,075	GTTAGGTIAGAT ATGTTGGGTAGTT AGGGTTT	CATAAACCCCTA ACCACCCCCAAA	both placenta and buffy coat are methylated
229639_s_at	SLC19A1	AI858077	2	SLC19A1 region A	chr21:45,780,108-45,780,457	TTTTAGTTTT GGTTGTAGGAGA GGTT	AATAAACACCTTA TAATTCAACCCAA AAAA	both placenta and buffy coat are methylated
				SLC19A1	chr21:45,780,66	GTTATTGGGATA	ACACATAATACTC	both placenta and buffy coat

Gene Name	Common Genebank	# of COBRA assay regions	Sequence name	(hg18) UCSC Mar 2006	F-primer (5' - 3')	R-primer (5' - 3')	Conclusion	
		region B	0-45,780,989	GTGGAGGTGTTG TT	ACCCCTATAAATA ACCTTAA		are methylated	
236988_x_at	SLC19A1	region C	chr21:45,786,58 9-45,786,920	TYGTGGTGGGA GGGTGT TT	CCCCCACACTCAC CTCACAAA		both placenta and buffy coat are unmethylated	
	ITGB2	W68403	1	ITGB2	chr21:45,155,72 7-45,156,027	GTGTGTATTGGAA ATAAGGGTA GGATI	TACACTCTACCCC ATATAACCCAAA	both placenta and buffy coat are methylated
		1	ITGB2	chr21:45,154,85 0-45,155,200	AGGGGAGTTTA TGGGTGGTTAGTT	CACCACCCCAAA TACAAAAAAA	both placenta and buffy coat are unmethylated	
240406_at	USP16	AI022850	1	USP16	chr21:29,318,79 3-29,319,027	TGGGGTGGGTGGT GGTTTAGTT	CTCCCAACRCAA AAACCAAA	both placenta and buffy coat are unmethylated
		2	USP16	chr21:29,345,68 9-29,346,141	AAGTGAGGATAT TATAAAGGATT TTAAAGGATT AAAGAATT TT	TAATAAATAATCA TATACTTATCAT ATACCTCAATATA	both placenta and buffy coat are methylated	
			USP16	chr21:29,346,42 2-29,346,772	GTAGATTGGTAT ATGGAAATTATA TTATGGTTAGTT T	CTCCCCACAATTCC CACATATCATAA	both placenta and buffy coat are methylated	
240809_at	C21orf12	AI138282	1	C21orf12 1 region A	chr21:42,314,58 8-42,314,901	GGGGTTATTATT TGGTGATAGTGG TATATA TT	ATCCCTACCCATT ATTCCCTCTTTACA AA	both placenta and buffy coat are unmethylated
			1	C21orf12 1 region B	chr21:42,302,12 6-42,302,508	TTTTATGAGAAAT TTTTTAAAATGG ATTAAATT	CATCCCAACACAC CRCTAAAA	both placenta and buffy coat are methylated

Table 3A. Detection of *HLCS*, *RASSF1A*, *ZFY* and *ZFX* in the mock- and *Bst*UI-digested DNA samples.

Sample	Copy number			
	<i>HLCS</i>	<i>RASSF1A</i>	<i>ZFY</i>	<i>ZFX</i>
Maternal blood 1_mock	487	464	0	455
Maternal blood 1_BstUI	6	0	0	459
Maternal blood 2_mock	374	321	0	397
Maternal blood 2_BstUI	17	0	0	437
Placenta 1_mock	295	287	158	171
Placenta 1_BstUI	196	187	164	177
Placenta 2_mock	158	113	93	64
Placenta 2_BstUI	106	81	90	95

The copy numbers were corrected for the Poisson distribution using the formula in the text.

5

Table 3B. Detection of *HLCS* and *RASSF1A* in the untreated and *Bst*UI-digested DNA samples.

Replicate	Sample	Copy number	
		<i>HLCS</i>	<i>RASSF1A</i>
1	Post-plasma 1_untreated	172	109
1	Post-plasma 1_Bst UI	9	1
2	Post-plasma 1_Bst UI	6	0
1	Post-plasma 2_untreated	106	66
1	Post-plasma 2_Bst UI	4	3
2	Post-plasma 2_Bst UI	2	1

The copy numbers were corrected for the Poisson distribution using the formula in the text.

10

Table 4. Information on the samples used for the digital PCR analysis. (A) Placental tissue samples, (B) Maternal plasma samples.

5 (A)

Trimester	Euploid sample	Number of panel	Copy number				
			HLCS	RASSF1A	ZFY	ZFX	HLCS/RASSF1A
3rd	N2541	4	1187	1165	994	1007	1.02
3rd	N2560	4	890	731	685	587	1.22
3rd	N2552	4	1156	829	806	703	1.40
3rd	N259K	4	957	712	673	670	1.35
3rd	N2582	4	934	781	623	569	1.20
1st	V2863	4	714	752	647	581	0.95
1st	V2894	4	625	604	481	469	1.03
1st	V2851	4	799	611	609	655	1.31
1st	V3031	4	1007	658	709	650	1.53
1st	V3104	4	1059	729	683	665	1.45

Trimester	T21 sample	Number of panel	Copy number				
			HLCS	RASSF1A	ZFY	ZFX	HLCS/RASSF1A
2nd	N0349	4	2028	1031	1022	676	1.97
2nd	N0435K	4	899	538	448	424	1.67
2nd	N0764L	4	990	747	576	571	1.33
2nd	N1519K	4	891	507	424	356	1.76
2nd	N2770K	4	868	592	517	511	1.47
2nd	N3265K	4	1004	605	469	410	1.66
1st	N0234L	4	964	764	558	513	1.26
1st	N0456E	4	717	459	316	308	1.56
1st	N0891L	4	955	655	520	491	1.46
1st	N2849K	4	848	562	423	600	1.51
1st	N3168K	4	825	625	444	406	1.32
1st	N3228K	4	1126	761	519	475	1.48

(B)

Trimester	Euploid sample	Plasma vol.	Number of panel	Copy number			
				HLCS	ZFY	ZFX	HLCS/ZFY
3rd	M3885	4 mL	6	296	147	1086	2.01
3rd	M3915	3.4 mL	6	820	352	3941	2.33
3rd	M3936	3.8 mL	6	694	283	3951	2.46
3rd	M4028	4.8 mL	6	2123	869	5679	2.44
3rd	M4029	4.8 mL	6	342	146	1259	2.34
3rd	M4039	4 mL	6	752	378	3380	1.99
3rd	M4055	4 mL	6	475	285	2612	1.67
3rd	M4060	3.6 mL	6	681	383	3882	1.78
2nd	M3364	4.4 mL	12	134	58	1782	2.30
2nd	M3367	4.6 mL	12	182	107	958	1.71
2nd	M3377	4.8 mL	12	208	76	1830	2.73
2nd	M3414	5.1 mL	12	144	83	979	1.73
2nd	M4046	5.5 mL	12	152	58	1633	2.62
2nd	M4048	5.8 mL	12	114	52	1392	2.18
2nd	M4096	6 mL	12	260	85	2308	3.04
2nd	M4103	6 mL	12	179	86	1608	2.07
1st	M4049	5.2 mL	12	105	54	1419	1.93
1st	M4050	5.8 mL	12	146	75	1054	1.94
1st	M4095	5.2 mL	12	124	62	1332	1.99
1st	M4232	6.6 mL	12	145	64	954	2.26
1st	M4265	6.1 mL	12	111	63	1685	1.75
1st	M4266	6.2 mL	12	1556	626	9078	2.49
1st	M4541	8.8 mL	18	282	121	2911	2.34
1st	M4621	9.1 mL	12	287	121	2062	2.38

Trimester	T21 sample	Plasma vol.	Number of panel	Copy number			
				Copy number HLCS	Copy number ZFY	Copy number ZFX	HLCS/ZFY
2nd	M2770	7.4 mL	12	731	248	3504	2.95
2nd	M3870	6.6 mL	12	370	95	1541	3.88
2nd	M4183	8.8 mL	18	695	231	2520	3.01
1st	M2948	6 mL	12	705	224	5322	3.15
1st	M4101	6.4 mL	12	630	162	3368	3.88

The number of copy of each locus and the ratios of *HLCS* to *RASSF1A* and *HLCS* to *ZFY* are shown. The copy numbers were corrected for the Poisson distribution using the formula in the text.

10

Table 5. Oligonucleotide sequences for the *HLCS*, *TMED8-C/G* SNP and *beta-actin* assays.

Assay	Oligonucleotide	Sequences (5' to 3')	PCR amplicon length (bp)
<i>HLCS</i>	Forward primer	CCGTGTGGCCAGAGGTG	96
	Reverse primer	AAAGGGCCAGGTGGGA	
	TaqMan probe (FAM)	FAM-AGGATTGGGGCTGCGC(MGB) ^a	
	TaqMan probe (VIC)	VIC-AGGATTGGGGCTGCGC(MGB) ^b	
<i>TMED8-C/G</i>	Forward primer	TGGTAAGACTCTTAGAAATCACAGATGT	96
		T	
	Reverse primer	GTATCCCAACTAACATCATTATTATGGTCA	
	TaqMan probe_ <i>TMED8-C</i>	FAM-CCCCTATCATGAGAAAT(MGB)	
<i>Beta-actin</i>	TaqMan probe_ <i>TMED8-G</i>	VIC-CCCCTATGATGAGAAAT(MGB)	
	Forward primer	CCACCACCGCCGAGAC	96
	Reverse primer	TGGCCGGGCTTACCTGG	
	TaqMan probe	FAM-AGCACAGAGCCTGCC(MGB)	

^a FAM = 6-carboxyfluorescein, MGB = minor-groove binding.

^b VIC = Applied Biosystems[®] proprietary dye.

5 **Table 6.** Ratio of *HLCS* to *TMED8-C* allele in (A) mock-digested placental DNA, and (B) *Bst*UI-digested placental DNA samples. 1T, first trimester; 2T, second trimester; 3T, third trimester.

(A)

Sample type	Sample	Fetal genotype	Maternal genotype	Copies per reaction		
				<i>HLCS</i>	<i>TMED8-C</i>	<i>HLCS/TMED8-C</i>
1T.euploid	V0057	CG	GG	2050	857	2.39
1T.euploid	V0360	CG	GG	866	343	2.52
1T.euploid	V3104	CG	GG	1877	794	2.36
3T.euploid	PLN74	CG	GG	3110	1502	2.07
3T.euploid	N0425	CG	GG	5695	2786	2.04
3T.euploid	N0426	CG	GG	4117	2103	1.96
1T.T21	N0456	CG	GG	1574	489	3.22
1T.T21	N0891	CG	GG	2011	621	3.24
1T.T21	N3634	CG	GG	3867	1193	3.24
2T.T21	N1519	CG	GG	2030	606	3.35
3T.euploid	N2541	GG		4226	0	N/A

(B)

Sample type	Sample	Fetal genotype	Maternal genotype	Copies per reaction		
				HLCS	TMED8-C	HLCS/TMED8-C
1T.euploid	V0057	CG	GG	1927	1001	1.92
1T.euploid	V0360	CG	GG	887	489	1.82
1T.euploid	V3104	CG	GG	1909	1035	1.84
3T.euploid	PLN74	CG	GG	3076	1504	2.05
3T.euploid	N0425	CG	GG	4364	2896	1.51
3T.euploid	N0426	CG	GG	3653	2669	1.37
1T.T21	N0456	CG	GG	1866	671	2.78
1T.T21	N0891	CG	GG	2350	955	2.46
1T.T21	N3634	CG	GG	4084	1493	2.73
2T.T21	N1519	CG	GG	3141	1001	3.14
3T.euploid	N2541	GG		4402	0	N/A

5 **Table 7.** Ratio of *HLCS* to *TMED8-G* allele in (A) mock-digested placental DNA, and (B) *Bst*UI-digested placental DNA samples. 1T, first trimester; 2T, second trimester; 3T, third trimester.

(A)

Sample type	Sample	Fetal genotype	Maternal genotype	Copies per reaction		
				HLCS	TMED8-G	HLCS/TMED8-G
1T.euploid	V0207	CG	CC	230	66	3.50
1T.euploid	V0208	CG	CC	220	98	2.25
1T.euploid	V0284	CG	CC	966	474	2.04
1T.euploid	V0488	CG	CC	498	226	2.20
1T.euploid	V0492	CG	CC	570	322	1.77
1T.euploid	V0503	CG	CC	1095	569	1.92
1T.euploid	V0580	CG	CC	892	397	2.25
1T.euploid	V3031	CG	CC	1993	925	2.15
3T.euploid	N0069	CG	CC	4858	2416	2.01
3T.euploid	PLN113	CG	CC	2136	886	2.41
3T.euploid	PLN114	CG	CC	3973	1909	2.08
3T.euploid	N0333	CG	CC	3144	1301	2.42
3T.euploid	N0524	CG	CC	5213	2817	1.85
3T.euploid	N0527	CG	CC	5376	2840	1.89
1T.T21	N3228	CG	CC	6787	2239	3.03
1T.T21	N3438	CG	CC	4349	1236	3.52
1T.T21	N4101	CG	CC	5787	1902	3.04
2T.T21	N2913	CG	CC	4907	1027	4.78
2T.T21	N4183	CG	CC	4673	1651	2.83
3T.euploid	N2582	CC		2177	0	N/A

(B)

Sample type	Sample	Fetal genotype	Maternal genotype	Copies per reaction		
				HLCS	TMED8-G	HLCS/TMED8-G
1T.euploid	V0207	CG	CC	246	163	1.52
1T.euploid	V0208	CG	CC	262	172	1.52
1T.euploid	V0284	CG	CC	443	463	0.96
1T.euploid	V0488	CG	CC	469	333	1.41
1T.euploid	V0492	CG	CC	501	308	1.63
1T.euploid	V0503	CG	CC	1144	755	1.52
1T.euploid	V0580	CG	CC	615	503	1.22
1T.euploid	V3031	CG	CC	2249	1290	1.74
3T.euploid	N0069	CG	CC	3389	2446	1.39
3T.euploid	PLN113	CG	CC	2001	969	2.07
3T.euploid	PLN114	CG	CC	2751	2019	1.36
3T.euploid	N0333	CG	CC	2499	1613	1.55
3T.euploid	N0524	CG	CC	3579	2834	1.26
3T.euploid	N0527	CG	CC	3250	3049	1.07
1T.T21	N3228	CG	CC	5448	2753	1.98
1T.T21	N3438	CG	CC	5733	2272	2.52
1T.T21	N4101	CG	CC	4610	2272	2.03
2T.T21	N2913	CG	CC	4057	1991	2.04
2T.T21	N4183	CG	CC	4517	1196	3.78
3T.euploid	N2582	CC		1439	0	N/A

5 **Table 8.** Digestion efficiency evaluated by *beta-actin* real-time qPCR analysis. (A) Samples for HLCS to TMED8-C ratio analysis. (B) Samples for HLCS to TMED8-G ratio analysis. 1T, first trimester; 2T, second trimester; 3T, third trimester.

(A)

Sample type	Sample	Copies per reaction		
		Mock	Bst UI	% digested
1T.euploid	V0057	1845	5	99.7
1T.euploid	V0360	856	6	99.3
1T.euploid	V3104	1852	8	99.5
3T.euploid	PLN74	2937	5	99.8
3T.euploid	N0425	4845	24	99.5
3T.euploid	N0426	4283	33	99.2
1T.T21	N0456	1022	3	99.7
1T.T21	N0891	1315	13	99.0
1T.T21	N3634	2275	15	99.3
2T.T21	N1519	1607	28	98.3
3T.euploid	N2541	3974	58	98.5

(B)

Sample type	Sample	Copies per reaction		
		Mock	BstUI	% digested
1T.euploid	V0207	131	1	98.9
1T.euploid	V0208	200	7	96.8
1T.euploid	V0284	856	2	99.8
1T.euploid	V0488	220	2	99.3
1T.euploid	V0492	560	7	98.8
1T.euploid	V0503	1036	2	99.8
1T.euploid	V0580	837	7	99.2
1T.euploid	V3031	2154	13	99.4
3T.euploid	N0069	4977	17	99.7
3T.euploid	PLN113	1873	31	98.3
3T.euploid	PLN114	3569	15	99.6
3T.euploid	N0333	2982	10	99.7
3T.euploid	N0524	5583	18	99.7
3T.euploid	N0527	5667	14	99.8
1T.T21	N3228	3649	26	99.3
1T.T21	N3438	3136	20	99.4
1T.T21	N4101	2748	16	99.4
2T.T21	N2913	2822	19	99.3
2T.T21	N4183	3544	9	99.7
3T.euploid	N2582	2341	12	99.5

5 **Table 9.** Specificity of the *TMED8*-C/G SNP assays. Genotypes of the placentas are indicated after the sample number. The copy numbers were corrected for Poisson distribution. (A) *HLCS* and *TMED8*-C duplex assay. (B) *HLCS* and *TMED8*-G duplex assay.

(A)

Sample	Copies		
	HLCS	<i>TMED8</i> -C	No. of PCR wells analyzed
Placenta1_CG	41	29	192
Placenta2_CG	95	46	192
Placenta3_CC	76	87	192
Placenta4_CC	28	20	192
Placenta5_GG	39	0	192
Placenta6_GG	13	0	192

10

(B)

Sample	Copies		
	HLCS	<i>TMED8</i> -G	No. of PCR wells analyzed
Placenta1_CG	82	37	192
Placenta2_CG	76	40	192
Placenta3_CC	192	0	384
Placenta4_CC	83	0	384
Placenta5_GG	51	53	192
Placenta6_GG	45	47	192

Table 10. Primer sequences for the PCR amplification of bisulfite-converted DNA involved in the analysis by the Epityper and bisulfite sequencing for *Marker 18A* and *beta-actin*.

Target	Assays	Directions	Sequences (5' to 3')
<i>Marker 18A</i>	Bisulfite sequencing	Forward	5' - aggaaggagGGTTATTGGGGTAGTAGG - 3'
		Reverse	5' - cagtaatacgaactataggagaaggctAAACTCAAACAAACACTC - 3'
<i>Marker 18A</i>	Epityper	Forward	5' - aggaaggagGGTTATTGGGGTAGTAGG - 3'
		Reverse	5' - cagtaatacgaactataggagaaggctAAACTCAAACAAACACTC - 3'
<i>Beta-actin</i>	Bisulfite sequencing	Forward	5' - TTTATTTTGYGATTTTATTGGTAAGAGTT - 3'
		Reverse	5' - AACACAAAACCTCRCCTTACC - 3'

Table 11A. Reaction conditions for bisulfite sequencing analysis.

<u>Bisulfite PCR reaction conditions</u>		Final concentration	Thermal profile	
10X buffer II	1X			
MgCl ₂	3mM			
dNTP	200μM			
Forward primer	200nM		95°C	10 min
Reverse primer	200nM		95°C	40s
AmpliTaq Gold	1U		Specific annealing temperature	45s
Bisulfite-converted DNA	50 ng		72°C	45s
Total reaction volume	25 μL		72°C	7 min
<u>Colony PCR assays</u>		Final concentration	Thermal profile	
10X buffer II	1X			
MgCl ₂	4 mM		95°C	10 min
dNTP	200μM		95°C	1 min
SP6 / T7 primers	Each 100nM		55°C-0.5°C per cycle	1.5 min
AmpliTaq Gold	1U		72°C	1 min
Template DNA	Colony		95°C	18 cycles
Total reaction volume	25 μL		50°C	1 min
			72°C	1.5 min
			72°C	1 min
			72°C	7 min
<u>Sequencing reaction</u>		Final concentration	Thermal profile	
Big Dye v. 3.1	2.5X			
5x BigDye sequencing buffer	1X		96°C	10s
Primer (SP6)	250nM		50°C	5s
Water	variable		60°C	4 min
Product from colony PCR reaction	1.5 μL			
Total reaction volume	10 μL			

Table 11B. Reaction conditions for the Epityper analysis.

Epityper assay	
PCR amplification	Final concentration
10X Hot Star Buffer	1X
dNTP	200 μM
Forward primer	200 nM
Reverse primer	200 nM
Hot Star Taq	0.2 unit
Bisulfite-converted DNA	50 ng
Total reaction volume	5 μL
	Thermal profile
	94°C 15 min
	94°C 20s 45 cycles
	Specific annealing temperature 30s
	72°C 1 min
	72°C 3 min
Dephosphorylation	Volume per reaction
RNase-free water	1.7 μL
Shrimp alkaline phosphatase (SAP)	0.3 μL
Total reaction volume	2 μL
	Thermal profile
	37°C 40 min
	85°C 5 min
This mixture is added to the product of the PCR amplification	
In vitro transcription and RNase A cleavage	Final concentration
RNase-free water	variable
5X T7 Polymerase Buffer	0.64X
T-cleavage mix (or C-cleavage)	0.24 μL
DTT	3.14 mM
T7 RNA & DNA Polymerase	22 units
RNase A	0.09 mg/mL
SAP-treated PCR product	2 μL
Total reaction volume	5 μL
Sample conditioning	Thermal profile
	37°C 3 hours
Add 20 μL water and 6 mg Clean Resin to each well in a 384-well setting. Rotate for 10 minutes and spin down for 5 minutes at 3,200g. The samples are then ready to be dispensed onto the SpectroCHIP® bioarray.	

Table 12. Oligonucleotide sequences for the *Marker 18A*, *ZFY*, and *beta-actin* qPCR / digital PCR assays.

Assay	Oligonucleotide	Sequences (5' to 3')	PCR amplicon length (bp)
<i>Marker 18A</i>	Forward primer	5'- AGGCCCTGCGCAGGGTGG -3'	83 bp
	Reverse primer	5'- CACTCGCTGAGCGCTCCCC -3'	
	TaqMan probe (FAM)	5'- FAM- CGCCCCGGCACAGGGCTCG -BHQ1 - 3'	
<i>ZFY</i>	Forward primer	5' - CAAGTGCTGGACTCAGATGTTAAGT - 3'	87 bp
	Reverse primer	5' - TGAAGTAATGTCAGAAAGCTAAACATCA - 3'	
	TaqMan probe (VIC)	5' - VIC- TCTTTACCACTGCAC -MGB - 3'	
<i>Beta-actin</i>	Forward primer	5' - CCCCCATCTCGGGAGGC - 3'	88 bp
	Reverse primer	5' - GTCTGGGGCGAGCGG - 3'	
	TaqMan probe (VIC)	5' - VIC- AGGGGGCTTCTCCGGGC -MGB - 3'	

NOTE: FAM is 6-carboxyfluorescein, VIC is Applied Biosystems® proprietary dye, BHQ1 is Black-hole quencher 1, and MGB is minor groove binding.

Table 13A. Reaction conditions for bisulfite sequencing analysis.

<u>Bisulfite PCR reaction conditions</u>		Thermal profile		
10X buffer II	Final concentration 1X			
MgCl ₂	3mM			
dNTP	200µM	95°C	10 min	
Forward primer	200nM	95°C	40s	40 cycles
Reverse primer	200nM	Specific annealing temperature	45s	
AmpliTaq Gold	1U	72°C	45s	
Bisulfite-converted DNA	50 ng	72°C	7 min	
Total reaction volume	25 µL			

<u>Colony PCR assays</u>		Thermal profile		
10X buffer II	Final concentration 1X	95°C	10 min	
MgCl ₂	4 mM	95°C	1 min	10 cycles
dNTP	200µM	55°C -0.5°C per cycle	1.5 min	
SP6 / T7 primers	Each 100nM	72°C	1 min	
AmpliTaq Gold	1U	95°C	1 min	18 cycles
Template DNA	Colony	50°C	1.5 min	
Total reaction volume	25 µL	72°C	1 min	
		72°C	7 min	

<u>Sequencing reaction</u>		Thermal profile		
Big Dye v. 3.1	Final concentration 2.5X	96°C	10s	25 cycles
5x BigDye sequencing buffer	1X	50°C	5s	
Primer (SP6)	250nM	60°C	4 min	
Water	variable			
Product from colony PCR reaction	1.5 µL			
Total reaction volume	10 µL			

Table 13B. Reaction conditions for the Epityper analysis.

Epityper assay			
PCR amplification			
	Final concentration	Thermal profile	
10X Hot Star Buffer	1X	94°C	15 min
dNTP	200µM	94°C	20s
Forward primer	200nM	Specific annealing temperature	45 cycles
Reverse primer	200nM	30s	
Hot Star Taq	0.2 unit	72°C	1 min
Bisulfite-converted DNA	50 ng		
Total reaction volume	5 µL	72°C	3 min
Dephosphorylation			
	Volume per reaction	Thermal profile	
RNase-free water	1.7µL	37°C	40 min
Shrimp alkaline phosphatase (SAP)	0.3µL	85°C	5 min
Total reaction volume	2 µL		
This mixture is added to the product of the PCR amplification			
In vitro transcription and RNase A cleavage			
	Final concentration	Thermal profile	
RNase-free water	variable	37°C	3 hours
5X T7 Polymerase Buffer	0.64X		
T-cleavage mix (or C-cleavage)	0.24µL		
DTT	3.14mM		
T7 RNA & DNA Polymerase	22 units		
RNase A	0.09 mg/mL		
SAP-treated PCR product	2 µL		
Total reaction volume	5 µL		
Sample conditioning			
Add 20 µL water and 6 mg Clean Resin to each well in a 384-well setting. Rotate for 10 minutes and spin down for 5 minutes at 3,200g. The samples are then ready to be dispensed onto the SpectroCHIP® bioarray.			

Table 14. Oligonucleotide sequences for the *Marker 13A* and *ZFY* digital PCR assays, respectively.

Assay	Oligonucleotide	Sequences (5' to 3')	PCR amplicon length (bp)
<i>Marker 13A</i>	Forward Primer	5' - GAAATAAACGGGGAACATCTTG - 3'	103 bp
	Reverse primer	5' - TCTCACGGAACCGTAGACTAGGAA - 3'	
	TaqMan probe (FAM)	5' - FAM- ACAGAGCAGGAAGCCGATGTGACTGC-BHQ1- 3'	
<i>ZFY</i>	Forward Primer	5' - CAAGTGCTGGACTCAGATGTAAC TG - 3'	87 bp
	Reverse primer	5' - TGAAGTAATGTCAGAAGCTAAACATCA - 3'	
	TaqMan probe (VIC)	5' - VIC-TCTTTACCCACACTGCAC -MGB - 3'	

NOTE: FAM is 6-carboxyfluorescein, VIC is Applied Biosystems® proprietary dye, BHQ1 is Black-hole quencher 1, and MGB is minor groove binding.

WHAT IS CLAIMED IS:

1. A method for detecting a chromosomal aneuploidy in a fetus carried by a pregnant woman, comprising the steps of:

(a) determining in a biological sample taken from the pregnant woman the amount of a methylation marker of fetal origin, wherein the methylation marker is located on a chromosome relevant to the chromosomal aneuploidy or within a section of a chromosome relevant to the chromosomal aneuploidy, and wherein the methylation marker of fetal origin is distinguished from its counterpart of maternal origin due to differential DNA methylation;

(b) determining the amount of a genetic marker of fetal origin in the sample, wherein the genetic marker is located on a reference chromosome, and wherein the genetic marker of fetal origin is distinguished from its counterpart of maternal origin in the sample due to difference in polynucleotide sequence, or the genetic marker does not exist in the maternal genome;

(c) determining the ratio of the amounts from (a) and (b); and

(d) comparing the ratio with a standard control, wherein the ratio higher or lower than the standard control indicates the presence of the chromosomal aneuploidy in the fetus.

2. The method of claim 1, wherein the methylation marker of fetal origin is from the placenta.

3. The method of claim 1, wherein the counterpart of maternal origin is from the pregnant woman's blood cells.

4. The method of claim 1, wherein the methylation marker of fetal origin is more methylated than its counterpart of maternal origin.

5. The method of claim 1, wherein the methylation marker of fetal origin is less methylated than its counterpart of maternal origin.

6. The method of claim 1, wherein the sample is maternal whole blood, serum, plasma, urine, amniotic fluid, genital tract lavage fluid, placental-tissue sample, chorionic villus sample, or a sample containing fetal cells isolated from maternal blood.

7. The method of claim 1, wherein the sample contains fetal nucleic acids.

8. The method of claim 1, wherein the methylation marker is a part of, or in proximity to, the *Holocarboxylase Synthetase (HLCs)* gene.

9. The method of claim 8, wherein the methylation marker is the putative promoter of the *HLCs* gene.

10. The method of claim 1, wherein the methylation marker is *Marker 18A* or *Marker 13A*.

11. The method of claim 1, wherein step (a) comprises treating the sample with a reagent that differentially modifies methylated and unmethylated DNA.

12. The method of claim 11, wherein the reagent comprises bisulfite or a protein or chemical that binds to DNA based on methylation status.

13. The method of claim 11, wherein the reagent comprises an enzyme that preferentially cleaves methylated DNA.

14. The method of claim 11, wherein the reagent comprises an enzyme that preferentially cleaves unmethylated DNA.

15. The method of claim 1, wherein the genetic marker does not exist in the maternal genome.

16. The method of claim 15, wherein the genetic marker is located on the Y chromosome.

17. The method of claim 1, wherein the genetic marker of fetal origin is distinguished from the genetic marker of maternal origin due to genetic polymorphism.

18. The method of claim 17, wherein the polymorphism comprises a single nucleotide polymorphism (SNP), simple tandem repeat polymorphism, or insertion-deletion polymorphism.

19. The method of claim 1, wherein the genetic marker is the *Zinc-Finger protein, Y-linked (ZFY)* gene.

20. The method of claim 1, wherein the genetic marker comprises SNP rs6636 in *TMED8* gene.

21. The method of claim 1, wherein more than one methylation marker or genetic marker is used.

22. The method of claim 1, wherein step (a) or (b) comprises amplification of the methylation marker or genetic marker.

23. The method of claim 22, wherein the amplification is by a polymerase chain reaction (PCR).

24. The method of claim 23, wherein the PCR is a methylation-specific PCR.

25. The method of claim 22, wherein the amplification is a nucleic acid sequence-specific amplification.

26. The method of claim 1, wherein step (a) or (b) is performed by molecular counting.

27. The method of claim 1, wherein step (a) or (b) comprises digital polymerase chain reaction, real-time quantitative polymerase chain reaction, array capture, a nucleic acid sequence-based detection, massively parallel genomic sequencing, single molecule sequencing, or multiplexed detection of polynucleotide with color-coded probe pairs.

28. The method of claim 1, wherein step (a) or (b) comprises mass spectrometry or hybridization to a microarray, fluorescence probe, or molecular beacon.

29. The method of claim 1, wherein the chromosome relevant to the chromosomal aneuploidy is chromosome 13, 18, 21, or X.

30. The method of claim 1, wherein the ratio being higher or lower than the standard control by at least 1 standard deviation indicates the presence of the chromosomal aneuploidy in the fetus.

31. The method of claim 1, wherein the ratio being higher or lower than the standard control by at least 2 standard deviations indicates the presence of the chromosomal aneuploidy in the fetus.

32. The method of claim 1, wherein the ratio being higher or lower than the standard control by at least 3 standard deviations indicates the presence of the chromosomal aneuploidy in the fetus.

Figure 1

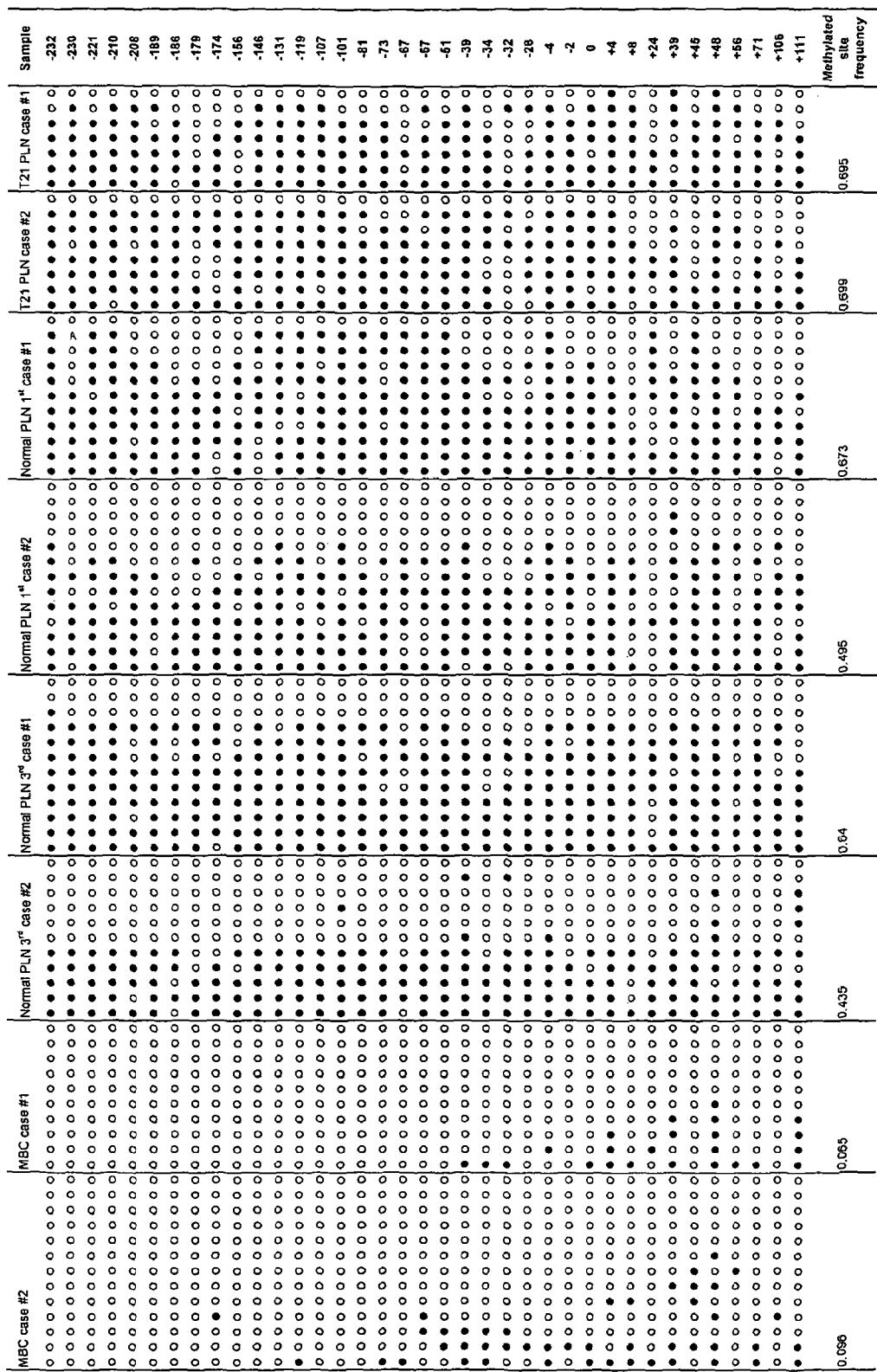
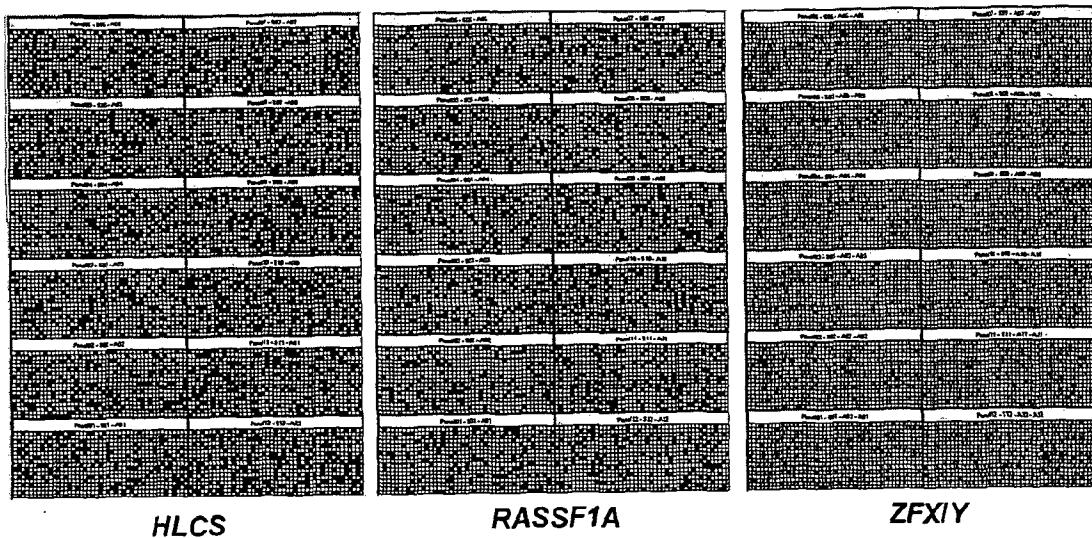


Figure 2

(A)



(B)

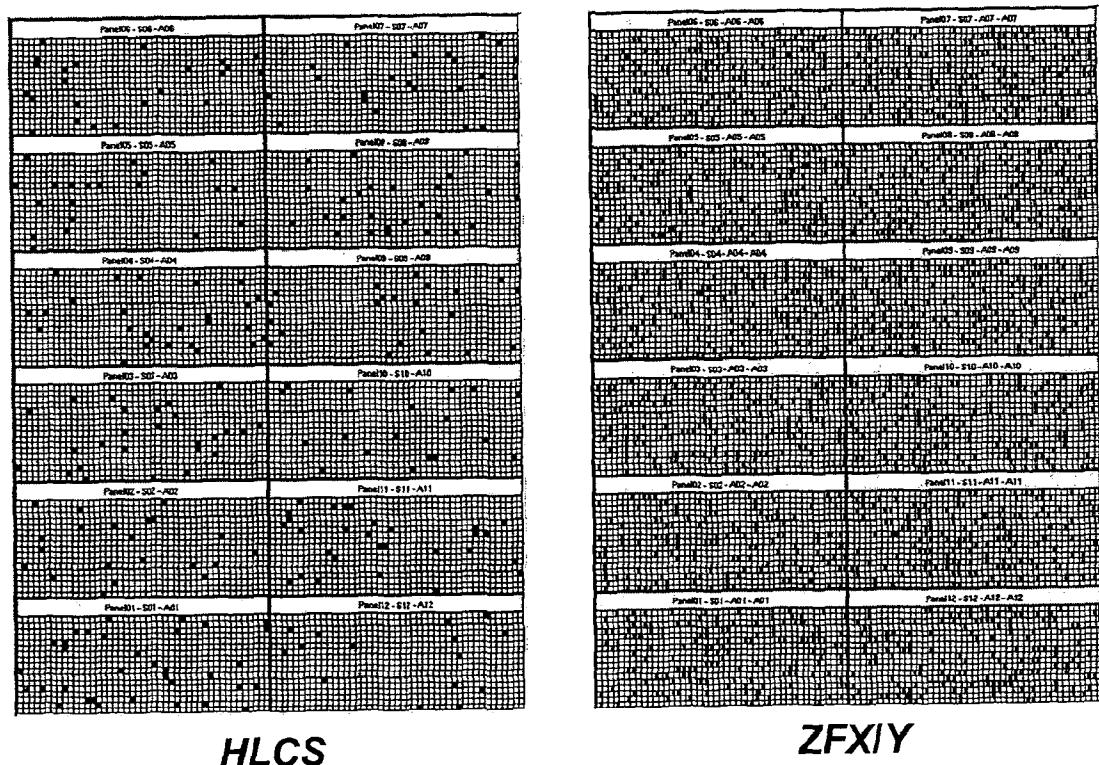
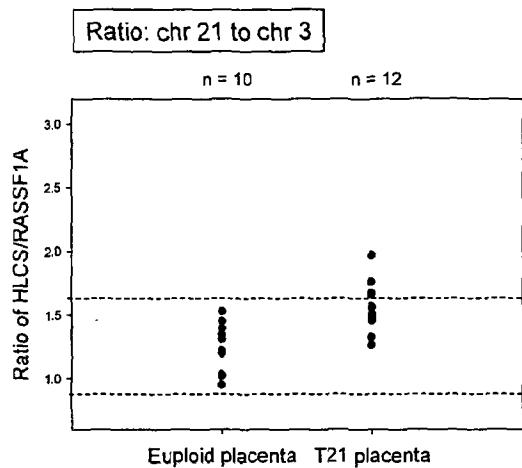
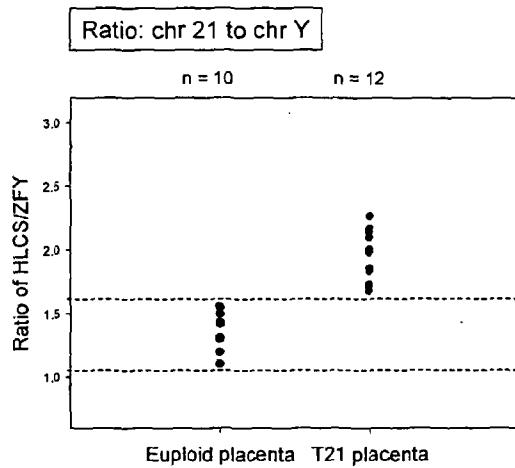


Figure 3**A**

----- Normal reference range: 0.86-1.63

P = 0.005, Mann-Whitney Rank Sum Test

B

----- Normal reference range: 1.08 – 1.62

P = <0.001, Mann-Whitney Rank Sum Test

Figure 4

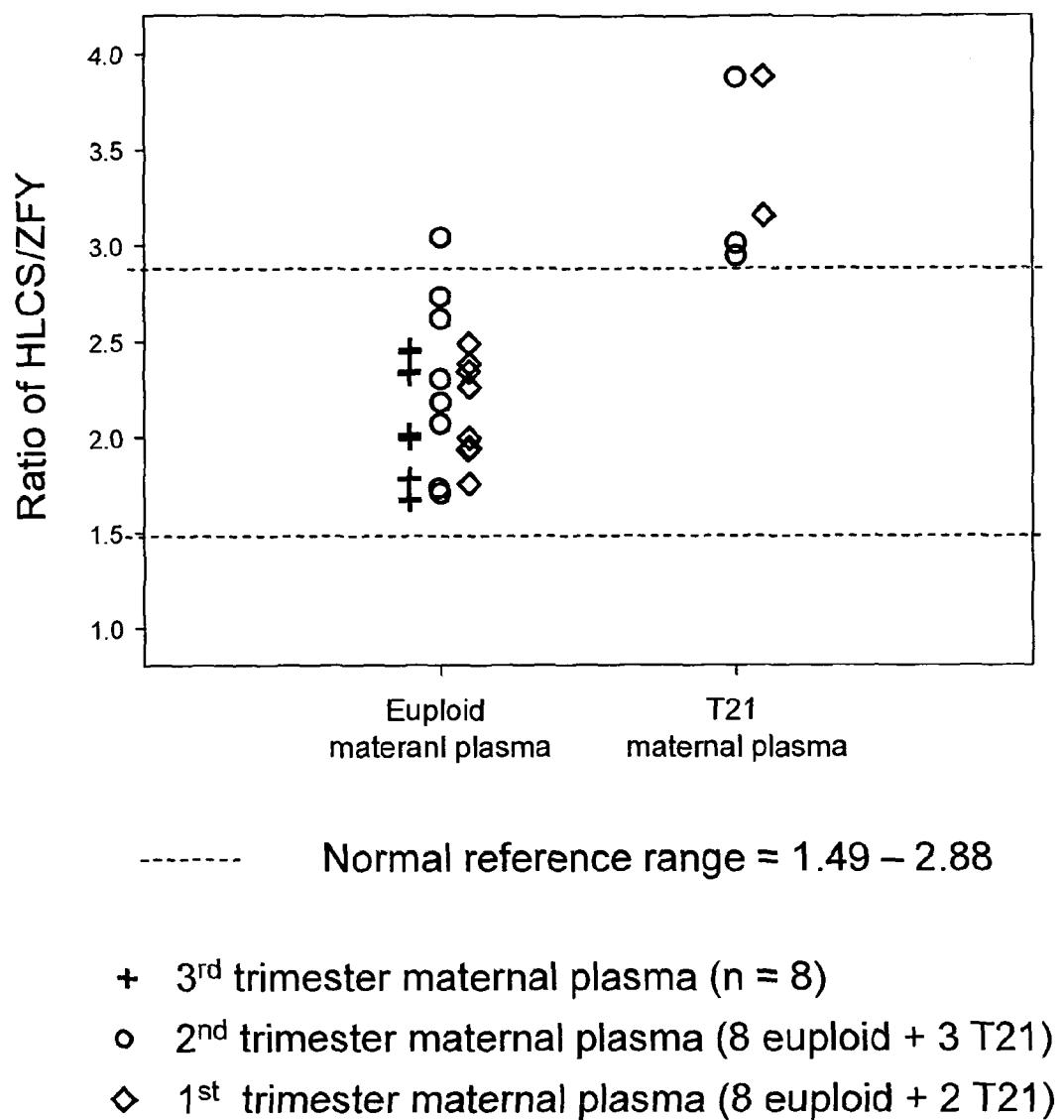


Figure 5***HLCS***

>chr21:37274960-37275055 96bp
CCGTGTGCCAGAGGTGgcagggcgcggcctgagcgggctggggcg
ggcaggatttggggctgcgccgaggggcTCCCGACCTGGCCCTT

RASSF1A

>chr3:50353111-50353206 96bp
AGCTGGCACCCGCTGGgcgcgcctggaaggccgacccggctggagcg
gccaaacgcgcctgcgcatcgcgggggcacCGCGTGCAAACCCACAC

ZFY

>chrY:2889345+2889431 87bp
CAAGTGCTGGACTCAGATGTACTGaagaagttttttaccacactgcac
agtcccagaTGATGTTTAGCTGACATTACTTCA

ZFX

>chrX:24107448+24107534 87bp
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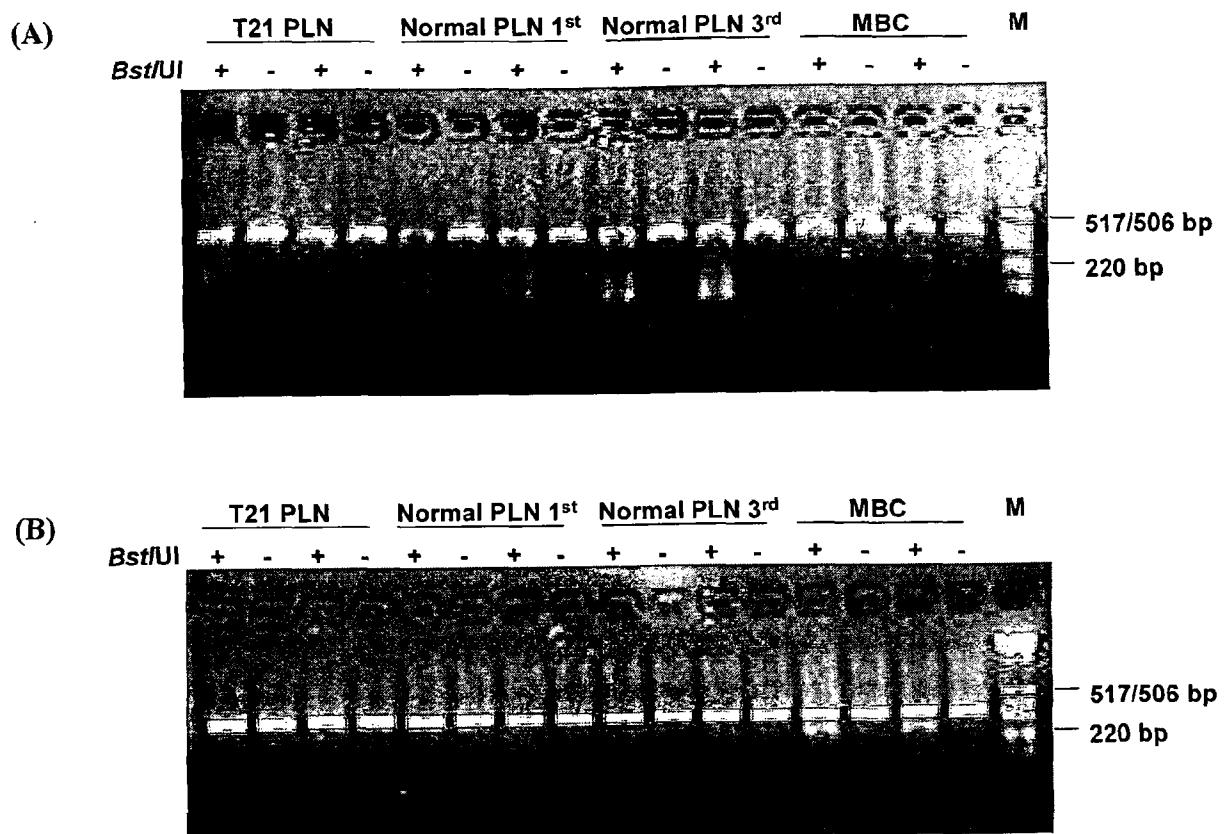
Figure 6

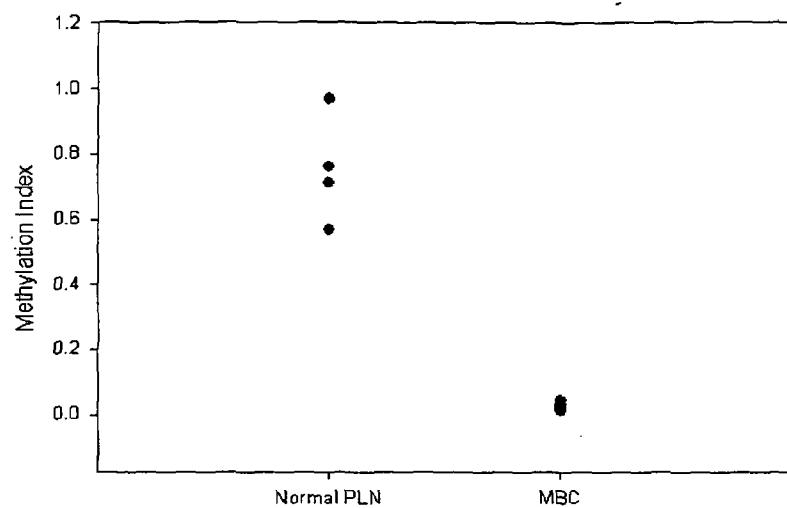
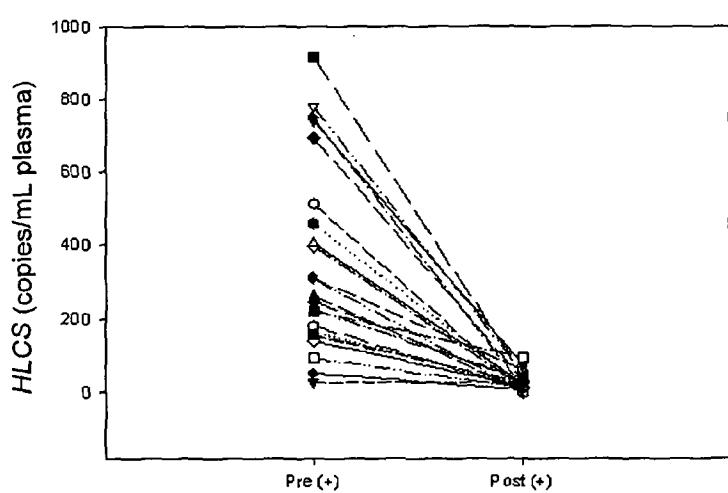
Figure 7

Figure 8

(A)



(B)

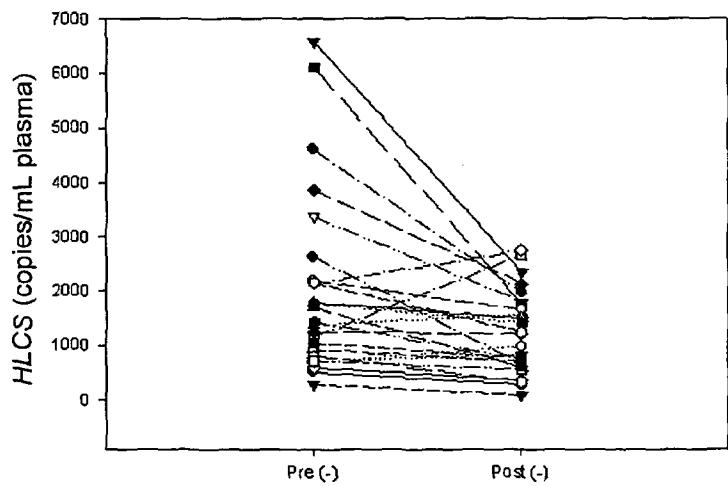
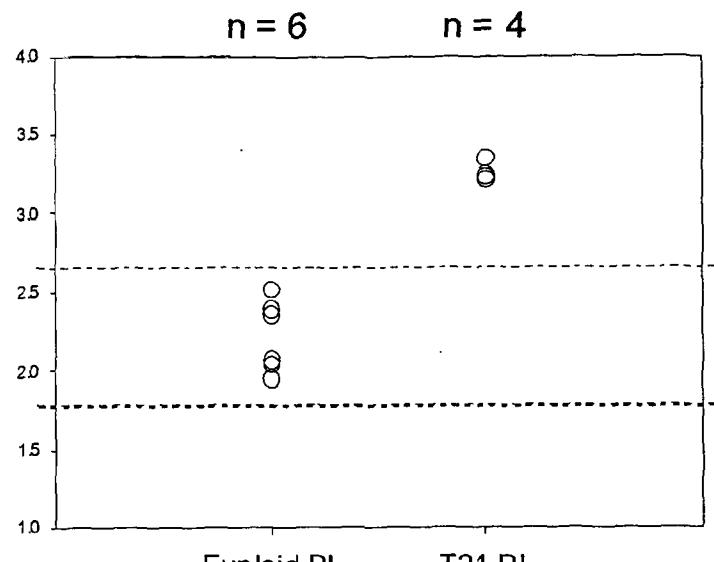
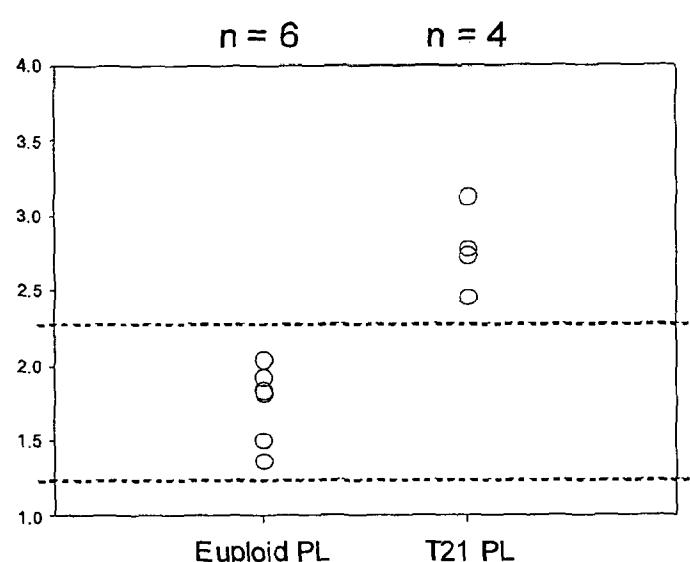


Figure 9**(A)**

Normal reference range = 1.78-2.66

(B)

Normal reference range = 1.24-2.26

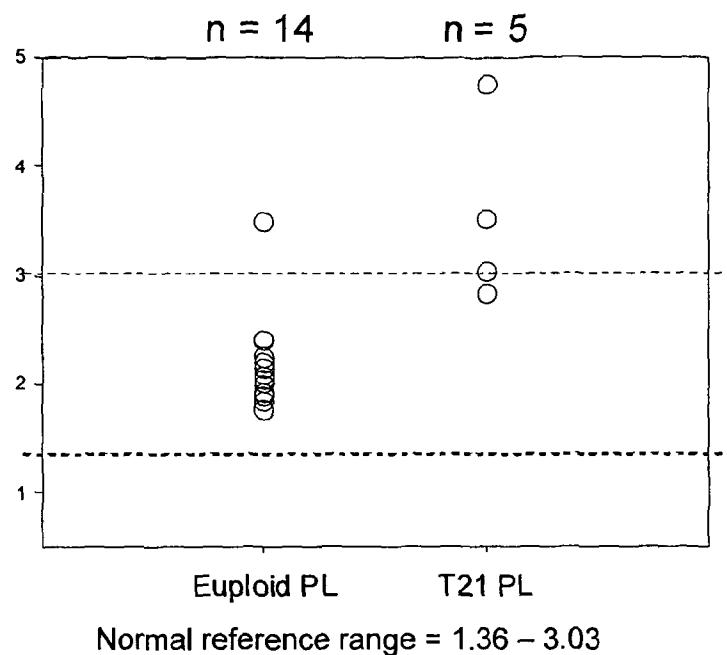
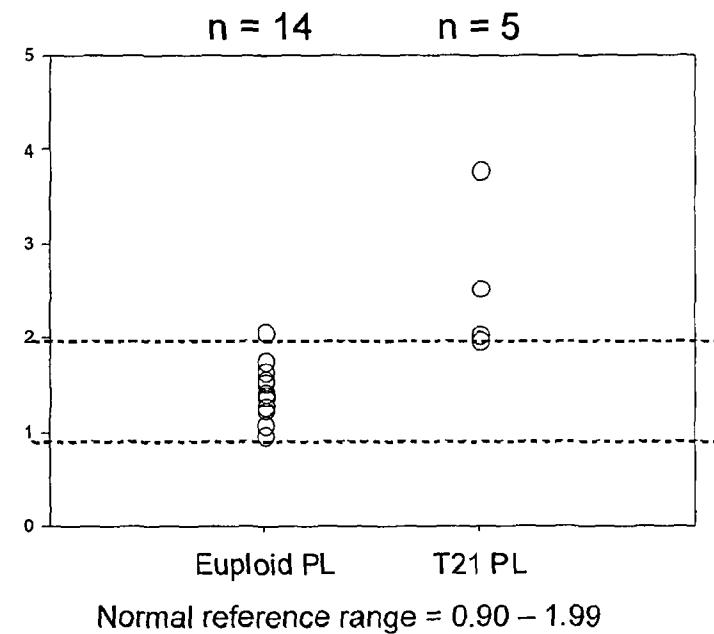
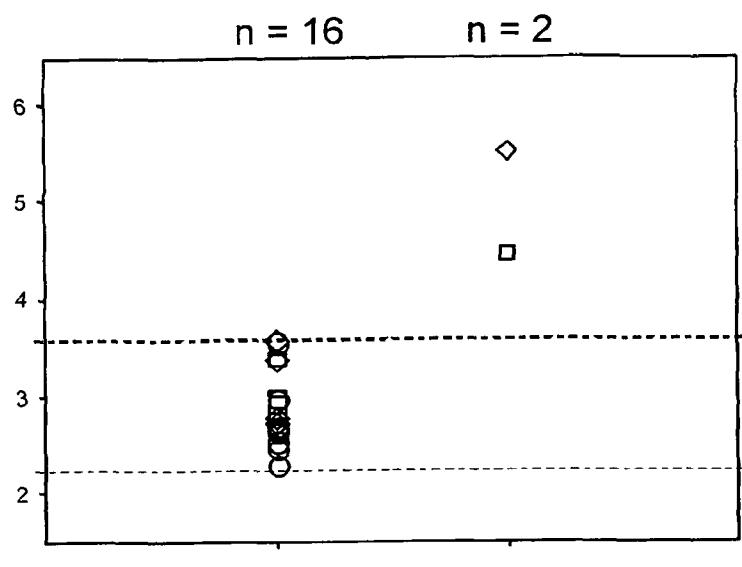
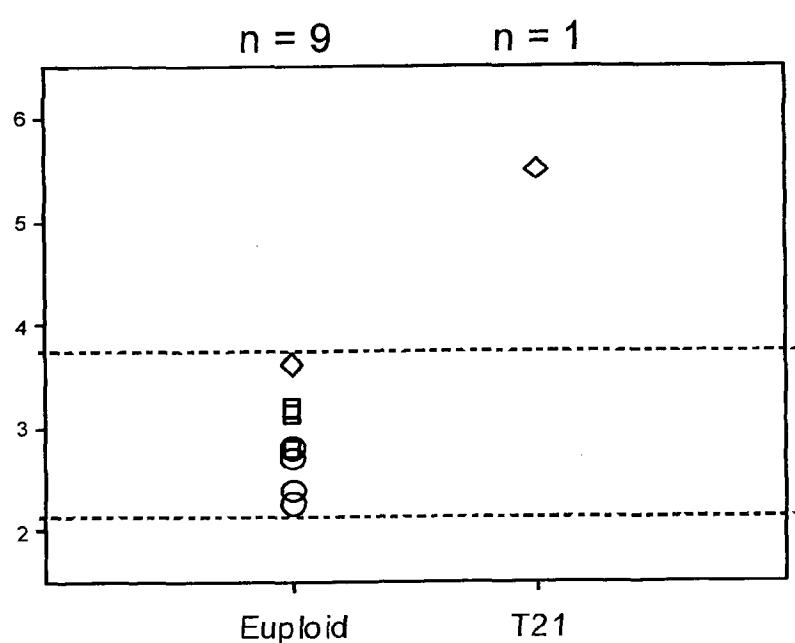
Figure 10**(A)****(B)**

Figure 11**(A)**

Normal reference range = 2.12 – 3.65

(B)

Normal reference range = 2.06 – 3.68

○ 3rd-trimester maternal plasma□ 2nd-trimester maternal plasma◇ 1st-trimester maternal plasma

Figure 12

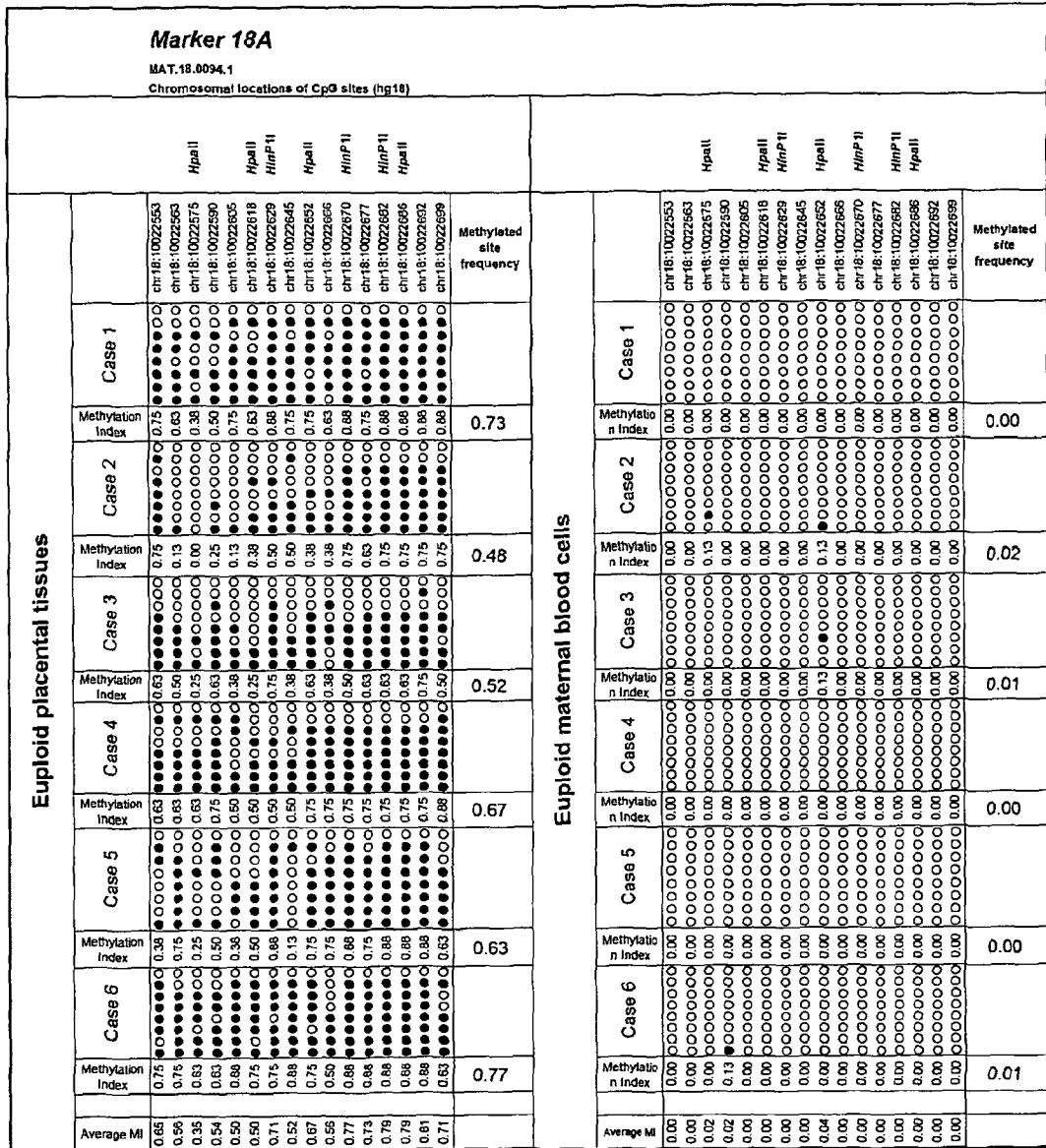


Figure 13

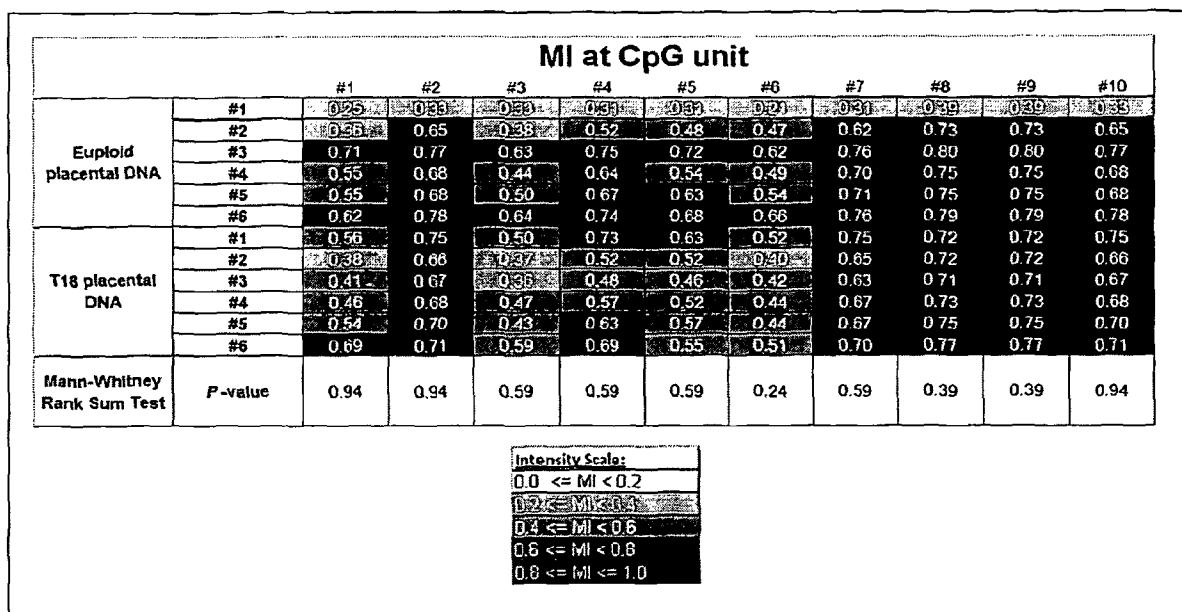


Figure 14A

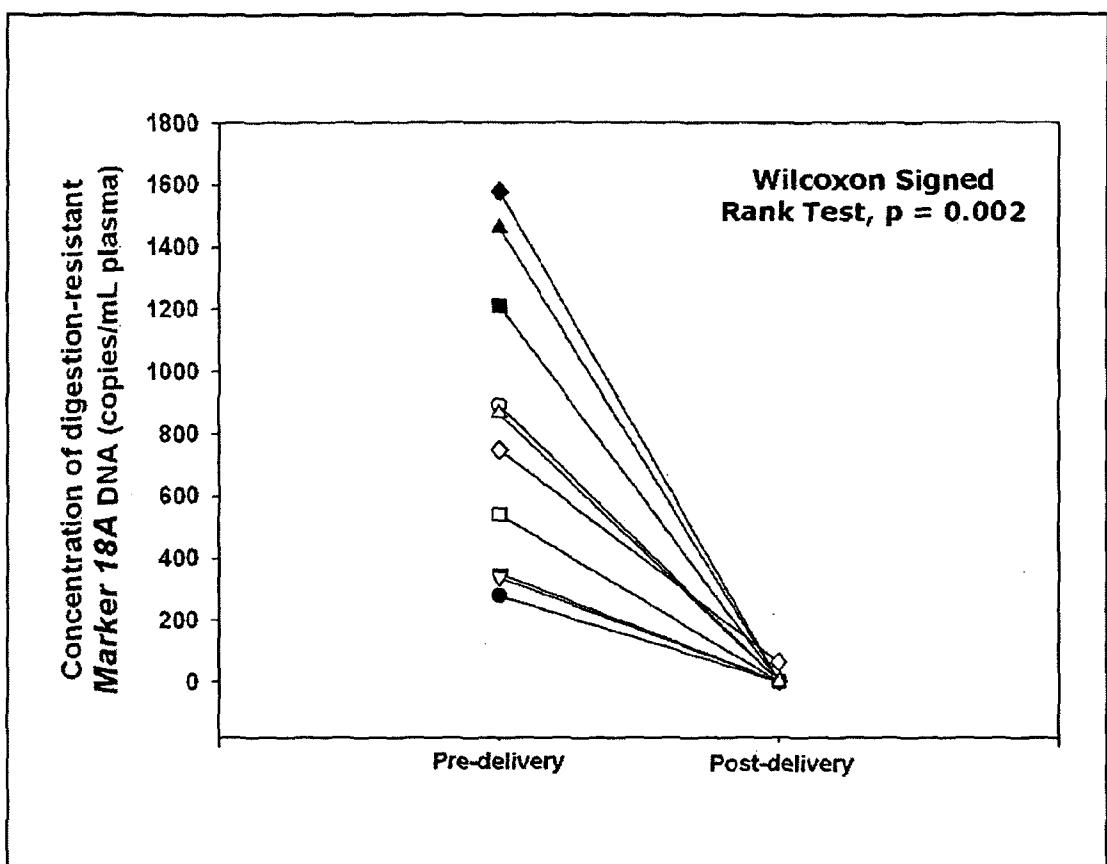


Figure 14B

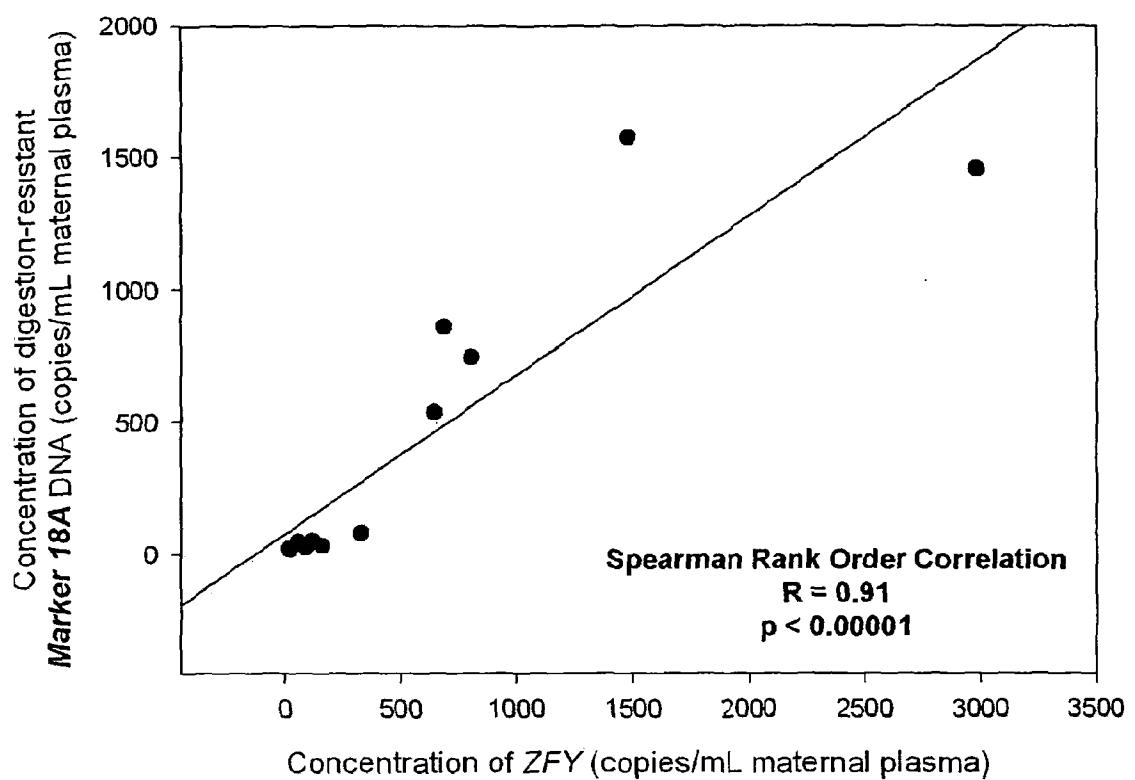


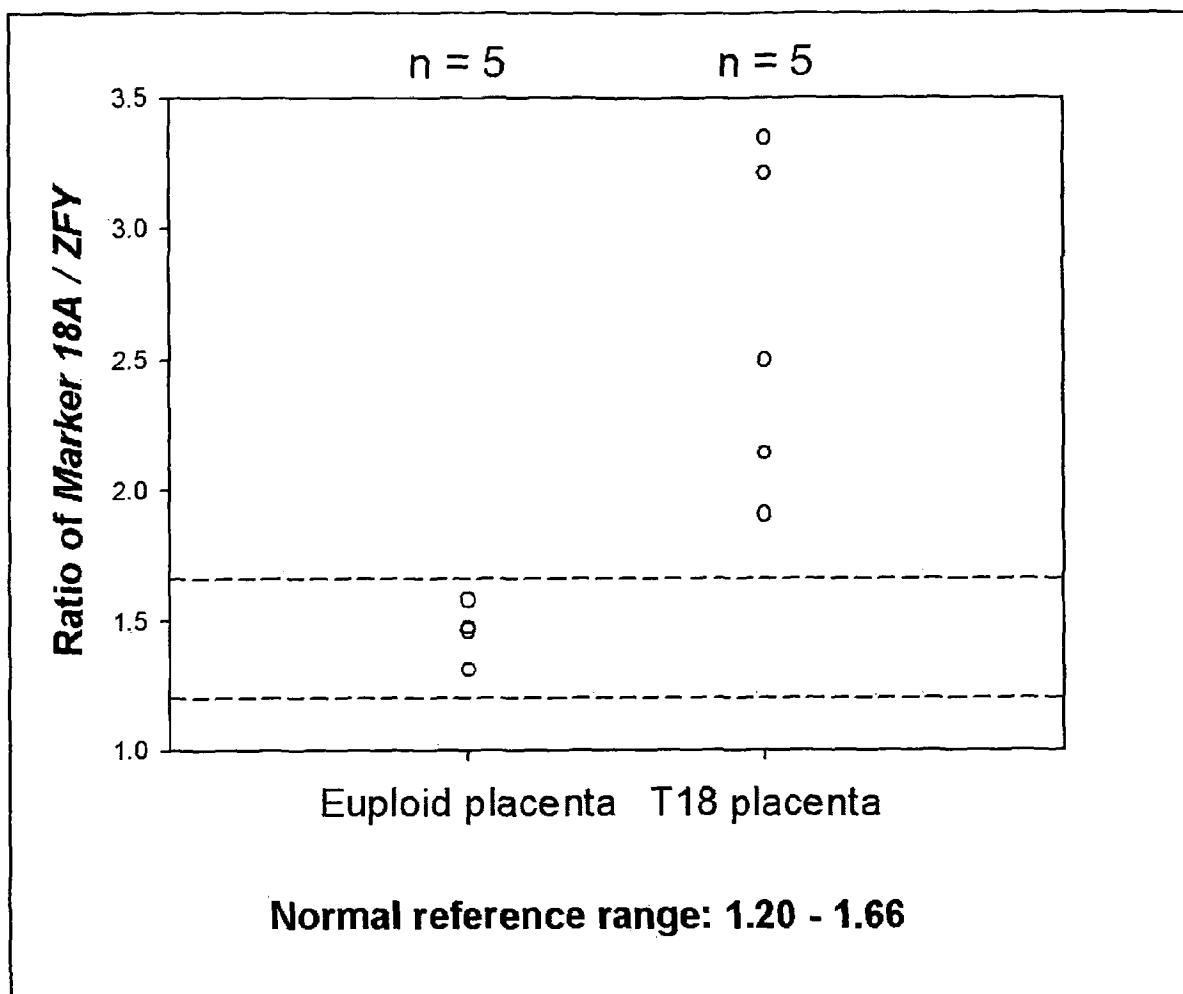
Figure 15

Figure 16

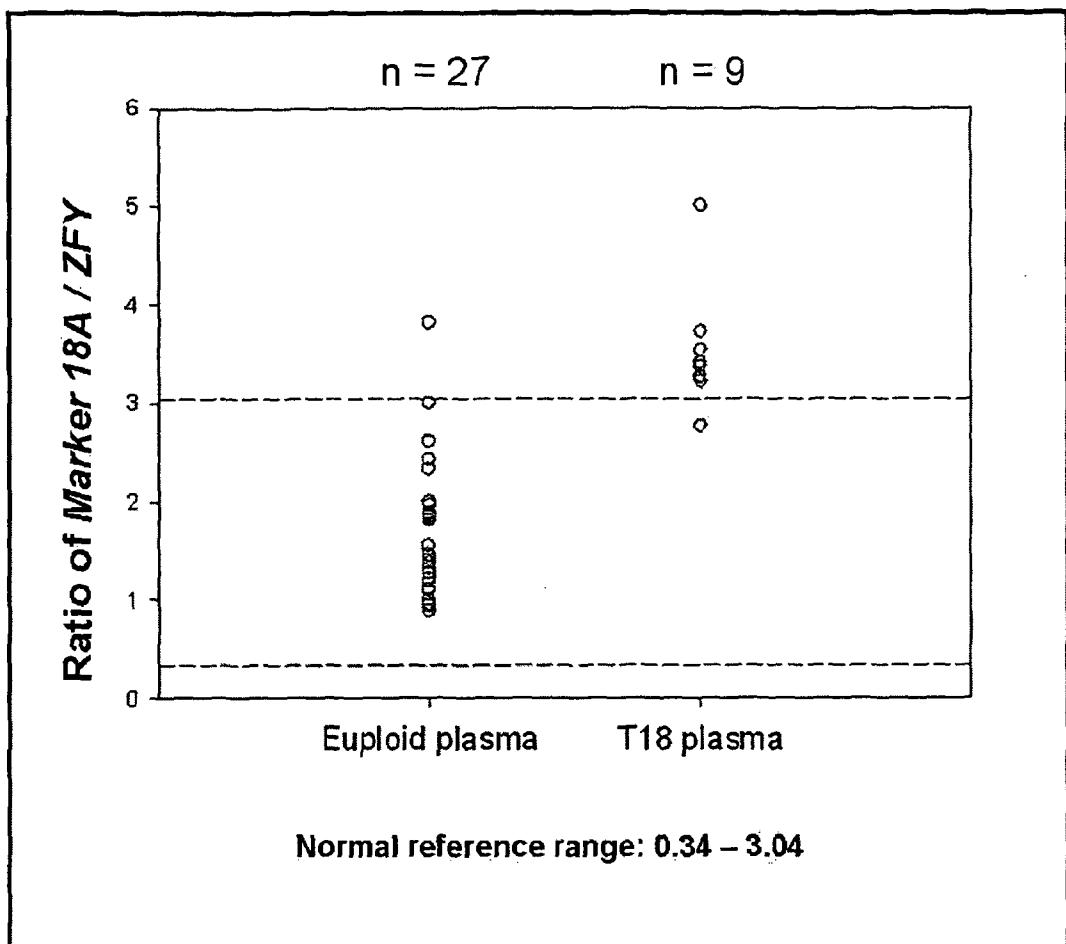
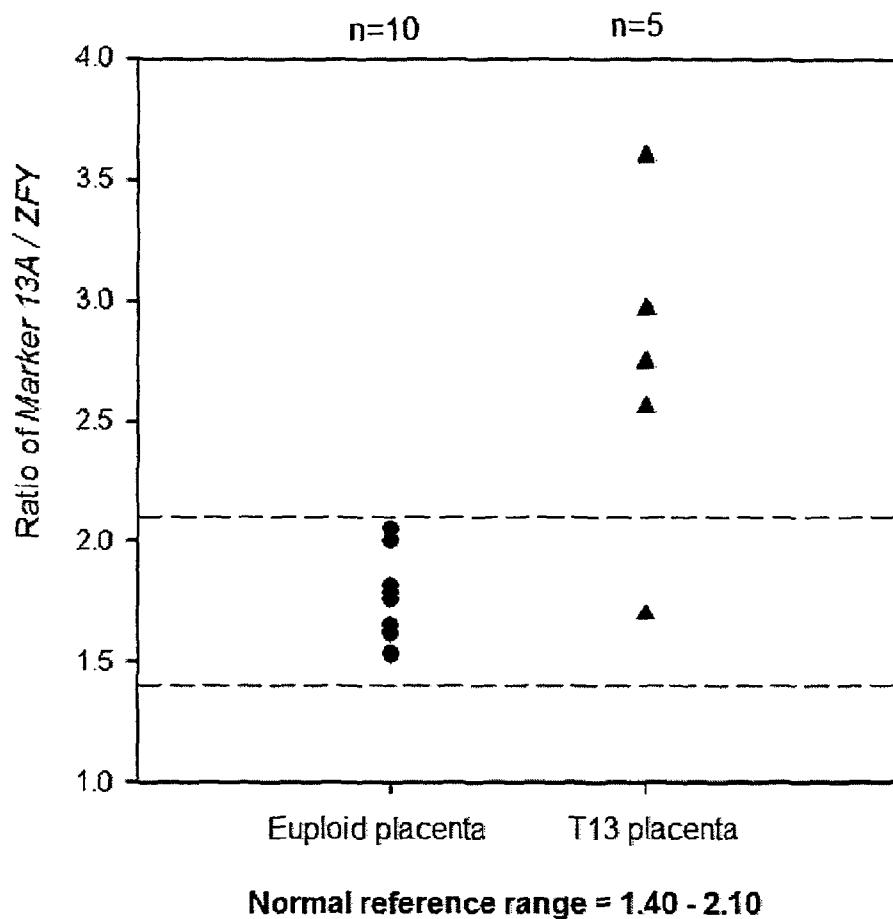


Figure 17

Figure 18

Figure 19

MI at CpG unit						
	#1	#2	#3	#4	#5	
Euploid placental DNA	#1	0.53	0.70	0.87	0.68	0.70
	#2	0.41	0.52	0.73	0.63	0.52
	#3	0.71	0.76	0.84	0.73	0.76
	#4	0.52	0.70	0.78	0.67	0.70
	#5	0.53	0.69	0.82	0.66	0.69
T13 placental DNA	#1	0.51	0.76	0.85	0.63	0.76
	#2	0.45	0.86	0.85	0.69	0.86
	#3	0.44	0.74	0.91	0.59	0.74
	#4	0.52	0.73	0.80	0.67	0.73
	#5	0.63	0.79	0.86	0.75	0.79
Mann-Whitney Rank Sum Test	p -value	0.42	0.03	0.22	1.00	0.03

Figure 20

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2010/001327

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

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

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

EPO-Internal, BIOSIS, MEDLINE, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2005/035725 A2 (UNIV BOSTON [US]; CANTOR CHARLES R [US]; DING CHUNMING [US]) 21 April 2005 (2005-04-21) paragraph [0025] – paragraph [0103]; claims 1,3,21 ----- -----	1-7, 11-14, 18,21-32
X	WO 2007/132167 A2 (UNIV HONG KONG CHINESE [CN]; LO YUK MING DENNIS [CN]; CHIU ROSSA WAI K) 22 November 2007 (2007-11-22) the whole document ----- -----	1-32

Further documents are listed in the continuation of Box C.

See patent family annex.

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Date of the actual completion of the international search 6 October 2010	Date of mailing of the international search report 19/10/2010
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Botz, Jürgen

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2010/001327

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TONG YU K ET AL: "Noninvasive prenatal detection of fetal trisomy 18 by epigenetic allelic ratio analysis in maternal plasma: Theoretical and empirical considerations" CLINICAL CHEMISTRY, vol. 52, no. 12, December 2006 (2006-12), pages 2194-2202, XP002603754 ISSN: 0009-9147 the whole document -----	1-32
X	WO 2007/103910 A2 (UNIV COLUMBIA [US]; BROWN STEPHEN [US]) 13 September 2007 (2007-09-13) the whole document -----	1-32
A	WO 2006/097049 A1 (UNIV HONG KONG CHINESE [CN]; UNIV BOSTON [US]) 21 September 2006 (2006-09-21) the whole document -----	1-32

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/GB2010/001327

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