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(54) **Title:** METHODS OF CANCER DETECTION USING EXTRAEMBRYONICALLY METHYLATED CPG ISLANDS

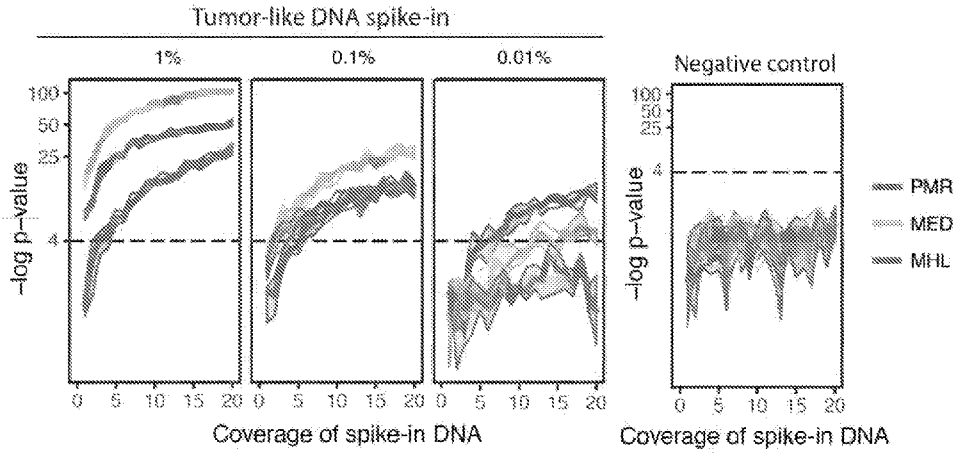

FIG. 2C

(57) **Abstract:** The present invention relates to methods of characterizing cell-free DNA (cfDNA), detecting cancer, detecting the eradication of cancer, and determining a probability distribution of haplotypes. The methods use the data from genomic sequences from CpG Islands (CGI) methylated in the genome of extraembryonic ectoderm (ExE) to determine a proportion of fully methylated haplotypes in order to characterize the cfDNA sample and detect certain cancers.

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METHODS OF CANCER DETECTION USING EXTRAEMBRYONICALLY METHYLATED CPG ISLANDS

RELATED APPLICATION(S)

[0001] This application claims priority to U.S. Provisional Application No. 63/126,863, filed on December 17, 2020, and U.S. Provisional Application No. 63/246,306, filed on September 20, 2021, the entire teachings of which are incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

[0002] The overwhelming majority of cancer-related deaths result from complications of metastatic disease. Modern anti-cancer therapies generally fail on metastatic disease due to tumor evolution [1], allowing heterogeneous cancer cell populations to acquire novel traits that enable them to escape from therapies, colonize new sites, and become more aggressive over time. Early diagnosis of disease leads to much-improved prognosis compared to advanced stage disease and can be based on imaging- or blood-based testing [2]. Although serum-based protein biomarkers such as carcinoma antigen-125 (CA-125) [3], carcinoembryonic antigen (CEA) [4], and prostate-specific antigen (PSA) [5] have been used to track the progression of specific cancer types, they lack the sensitivity and specificity necessary for detection of early stage diseases.

[0003] Liquid biopsies based on the analysis of cell-free DNA (cfDNA) have received much interest due to their promise to identify cancer-causing mutations in the plasma of patients with early stage disease. However, inter- and intra-tumor heterogeneity limit the sensitivity of these methods since recurrent clonal mutations are rare. More recent advances are based on methylation profiling of cfDNA in order to detect and classify reads stemming from a certain tumor type. These approaches are promising but need to be optimized for each tumor type. There is, therefore, a need to provide innovative methods for cancer detection with higher sensitivity due to tumor heterogeneity.

SUMMARY OF THE INVENTION

[0004] Cancer screening methods were discovered by detecting certain pan-cancer methylation signatures of cfDNA. Specifically, the pan-cancer methylation signature is based on loci preferentially methylated in extraembryonic ectoderm that is different from epiblast and that is present across most human cancer types.

[0005] Based on these findings, an ultra-sensitive identification of tumor-derived cfDNA was developed that allows non-invasive early diagnosis of human cancer. Computation analysis of methylation haplotypes identified from individual bisulfite-converted reads reduced background signal stemming from normal cell types. The result provides an ability to detect the extraembryonic methylation signature in plasma samples of patients with various stages of cancerous disease. The present invention improves over previous screening methods by providing an ultra-sensitive, non-invasive pan-cancer diagnosis of disease based on plasma cell-free methylation patterns.

[0006] In an embodiment, the invention is directed to a method of characterizing a cell-free DNA (cfDNA) sample from a subject, comprising receiving sequencing data comprising reads of methylation sequences for a genomic sequence from the cfDNA sample, wherein the genomic sequence comprises a plurality of CpG Islands (CGI) methylated in the genome of extraembryonic ectoderm (ExE) and not methylated in corresponding epiblast or adult tissue, determining a proportion of haplotypes of the genomic sequence that are fully methylated, and characterizing the cfDNA sample as comprising fully methylated cfCDNA if the proportion of haplotypes is greater than a significance threshold.

[0007] In certain embodiments, each haplotype comprises five CGI methylated in the genome of ExE and not methylated in corresponding epiblast or adult tissue. In certain embodiments, the cfDNA sample comprises between 0.01% and 0.1% tumor DNA. In certain embodiments, the sequencing data comprises sequence information for less than 0.3% of the genome of the subject. In certain embodiments, the sequencing data comprises sequence information substantially limited to one or more regions of the subject's genome having a plurality of CGI methylated in the genome of ExE and not methylated in corresponding epiblast or adult tissue. In certain embodiments, the fully methylated haplotypes are compared to one or more pre-established fully

methylated haplotype signatures and the cfDNA sample is further characterized as corresponding or not corresponding to the pre-established fully methylated haplotype signature. In certain embodiments, the pre-established fully methylated haplotype signature has been identified via a method comprising random forest, support vector machine, or deep learning analysis. In certain embodiments, the sequencing data comprising reads of methylation sequences for a genomic sequence from the cfDNA sample has been enriched for sequences comprising methylation. In certain embodiments, the enrichment comprises an MBD2 protein-based enrichment method. In certain embodiments, the cfDNA sample was obtained from plasma, urine, stool, menstrual fluid, or lymph fluid. In some embodiments, the method further comprises a step of determining a tissue of origin from the sequencing data.

[0008] In an embodiment, the invention is directed to a method for detecting cancer in a subject, comprising receiving sequencing data comprising reads of methylation sequences for a genomic sequence from a cfDNA sample from the subject, wherein the genomic sequence comprises a plurality of CpG Islands (CGI) methylated in the genome of extraembryonic ectoderm (ExE) and not methylated in corresponding epiblast or adult tissue, determining a proportion of haplotypes of the genomic sequence that are fully methylated, and detecting cancer in the subject if the proportion of fully methylated haplotypes is greater than a significance threshold.

[0009] In certain embodiments, each haplotype comprises five CGI methylated in the genome of ExE and not methylated in corresponding epiblast or adult tissue. In certain embodiments, the cfDNA sample comprises between 0.01% and 0.1% tumor DNA. In certain embodiments, the sequencing data comprises sequence information for less than 0.3% of the genome of the subject. In certain embodiments, the sequencing data comprises sequence information substantially limited to one or more regions of the subject's genome having a plurality of CGI methylated in the genome of ExE and not methylated in corresponding epiblast or adult tissue. In certain embodiments, the fully methylated haplotypes are compared to one or more pre-established fully methylated haplotype signatures corresponding to one or more tumor types, and the presence or absence of the one or more tumor types are detected in the subject.

[0010] In certain embodiments, the one or more tumor types comprise one or more of acute myeloid leukemia, bladder cancer, breast cancer, colon cancer, esophageal cancer, kidney

cancer, liver cancer, lung cancer, ovarian cancer, pancreatic cancer, prostate cancer, or stomach cancer. In certain embodiments, the pre-established fully methylated haplotype signatures corresponding to one or more tumor types have been identified via a method comprising random forest, support vector machine, or deep learning analysis. In certain embodiments, the sequencing data comprising reads of methylation sequences for a genomic sequence from the cfDNA sample has been enriched for sequences comprising methylation. In certain embodiments, the enrichment comprises an MBD2 protein-based enrichment method. In certain embodiments, the cfDNA sample was obtained from plasma, urine, stool, menstrual fluid, or lymph fluid. In certain embodiments, the presence of cancer is detected in the sample with 100% sensitivity and 95% specificity. In certain embodiments, the cancer is stage I or stage III. In certain embodiments, the cancer is selected from the group comprising adenocarcinoma, acute myeloid leukemia, bladder cancer, breast cancer, colon cancer, esophageal cancer, kidney cancer, liver cancer, lung cancer, ovarian cancer, pancreatic cancer, prostate cancer, stomach cancer, and uterine cancer. In certain embodiments, the method further comprises a step of treating the subject for cancer when cancer is detected in the subject. In some embodiments, the method further comprises a step of determining a tissue of origin from the sequencing data.

[0011] In an embodiment, the invention is directed to a method of detecting eradication of cancer from a subject, comprising receiving sequencing data comprising reads of methylation sequences for a genomic sequence from a cfDNA sample from a subject after a cancer treatment, wherein the genomic sequence comprises a plurality of CGIs methylated in the genome of ExE and not methylated in corresponding epiblast or adult tissue, determining a proportion of haplotypes of the genomic sequence that are fully methylated, and detecting cancer in the subject if the proportion of fully methylated haplotypes is greater than a significance threshold, wherein if cancer is not detected in the subject then the cancer has been eradicated from the subject.

[0012] In certain aspects, the genomic sequence comprises a contiguous sequence of about 8 megabases of the human genome comprising a plurality of CGIs methylated in the genome of ExE. In certain embodiments, the genomic sequence comprises a contiguous sequence of about 8 megabases of the human genome comprising a plurality of CGIs methylated in the genome of extraembryonic ectoderm (ExE). In certain embodiments, the genomic sequence comprises 50-75 CGIs methylated in the genome of ExE. In certain embodiments, the genomic sequence

comprises a contiguous sequence of about 8 megabases of the human genome comprising a plurality of CGIs methylated in the genome of ExE. In certain embodiments, the genomic sequence comprises 50-75 CGIs methylated in the genome of ExE. In certain aspects, the genomic sequence comprises one or more sequences provided in Table 3.

[0013] In an embodiment, the invention is directed to a method of determining a probability distribution of haplotypes comprising receiving sequencing data comprising reads of methylation sequences for a genomic sequence from the cfDNA sample, wherein the genomic sequence comprises a plurality of CpG Islands (CGI) methylated in the genome of extraembryonic ectoderm (ExE) and not methylated in corresponding epiblast or adult tissue, assigning a training or validation set based on the methylated ExE CGI data applying a machine learning method to estimate the probability distribution of all haplotypes across ExE sites, and determining one or more classifications of tumor versus normal samples based on a prediction score obtained from the machine learning method.

[0014] In certain embodiments, the machine learning method is random forest. In certain embodiments, the machine learning method is a support vector machine. In certain embodiments, the machine learning method is deep learning. In certain embodiments, the method further comprises the method step of evaluating the performance of the prediction comprising performing an in silico simulation by comparing randomly sampled sequencing reads from epiblast or adult tissue with the ExE reads. In some embodiments, the method further comprises a step of determining a tissue of origin from the sequencing data.

[0015] Some aspects of the present disclosure are directed to a method of determining a tissue origin comprising receiving targeted bisulfite sequencing data comprising reads of methylation sequences for a genomic sequence from a cfDNA sample, wherein the genomic sequence comprises a plurality of CpG Islands (CGI) methylated in the genome of extraembryonic ectoderm (ExE) and not methylated in corresponding epiblast or adult, and determining a tissue of origin by calculating a relative abundance of haplotypes from the methylated genomic regions by defining a tissue-specific index (TSI) for each haplotype. In some embodiments, the TSI is calculated by the formula:

$$TSI = \frac{\sum_{j=1}^{n} 1 - \frac{10^{PKR(j)}}{10^{PKR(\max)}}}{n-1}$$

wherein n is the number of tissues, PKR(j) is the fraction of a specific haplomer in tissue, and j and PKR max are PKR of the highest methylated tissue. In some embodiments, the sequencing data comprises one or more sequences provided in Table 2.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] FIG. 1A shows Mouse E6.5 conceptus that was used to characterize DNA methylation landscapes of embryonic and extraembryonic tissues by comparing Epiblast and ExE (Extraembryonic Ectoderm).

[0017] FIG. 1B shows ExE Hyper CGIs that are genetically more conserved. Mean conservation scores (phyloP30-way) were plotted as a function of distance to center of CGIs. Only CGIs that are close to TSS (+/- 2000 bp) were included.

[0018] FIG. 1C shows mouse ExE hyper CGIs that were lifted over to orthologous CGIs in human.

[0019] FIG. 1D shows ExE hyper CGIs that accurately differentiate cancer from normal samples. 13 TCGA cancer types that contain matched normal tissues were used to test performance of ExE hyper CGIs in cancer prediction. Half samples were randomly chosen to be trained by SVM with Gaussian kernel, the resulting model was used to predict the rest half samples either as tumor or normal. The results were presented as a ROC curve and the area under curve (AUC) is shown.

[0020] FIG. 1E shows cancer is genetically heterogeneous and epigenetically homogeneous. The results from FIG. 1D are further summarized to show the fraction of samples in each cancer type that was correctly predicted by ExE hyper CGIs. In parallel, the fraction of samples that contain TP53 mutations are also shown.

[0021] FIG. 2A shows an illustration of DNA methylation haplotypes. The methylation pattern of CpGs on each sequencing fragment represents a discrete DNA methylation haplotype, which can be classified as unmethylated reads, discordant reads, or fully methylated reads. Proportion of fully methylated reads (PMR) is defined as fraction of fully methylated reads.

[0022] FIG. 2B shows that using proportion of fully methylated reads (PMR) significantly reduces background noise in normal cells. Sequencing reads from public WGBS data at OTX2 locus were aggregated to increase coverage for tumor and normal samples, respectively.

[0023] FIG. 2C shows an in silico simulation. Sequencing reads from ExE (tumor-like) were spiked into reads from Epiblast (normal-like). The fraction of ExE-derived reads represent 1%, 0.1% or 0.01% in three sets, respectively. In negative controls, all reads were randomly sampled from Epiblast. Prediction results were shown for PMR, MHL and mean methylation-based methods.

[0024] FIG. 3A shows a general workflow of targeted bisulfite sequencing used. MBD enrichment is optional but could be used to specifically enrich methylated reads.

[0025] FIG. 3B shows evenness of hybrid capture. On-target coverage was normalized by mean coverage in designed regions. This curve describes the fraction of loci that have coverage higher than pre-defined threshold.

[0026] FIG. 3C shows efficiency of targeted sequencing. To assess efficiency of targeted sequencing, the same biological sample was profiled by WGBS and targeted BS. Normalized coverages were shown as a function of distance to center of designed CGIs.

[0027] FIG. 3D shows enrichment of methylated haplotypes by proteins with methyl-CpG binding domain (MBD). Enrichment efficiency is measured by proportion of methylated reads.

[0028] FIG. 4A shows the correlation of normalized counts between two assays, targeted-BS with and without MBD enrichment. Targeted-BS was performed on 4 samples (HuES64, HCT116, normal uterus and uterus cancer) in two conditions, with or without MBD enrichment. Correlation of normalized counts between two assays were assessed for each type of DNA

methylation haplotye. All 32 DNA methylation haplotyes were grouped into 6 classes based on length of fully methylated *k*-mers.

[0029] FIG. 4B shows normalized coverage of fully methylated reads that were compared between two assays, targeted BS with and without MBD enrichment, for uterus cancer and uterus normal. Pearson correlation coefficient is also shown in the figure.

[0030] FIG. 4C shows normalized coverage of fully methylated reads were compared between two assays, targeted-BS and WGBS, for uterus cancer and normal uterus. Pearson correlation coefficient is also shown in figure.

[0031] FIG. 5A shows ultra-sensitive detection of cancer in a dilution sample of HuES64 DNA mixed with HCT116.

[0032] FIG. 5B shows ultra-sensitive detection of cancer in a dilution sample of HuES64 DNA mixed with colon cancer DNA spike-in.

[0033] FIG. 5C shows ultra-sensitive detection of cancer in a dilution sample of normal uterus DNA mixed with uterus cancer DNA spike-in. Fractions of spike-in in all three experiments include 1%, 0.1% and 0.01%. NMR-based was used to predict the presense of spike-in using increasing numbers of top ranking markers.

[0034] FIG. 6 shows ExE hyper CGIs accurately differentiate cancer from normal samples. 13 TCGA cancer types that contain matched normal tissues were used to test performance of ExE hyper CGIs in cancer prediction. The pan-cancer cohort consists of 685 tumor samples and 710 normal samples, which were subdivided into a training and a validation set with equal sample size. Random forest (RF) was implemented using the 'randomForest' function of the 'randomForest' R package, using default parameter settings. False positive rate and true positive rate were calculated using the 'roc' function of the 'pROC' R package, based on the 'out-of-bag' votes for the training data. RF was able to classify tumor samples with high specificity, and high sensitivity (AUC = 0.98).

[0035] FIG. 7 shows a comparison of proportion of fully methylated reads (PMR) with three other metrics used in the literature. Five patterns of methylation haplotype combinations

(schematic) are used to illustrate the differences between methylation frequency, number of haplotypes, methylation haplotype load (MHL), and PMR.

[0036] FIG. 8 shows a schematic illustration of the method for quantification DNA methylation by PMR. Sixteen DNA methylation haplotypes were shown to represent schematic sequencing reads aligned to a locus. For a DNA methylation haplotype, fully methylated *k*-mers and total number *k*-mers were counted for a given width of k-mer. PMR is then defined as proportion of fully methylated *k*-mer across all reads aligned in a locus.

[0037] FIG. 9A shows cancer prediction using mean methylation on simulated data. To evaluate the performance of mean methylation in terms of cancer prediction, in silico simulations were performed by randomly sampling sequencing reads from normal-like tissue epiblast as well as tumor-like tissue ExE as a spike-in. The fraction of spike-in ranged from 0.01% to 1%, which matches the fraction of ctDNA in cell-free DNA. ExE was compared to epiblast to identify CGIs that have higher mean methylation in ExE, as indicated in red.

[0038] FIG. 9B shows simulated samples that were compared to epiblast using CGIs defined in previous step, the resulting mean methylation difference was represented as a boxplot for each spike-in group.

[0039] FIG. 9C shows the number of CGIs with increased or decreased mean methylation that were counted, respectively, and significance p-value that was estimated by one-sided binomial test to predict presence of ExE DNA.

[0040] FIG. 10A shows cancer prediction using MHL on simulated data within silico simulations by randomly sampling sequencing reads from normal-like tissue epiblast as well as tumor-like tissue ExE as a spike-in to evaluate the performance of MHL in terms of cancer prediction. The fraction of spike-in ranged from 0.01% to 1%, which matches the fraction of ctDNA in cell-free DNA. ExE was compared to epiblast to identify CGIs that have higher MHL in ExE, as indicated in red.

[0041] FIG. 10B shows simulated samples that were compared to epiblast using CGIs defined in previous step, the resulting MHL difference was represented as a boxplot for each spike-in group.

[0042] FIG. 10C shows the number of CGIs with increased or decreased MHL that were counted, respectively, and significance p-value that was estimated by one-sided binomial test to predict presence of ExE DNA.

[0043] FIG. 11A shows in silico simulations were performed by randomly sampling sequencing reads from normal-like tissue epiblast as well as tumor-like tissue ExE as a spike-in to evaluate the performance of PMR in terms of cancer prediction. The fraction of spike-in ranged from 0.01% to 1%, which matches the fraction of ctDNA in cell-free DNA. ExE was compared to epiblast to identify CGIs that have higher PMR in ExE, as indicated in red.

[0044] FIG. 11B shows simulated samples that were compared to epiblast using CGIs defined in previous step, the resulting PMR difference was represented as a boxplot for each spike-in group.

[0045] FIG. 11C shows the number of CGIs with increased or decreased PMR that were counted, respectively, and significance p-value was estimated by one-sided binomial tes to predict presence of ExE DNA.

[0046] FIG. 12 shows an identification of optimal ***k***-mer length for PMR. PMR is a function of *k*- mer length. To identify the optimal *k*-mer for cancer prediction, simulated data with 0.01% ExE spike-in (Methods) using the PMR method were tested. Maximum sensitivity was achieved when *k*-mer length was set to 5.

[0047] FIG. 13 shows that MHL is a biased metric to measure DNA methylation across assays. Targeted-BS was performed on 4 samples (HuES64, HCT116, uterus cancer and uterus normal tissues) in two conditions, with or without MBD enrichment. MHL were compared between two assays, targeted-BS with and without MBD enrichment, for 4 samples respectively.

[0048] FIG. 14 shows PMR is a biased metric to measure DNA methylation across assays. Targeted-BS was performed on 4 samples (HuES64, HCT116, uterus cancer and uterus normal tissues) in two conditions, with or without MBD enrichment. PMR were compared between two assays, targeted-BS with and without MBD enrichment, for 4 samples respectively.

[0049] FIG. 15 shows NMR as an unbiased metric to measure DNA methylation across assays. Performance targeted-BS was performed on 4 samples (HuES64, HCT116, uterus cancer and

uterus normal tissues) in two conditions, with or without MBD enrichment. NMR were compared between two assays, targeted-BS with and without MBD enrichment, for 4 samples respectively. Pearson correlation coefficient of 0.99 is observed for all 4 samples.

[0050] FIG. 16A shows detection of cancer in dilution samples using targeted-BS with MBD enrichment. HuES64 DNA was mixed with HCT116 or colon cancer DNA spike-in, and normal uterus DNA was mixed with uterus cancer DNA spike-in. Fractions of spike-in in all three experiments include 1%, 0.1% and 0.01%. Experiment of FIG. 16A was performed in parallel with 1 μg input DNA.

[0051] FIG. 16B shows the parallel experiment with 50 ng DNA. NMR-based was used to predict the presence of spike-in using increasing numbers of top-ranking markers.

[0052] FIG. 17A shows an example of how the NMR-based cancer prediction pipeline works on HCT116 dilution data. HCT116 was compared to human ES cell (HuES64) to identify CGIs that have higher NMR in HCT116, with a cutoff of 0.1. Then, these CGIs were ranked descendingly based on the difference of NMR between HCT116 and HuES64. The top 200 CGIs were selected as markers. Scatter plots of NMR are shown in which selected markers were highlighted in red. NMR in test sample was compared to that in HuES64.

[0053] FIG. 17B shows boxplots of ΔNMR for 1%, 0.1% and 0.1% spike-in.

[0054] FIG. 17C shows, to test whether ΔNMR are statistically higher than zero, the number of markers that were counted with increased NMR (ΔNMR >0), decreased NMR (ΔNMR <0). P-values were calculated by one-sided binomial test.

[0055] FIG. 18A shows an example to show how the NMR-based cancer prediction pipeline works on colon cancer dilution data. Colon cancer was compared to normal colon to identify CGIs that have higher NMR in colon cancer, with a cutoff of 0.1. Then, these CGIs were ranked descendingly based on the difference of NMR between tumor samples and Hu64ES. The top 200 CGIs were selected as markers. Scatter plots of NMR (Normal) is shown.

[0056] FIG. 18B shows scatter plots of NMR(ES).

[0057] FIG. 18C shows boxplots of ΔNMR for 1%, 0.1% and 0.1% spike-in.

[0058] FIG. 18D shows, to test whether ΔNMR are statistically higher than zero, the number of markers that were counted with increased NMR (ΔNMR >0), decreased NMR (ΔNMR <0). P-values were calculated by one-sided binomial test.

[0059] FIG. 19 shows the identification of optimal *k*-mer length for NMR. NMR is a function of *k*-mer length. To identify the optimal *k*-mer for cancer prediction, colon cancer spike-in data was tested with 0.01% colon cancer DNA. Maximum sensitivity was achieved when *k*-mer length was set to 5.

[0060] FIG. 20A shows detection of cancer in dilution samples using mean methylation. HuES64 DNA was mixed with HCT116 or colon cancer DNA spike-in, and uterus normal DNA was mixed with uterus cancer DNA spike-in. Fractions of spike-in in all three experiments include 1%, 0.1% and 0.01%.

[0061] FIG. 20B shows MHL-based method to predict the presence of spike-in using increasing numbers of top-ranking markers.

[0062] FIG. 21 shows prediction fraction of tumor DNA in colon cancer cohort. The prediction result was shown for each sample as indicated by the vertical dash line.

[0063] FIG. 22 shows the prediction fraction of tumor DNA in breast cancer cohort. Prediction result was shown in figure for each sample as indicated by the vertical dash line.

[0064] FIG. 23 shows a diagram of different CGI regions that were analyzed for cancer screening methods.

DETAILED DESCRIPTION OF THE INVENTION

[0065] **Methods of characterizing a cell-free DNA (cfDNA) sample**

[0066] In one aspect, a method disclosed herein is directed to characterizing a cell-free DNA (cfDNA) sample from a subject, comprising receiving sequencing data comprising reads of methylation sequences for a genomic sequence from the cfDNA sample, wherein the genomic

sequence comprises a plurality of CpG Islands (CGI) methylated in the genome of extraembryonic ectoderm (ExE) and not methylated in corresponding epiblast or adult tissue, determining a proportion of haplotypes of the genomic sequence that are fully methylated, and characterizing the cfDNA sample as comprising fully methylated cfCDNA if the proportion of haplotypes is greater than a significance threshold.

[0067] In certain aspects, the genomic sequence comprises a contiguous sequence of about 8 megabases of the human genome comprising a plurality of CGIs methylated in the genome of ExE. In certain embodiments, the genomic sequence comprises a contiguous sequence of about 8 megabases of the human genome comprising a plurality of CGIs methylated in the genome of ExE and comprising bases 57,258,577-57,282,377 of chr14 (human). In certain embodiments, the genomic sequence comprises a contiguous sequence of up to 8 megabases of the human genome comprising a plurality of CGIs methylated in the genome of extraembryonic ectoderm (ExE). In certain embodiments, the genomic sequence comprises a contiguous sequence of 6.1 megabases of the human genome comprising a plurality of CGIs methylated in the genome of extraembryonic ectoderm (ExE). In certain aspects, the genomic sequence comprises one or more sequences provided in Table 3.

[0068] In certain embodiments, the genomic sequence comprises 50-75 CGIs methylated in the genome of ExE. In certain embodiments, the genomic sequence comprises a contiguous sequence of about 8 megabases of the human genome comprising a plurality of CGIs methylated in the genome of ExE. In certain embodiments, the genomic sequence comprises 50-75 CGIs methylated in the genome of ExE. In certain embodiments, the genomic sequence comprises up to 100 CGIs methylated in the genome of ExE. In certain embodiments, the genomic sequence comprises up to 500 CGIs methylated in the genome of ExE. In certain embodiments, the genomic sequence comprises up to 1000 CGIs methylated in the genome of ExE. In certain embodiments, the genomic sequence comprises up to 1500 CGIs methylated in the genome of ExE. In a more particular embodiment, the genomic sequence comprises about 1,265 CGIs hypermethylated in ExE tissues. In a more particular embodiment, the genomic sequence comprises about 473 CGIs hypermethylated in ExE tissues.

[0069] As used herein, the significance threshold refers to an observed significance value known as a significance prediction value (p-value) estimated by a one-sided binomial test to predict presence of ExE DNA. In certain embodiments, for a 5% fraction of ctDNA in cell-free DNA the P-value (i.e., the minimum p-value signifying significance) is $5.3x10^{-145}$. In certain embodiments, for a 1% fraction of ctDNA in cell-free DNA the P-value is $3.9x10^{-78}$. In certain embodiments, for a 0.1% fraction of ctDNA in cell-free DNA the P-value is $6.5x10^{-19}$. In certain embodiments, for a 0.01% fraction of ctDNA in cell-free DNA the P-value is $6.3x10^{-4}$. In certain embodiments, for a 5% fraction of ctDNA in cell-free DNA the P-value is $1.9x10^{-78}$. In certain embodiments, for a 1% fraction of ctDNA in cell-free DNA the P-value is $7.4x10^{-34}$. In certain embodiments, for a 0.1% fraction of ctDNA in cell-free DNA the P-value is $4.2x10^{-10}$. In certain embodiments, for a 0.01% fraction of ctDNA in cell-free DNA the P-value is $3.1x10^{-2}$. In certain embodiments, for a 5% fraction of ctDNA in cell-free DNA the P-value is $4.5x10^{-26}$. In certain embodiments, for a 1% fraction of ctDNA in cell-free DNA the P-value is $3.4x10^{-15}$. In certain embodiments, for a 0.1% fraction of ctDNA in cell-free DNA the P-value is $1.1x10^{-8}$. In certain embodiments, for a 0.01% fraction of ctDNA in cell-free DNA the P-value is $4.5x10^{-6}$. In certain embodiments, at a 1% fraction, the P-value is $1.3x10^{-58}$. In certain embodiments, at a 0.1% fraction, the P-value is $2.0x10^{-37}$. In certain embodiments, at a 0.01% fraction, the P-value is $3.9x10^{-9}$. In certain embodiments, at a 1% fraction, the P-value is $1.6x10^{-54}$. In certain embodiments, at a 0.1% fraction, the P-value is $3.3x10^{-26}$. In certain embodiments, at a 0.01% fraction, the P-value is $1.1x10^{-5}$.

[0070] In certain aspects, the cfDNA sample comprises between 0.01% and 0.1% of tumor DNA. In certain aspects, the cfDNA sample comprises 0.01% of tumor DNA. In certain aspects, the cfDNA sample comprises 0.02% of tumor DNA. In certain aspects, the cfDNA sample comprises 0.03% of tumor DNA. In certain aspects, the cfDNA sample comprises 0.04% of tumor DNA. In certain aspects, the cfDNA sample comprises 0.05% of tumor DNA. In certain aspects, the cfDNA sample comprises 0.06% of tumor DNA. In certain aspects, the cfDNA sample comprises 0.07% of tumor DNA. In certain aspects, the cfDNA sample comprises 0.08% of tumor DNA. In certain aspects, the cfDNA sample comprises 0.09% of tumor DNA. In certain aspects, the cfDNA sample comprises 0.1% of tumor DNA. In certain aspects, the cfDNA sample comprises 0.15% of tumor DNA. In certain aspects, the cfDNA sample comprises 0.2% of tumor DNA. In certain aspects, the cfDNA sample comprises 0.25% of tumor DNA. In

certain aspects, the cfDNA sample comprises 0.3% of tumor DNA. In certain aspects, the cfDNA sample comprises 0.35% of tumor DNA. In certain aspects, the cfDNA sample comprises 0.25% of tumor DNA. In certain aspects, the cfDNA sample comprises 0.3% of tumor DNA. In certain aspects, the cfDNA comprises 0.4% of tumor DNA. In certain aspects, the cfDNA comprises 0.5% or more of tumor DNA. In certain aspects, the cfDNA comprises 1% or more of tumor DNA. In certain aspects, the cfDNA comprises 1.5% or more of tumor DNA. In certain aspects, the cfDNA comprises 2% or more of tumor DNA. In certain aspects, the cfDNA comprises 3% or more of tumor DNA. In certain aspects, the cfDNA comprises 4% or more of tumor DNA. In certain aspects, the cfDNA comprises 5% or more of tumor DNA.

[0071] In certain aspects, the sequencing data comprises sequence information for less than 0.01% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 0.05% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 0.1% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 0.2% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 0.3% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 0.4% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 0.5% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 0.6% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 0.7% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 0.8% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 0.9% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 1% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 1.1% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 1.2% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 1.3% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 1.4% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 1.5% of the genome of the subject. In certain aspects, the sequencing data comprises sequence

information for less than 1.6% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 1.7% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 1.8% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 1.9% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 2% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 5% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 10% of the genome of the subject.

[0072] In certain aspects, each haplotype comprises five CGI methylated in the genome of ExE not methylated in corresponding epiblast or adult tissue. In certain aspects, each haplotype comprises four CGI methylated in the genome of ExE not methylated in corresponding epiblast or adult tissue. In certain aspects, each haplotype comprises three CGI methylated in the genome of ExE not methylated in corresponding epiblast or adult tissue. In certain aspects, each haplotype comprises two CGI methylated in the genome of ExE not methylated in corresponding epiblast or adult tissue. In certain aspects, each haplotype comprises one CGI methylated in the genome of ExE not methylated in corresponding epiblast or adult tissue. In certain aspects, each haplotype comprises six CGI methylated in the genome of ExE not methylated in corresponding epiblast or adult tissue. In certain aspects, each haplotype comprises seven CGI methylated in the genome of ExE not methylated in corresponding epiblast or adult tissue. In certain aspects, each haplotype comprises eight CGI methylated in the genome of ExE not methylated in corresponding epiblast or adult tissue. In certain aspects, each haplotype comprises nine CGI methylated in the genome of ExE not methylated in corresponding epiblast or adult tissue. In certain aspects, each haplotype comprises ten CGI methylated in the genome of ExE not methylated in corresponding epiblast or adult tissue.

[0073] In certain aspects, the sequencing data comprises sequence information substantially limited to one or more regions of the subject's genome having a plurality of CGI methylated in the genome of ExE and is not methylated in corresponding epiblast or adult tissue. In certain aspects, the one or more regions of the subject genome are about 1200 CGIs as a pan-cancer methylation signature (e.g., as shown in Table 3). In certain aspects, the one or more regions are

one to five CGI patterns representing a discrete DNA methylation haplotype. In certain aspects, the region is an 8 megabase region. In certain aspects, the 8 megabase region comprises CHR14:57,258,577-57,282,337. In certain aspects, the genomic regions comprise one or more sequences provided in Table 3.

[0074] In certain aspects, fully methylated haplotypes are compared to one or more pre-established fully methylated haplotype signatures. The cfDNA sample is further characterized as corresponding or not corresponding to the pre-established fully methylated haplotype signature. In some embodiments, the fully methylated haplotypes are globally normalized for the number of haplotypes in a region by total number of haplotypes across all regions (i.e., to obtain an NMR).

[0075] In certain aspects, the pre-established fully methylated haplotype signature has been identified via a method comprising random forest, support vector machine, or deep learning analysis. As used herein, random forest algorithm operates by constructing a multitude of decision trees at training time and outputting the classification or mean/average prediction/regression of the individual trees.

[0076] As used herein, support vector machine is a machine learning method that constructs a set of hyperplanes that can be used for classification, regression, or detection of multidimensional data. As used herein, deep learning analysis refers to a class of machine learning algorithms that use multiple layers to progressively extract higher-level features from the raw input.

[0077] In certain aspects, the sequencing data includes reads of methylation sequences for a genomic sequence from the cfDNA sample that has been enriched for methylation sequences. In certain aspects, the enrichment includes a methyl-DNA binding protein-based enrichment method. In certain aspects, the methyl-DNA binding protein of the enrichment method is a methyl-binding domain (MBD) selected from MBD1, MBD2, MBD3, and MBD4.

[0078] As used herein, "sample" is not limited and may be any suitable fluid disclosed herein. In some embodiments, the sample is blood, serum, plasma, urine, stool, menstrual fluid, lymph fluid, and other bodily fluids.

[0079] As used herein, "CpG" and "CpG dinucleotide" are used interchangeably and refer to a dinucleotide sequence containing an adjacent guanine and cytosine where the cytosine is located 5′ of guanine.

[0080] As used herein, "CpG island" or "CGI" refers to a region with a high frequency of CpG sites. The region is at least 200 bp, with a GC percentage greater than 50%, and an observed-to-expected CpG ratio greater than 60%.

[0081] As used herein, a "haplotype" refers to a combination of CpG sites found on the same chromosome. Similarly, a "DNA methylation haplotype" represents the DNA methylation status of CpG sites on the same chromosome.

[0082] In certain embodiments, a sample (e.g., a fluid sample) is screened using whole-genome bisulfite sequencing (WGBS), TCGA Illumina Infinium HumanMethylation450K BeadChip sequencing (TCGA), and/or reduced representation bisulfite sequencing (RRBS), or by other suitable methylation detection assays known in the art.

[0083] In certain embodiments, the inventions disclosed herein relate to methods of using proportion of concordantly methylated reads (PMR) (i.e., fully methylated haplotypes) to detect circulating tumor DNA (ctDNA) in a sample. In certain aspects, a methylation sequence for a sample is obtained and at least one CpG Island (CGI) is identified on the methylation sequence. PMR for the identified CpG Island is calculated and then compared to a control background of a normal tissue or epiblast. The presence of ctDNA is detected in the sample when the PMR of the sample is larger than the control background (e.g., signal is higher by bank sum test).

[0084] The presence of ctDNA may be detected in the cfDNA with a greater sensitivity and specificity than methods previously known by those of skill in the art. For example, ctDNA may be detected in the sample using PMR with a sensitivity of greater than 75%, 80%, 85%, 90%, 95%, or 99%. In certain aspects, ctDNA is detected in the sample using PMR with 100% sensitivity. ctDNA may be detected in the sample using PMR with a specificity of greater than 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95%. In certain aspects, ctDNA is detected in the sample using PMR with 95% specificity. In some aspects, ctDNA is detected in the sample using PMR with at least 90% sensitivity and at least 90% specificity. In some

aspects, ctDNA is detected in the sample using PMR with at least 100% sensitivity and at least 95% specificity.

[0085] As used herein, "sensitivity" measures the proportion of positives (i.e., the presence of ctDNA) that are correctly identified in the cfDNA.

[0086] As used herein, "specificity" measures the proportion of negatives (i.e., non-ctDNA) that are correctly identified in the cfDNA.

[0087] The amount of ctDNA detected in the sample may be measured and quantified. In some aspects, the sample comprises 0.005% to 1.5% ctDNA, 0.01% to 1% ctDNA, 0.05% to 0.5% ctDNA, 0.1% to 0.3% ctDNA. In some embodiments, the sample comprises 0.01% ctDNA. In certain aspects, the presence of 0.01% ctDNA is detected in cfDNA using PMR with about 100% sensitivity and about 95% specificity, with a p-value cutoff of 10^{-4}.

[0088] In some embodiments, the inventions disclosed herein relate to methods of screening for cancer by using PMR to detect ctDNA in a sample as described herein, wherein the presence of ctDNA in the sample is indicative of the subject having cancer.

[0089] The methods described herein may be applied to a subject who is at risk of cancer or at risk of cancer recurrence. The subject is not limited and may be any suitable subject. In some embodiments, the subject is an individual diagnosed with, suffering from, at risk of developing, or suspected of having cancer. In some embodiments, the subject is a human. In some embodiments, the subject is a non-human mammal. In some embodiments, the subject is a non-mammal vertebrate animal. In some embodiments, the subject is a common lab animal. A subject at risk of cancer may be, e.g., a subject who has not been diagnosed with cancer but has an increased risk of developing cancer. Determining whether a subject is considered "at increased risk" of cancer is within the skill of the ordinarily skilled medical practitioner. Any suitable test(s) and/or criteria can be used. For example, a subject may be considered "at increased risk" of developing cancer if any one or more of the following apply: (i) the subject has an inherited mutation or genetic polymorphism that is associated with increased risk of developing or having cancer relative to other members of the general population not having such mutation or genetic polymorphism (e.g., inherited mutations in certain TSGs are known to be associated with

increased risk of cancer); (ii) the subject has a gene or protein expression profile, and/or presence of particular substance(s) in a sample obtained from the subject (e.g., blood), that is/are associated with increased risk of developing or having cancer relative to the general population; (iii) the subject has one or more risk factors such as a family history of cancer, exposure to a tumor-promoting agent or carcinogen (e.g., a physical carcinogen, such as ultraviolet or ionizing radiation; a chemical carcinogen such as asbestos, tobacco or smoke components, aflatoxin, arsenic; a biological carcinogen such as certain viruses or parasites); (iv) the subject is over a specified age, e.g., over 60 years of age. A subject suspected of having cancer may be a subject who has one or more symptoms of cancer or who has had a diagnostic procedure performed that suggested or was consistent with the possible existence of cancer. A subject at risk of cancer recurrence may be a subject who has been treated for cancer and appears to be free of cancer, e.g., as assessed by an appropriate method.

[0090] As used herein, the phrase "cancer" is intended to broadly apply to any cancerous condition.

[0091] In certain aspects, the cancer is stage I, stage II, stage III, or stage IV. In certain aspects, the cancerous cells are present but have not spread to nearby tissue.

[0092] Illustrative examples of cancers include, but are not limited to, adrenal cancer, adrenocortical carcinoma, anal cancer, appendix cancer, astrocytoma, atypical teratoid/rhabdoid tumor, basal cell carcinoma, bile duct cancer, bladder cancer, bone cancer, brain/CNS cancer, breast cancer, bronchial tumors, cardiac tumors, cervical cancer, cholangiocarcinoma, chondrosarcoma, chordoma, colon cancer, colorectal cancer, craniopharyngioma, ductal carcinoma in situ (DCIS) endometrial cancer, ependymoma, esophageal cancer, esthesioneuroblastoma, Ewing´s sarcoma, extracranial germ cell tumor, extragonadal germ cell tumor, eye cancer, fallopian tube cancer, fibrous histiosarcoma, fibrosarcoma, gallbladder cancer, gastric cancer, gastrointestinal carcinoid tumors, gastrointestinal stromal tumor (GIST), germ cell tumors, glioma, glioblastoma, head and neck cancer, hemangioblastoma, hepatocellular cancer, hypopharyngeal cancer, intraocular melanoma, kaposi sarcoma, kidney cancer, laryngeal cancer, leiomyosarcoma, lip cancer, liposarcoma, liver cancer, lung cancer, non-small cell lung cancer, lung carcinoid tumor, malignant mesothelioma, medullary

carcinoma, medulloblastoma, menangioma, melanoma, Merkel cell carcinoma, midline tract carcinoma, mouth cancer, myxosarcoma, myelodysplastic syndrome, myeloproliferative neoplasms, nasal cavity and paranasal sinus cancer, nasopharyngeal cancer, neuroblastoma, oligodendroglioma, oral cancer, oral cavity cancer, oropharyngeal cancer, osteosarcoma, ovarian cancer, pancreatic cancer, pancreatic islet cell tumors, papillary carcinoma, paraganglioma, parathyroid cancer, penile cancer, pharyngeal cancer, pheochromocytoma, pinealoma, pituitary tumor, pleuropulmonary blastoma, primary peritoneal cancer, prostate cancer, rectal cancer, retinoblastoma, renal cell carcinoma, renal pelvis and ureter cancer, rhabdomyosarcoma, salivary gland cancer, sebaceous gland carcinoma, skin cancer, soft tissue sarcoma, squamous cell carcinoma, small cell lung cancer, small intestine cancer, stomach cancer, sweat gland carcinoma, synovioma, testicular cancer, throat cancer, thymus cancer, thyroid cancer, urethral cancer, uterine cancer, uterine sarcoma, vaginal cancer, vascular cancer, vulvar cancer, and Wilms Tumor. In some embodiments of the methods described herein, the cancer is adrenocortical carcinoma, bladder urothelial carcinoma, breast invasive carcinoma, cervical and endocervical cancers, cholangiocarcinoma, colon adenocarcinoma, colorectal adenocarcinoma, lymphoid neoplasm diffuse large B-cell lymphoma, esophageal carcinoma, FFPE Pilot Phase II, glioblastoma multiforme, glioma, head and neck squamous cell carcinoma, kidney chromophobe, pan-kidney cohort (KICH+KIRC+KIRP), kidney renal clear cell carcinoma, kidney renal papillary cell carcinoma, acute myeloid leukemia, brain lower grade glioma, liver hepatocellular carcinoma, lung adenocarcinoma, lung squamous cell carcinoma, mesothelioma, ovarian serous cystadenocarcinoma, pancreatic adenocarcinoma, pheochromocytoma and paraganglioma, prostate adenocarcinoma, rectum adenocarcinoma, sarcoma, skin cutaneous melanoma, stomach adenocarcinoma, stomach and esophageal carcinoma, testicular germ cell tumors, thyroid carcinoma, thymoma, uterine corpus endometrial carcinoma, uterine carcinosarcoma, and uveal melanoma. In other embodiments, the invention provides methods of treating a subject in need of treatment for cancer.

[0093] In some embodiments, PMR is used to detect ctDNA in a sample as described herein, where the presence of the ctDNA is indicative of the subject having cancer. The individual is then treated for cancer using any methods of treatment generally known to those of skill in the art (e.g., therapeutics or procedures).

[0094] For example, therapies or anticancer agents that may be used for treating the subject include anti-cancer agents, chemotherapeutic drugs, surgery, radiotherapy (e.g., γ-radiation, neutron beam radiotherapy, electron beam radiotherapy, proton therapy, brachytherapy, and systemic radioactive isotopes), endocrine therapy, biologic response modifiers (e.g., interferons, interleukins), hyperthermia, cryotherapy, agents to attenuate any adverse effects, or combinations thereof, useful for treating a subject in need of treatment for a cancer. Non-limiting examples of cancer chemotherapeutic agents that may be used include, e.g., alkylating and alkylating-like agents such as nitrogen mustards (e.g., chlorambucil, chlormethine, cyclophosphamide, ifosfamide, and melphalan), nitrosoureas (e.g., carmustine, fotemustine, lomustine, streptozocin); platinum agents (e.g., alkylating-like agents such as carboplatin, cisplatin, oxaliplatin, BBR3464, satraplatin), busulfan, dacarbazine, procarbazine, temozolomide, thioTEPA, treosulfan, and uramustine; antimetabolites such as folic acids (e.g., aminopterin, methotrexate, pemetrexed, raltitrexed); purines such as cladribine, clofarabine, fludarabine, mercaptopurine, pentostatin, thioguanine; pyrimidines such as capecitabine, cytarabine, fluorouracil, floxuridine, gemcitabine; spindle poisons/mitotic inhibitors such as taxanes (e.g., docetaxel, paclitaxel), vincas (e.g., vinblastine, vincristine, vindesine, and vinorelbine), epothilones; cytotoxic/anti-tumor antibiotics such anthracyclines (e.g., daunorubicin, doxorubicin, epirubicin, idarubicin, mitoxantrone, pixantrone, and valrubicin), compounds naturally produced by various species of Streptomyces (e.g., actinomycin, bleomycin, mitomycin, plicamycin) and hydroxyurea; topoisomerase inhibitors such as camptotheca (e.g., camptothecin, topotecan, irinotecan) and podophyllums (e.g., etoposide, teniposide); monoclonal antibodies for cancer therapy such as anti-receptor tyrosine kinases (e.g., cetuximab, panitumumab, trastuzumab), anti-CD20 (e.g., rituximab and tositumomab), and others for example alemtuzumab, aevacizumab, gemtuzumab; photosensitizers such as aminolevulinic acid, methyl aminolevulinate, porfimer sodium, and verteporfin; tyrosine and/or serine/threonine kinase inhibitors, e.g., inhibitors of Abl, Kit, insulin receptor family member(s), VEGF receptor family member(s), EGF receptor family member(s), PDGF receptor family member(s), FGF receptor family member(s), mTOR, Raf kinase family, phosphatidyl inositol (PI) kinases such as PI3 kinase, PI kinase-like kinase family members, cyclin dependent kinase (CDK) family members, Aurora kinase family members (e.g., kinase inhibitors that are on the market or have shown efficacy in at least one phase III trial in tumors, such as cediranib, crizotinib, dasatinib, erlotinib, gefitinib, imatinib, lapatinib, nilotinib,

sorafenib, sunitinib, vandetanib), growth factor receptor antagonists, and others such as retinoids (e.g., alitretinoin and tretinoin), altretamine, amsacrine, anagrelide, arsenic trioxide, asparaginase (e.g., pegasparagase), bexarotene, bortezomib, denileukin diftitox, estramustine, ixabepilone, masoprocol, mitotane, and testolactone, Hsp90 inhibitors, proteasome inhibitors (e.g,. bortezomib), angiogenesis inhibitors, e.g., anti-vascular endothelial growth factor agents such as bevacizumab (Avastin) or VEGF receptor antagonists, matrix metalloproteinase inhibitors, various pro-apoptotic agents (e.g., apoptosis inducers), Ras inhibitors, anti-inflammatory agents, cancer vaccines, or other immunomodulating therapies, etc. It will be understood that the preceding classification is non-limiting.

[0095] In some embodiments, the method further comprises a step of determining a tissue of origin from the sequencing data.

[0096] **Methods for Detecting Cancer**

[0097] In another aspect, a method as described herein is directed to a method for detecting cancer in a subject comprising receiving sequencing data comprising reads of methylation sequences for a genomic sequence from a cfDNA sample from the subject wherein the genomic sequence comprises a plurality of CpG Islands (CGI) methylated in the genome of extraembryonic ectoderm (ExE) and that are not methylated in corresponding epiblast or adult tissue, determining a proportion of haplotypes of the genomic sequence that are fully methylated, and detecting cancer in the subject if the proportion of fully methylated haplotypes is greater than a significance threshold.

[0098] The cancer is not limited and may be any cancer described herein. In certain aspects, the cancer is selected from acute myeloid leukemia, bladder cancer, breast cancer, colon cancer, esophageal cancer, kidney cancer, liver cancer, lung cancer, ovarian cancer, pancreatic cancer, prostate cancer, and stomach cancer.

[0099] In certain aspects, each haplotype comprises five CGI methylated in the genome of ExE not methylated in corresponding epiblast or adult tissue. In certain aspects, each haplotype comprises four CGI methylated in the genome of ExE not methylated in corresponding epiblast or adult tissue. In certain aspects, each haplotype comprises three CGI methylated in the genome

of ExE not methylated in corresponding epiblast or adult tissue. In certain aspects, each haplotype comprises two CGI methylated in the genome of ExE not methylated in corresponding epiblast or adult tissue. In certain aspects, each haplotype comprises one CGI methylated in the genome of ExE not methylated in corresponding epiblast or adult tissue. In certain aspects, each haplotype comprises six CGI methylated in the genome of ExE not methylated in corresponding epiblast or adult tissue. In certain aspects, each haplotype comprises seven CGI methylated in the genome of ExE not methylated in corresponding epiblast or adult tissue. In certain aspects, each haplotype comprises eight CGI methylated in the genome of ExE not methylated in corresponding epiblast or adult tissue. In certain aspects, each haplotype comprises nine CGI methylated in the genome of ExE not methylated in corresponding epiblast or adult tissue. In certain aspects, each haplotype comprises ten CGI methylated in the genome of ExE not methylated in corresponding epiblast or adult tissue.

[0100] In certain aspects, the cfDNA sample comprises between 0.01% and 0.1% of tumor DNA. In certain aspects, the cfDNA sample comprises 0.01% of tumor DNA. In certain aspects, the cfDNA sample comprises 0.02% of tumor DNA. In certain aspects, the cfDNA sample comprises 0.03% of tumor DNA. In certain aspects, the cfDNA sample comprises 0.04% of tumor DNA. In certain aspects, the cfDNA sample comprises 0.05% of tumor DNA. In certain aspects, the cfDNA sample comprises 0.06% of tumor DNA. In certain aspects, the cfDNA sample comprises 0.07% of tumor DNA. In certain aspects, the cfDNA sample comprises 0.08% of tumor DNA. In certain aspects, the cfDNA sample comprises 0.09% of tumor DNA. In certain aspects, the cfDNA sample comprises 0.1% of tumor DNA.

[0101] In certain aspects, the sequencing data comprises sequence information for less than 0.1% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 0.2% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 0.3% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 0.4% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 0.5% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 0.6% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 0.7% of the genome of the subject. In certain

aspects, the sequencing data comprises sequence information for less than 0.8% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 0.9% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 1% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 1.1% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 1.2% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 1.3% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 1.4% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 1.5% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 1.6% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 1.7% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 1.8% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 1.9% of the genome of the subject. In certain aspects, the sequencing data comprises sequence information for less than 2% of the genome of the subject.

[0102] In certain aspects, the sequencing data comprises sequence information substantially limited to one or more regions of the subject's genome having a plurality of CGI methylated in the genome of ExE and not methylated in corresponding epiblast or adult tissue.

[0103] In certain aspects, fully methylated haplotypes are compared to one or more pre-established fully methylated haplotype signatures corresponding to one or more tumor types. The method includes determining the presence or absence of the one or more tumor types that are detected in the subject.

[0104] In certain aspects, the pre-established fully methylated haplotype signatures corresponding to one or more tumor types have been identified via a method comprising random forest, support vector machine, or deep learning analysis.

[0105] In certain aspects, the sequencing data comprising reads of methylation sequences for a genomic sequence from the cfDNA sample has been enriched for sequences comprising

methylation. In certain aspects, the enrichment includes a methyl-DNA binding protein-based enrichment method. In certain aspects, the methyl-DNA binding protein of the enrichment method is a methyl-binding domain (MBD) selected from MBD1, MBD2, MBD3, and MBD4. In certain aspects, the enrichment method further comprises targeted bisulfite sequencing (targeted-BS). In certain aspects, up to 6.2 Mb of ExE hyper CGIs are enriched. In certain aspects, the enrichment method achieved greater than 50-fold enrichment compared to whole-genome bisulfite sequencing (WGBS). In certain aspects, the enrichment method achieved greater than 100-fold enrichment compared to WGBS. In certain aspects, the enrichment method achieved greater than 400-fold enrichment compared to WGBS.

[0106] In certain aspects, the cfDNA sample was obtained from plasma, urine, stool, menstrual fluid, or lymph fluid.

[0107] In certain aspects, the presence of cancer is detected in the sample with 100% sensitivity and 95% specificity. The presence of ctDNA may be detected in the cfDNA with a greater sensitivity and specificity than methods previously known by those of skill in the art. For example, ctDNA may be detected in the sample using PMR with a sensitivity of greater than 75%, 80%, 85%, 90%, 95%, or 99%. In certain aspects, ctDNA is detected in the sample using PMR with 100% sensitivity. ctDNA may be detected in the sample using PMR with a specificity of greater than 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95%. In certain aspects, ctDNA is detected in the sample using PMR with 95% specificity. In some aspects, ctDNA is detected in the sample using PMR with at least 90% sensitivity and at least 90% specificity. In some aspects, ctDNA is detected in the sample using PMR with at least 100% sensitivity and at least 95% specificity.

[0108] In certain aspects, the method further includes the step of treating the subject for cancer when cancer is detected in the subject. The method of treating is not limited and may be any method described herein. In some embodiments, the method of treating is with a chemotherapeutic agent. In some embodiments, the method further comprises a step of determining a tissue of origin from the sequencing data.

[0109] **Methods of Detecting Eradication of Cancer**

[0110] In another aspect, a method disclosed herein is directed to detecting eradication of a cancer from a subject, comprising receiving sequencing data comprising reads of methylation sequences for a genomic sequence from a cfDNA sample from a subject after a cancer treatment, wherein the genomic sequence comprises a plurality of CpG Islands (CGI) methylated in the genome of extraembryonic ectoderm (ExE) and not methylated in corresponding epiblast or adult tissue, determining a proportion of haplotypes of the genomic sequence that are fully methylated, and detecting cancer in the subject if the proportion of fully methylated haplotypes is greater than a significance threshold, wherein if cancer is not detected in the subject then the cancer has been eradicated from the subject. The cancer is not limited and may be any suitable cancer described herein. The subject is not limited and also may be any subject described herein. In some aspects, the subject is human.

[0111] In certain aspects, the genomic sequence comprises 1-1300 CGIs methylated in the genome of ExE. In certain aspects, the genomic sequence comprises 1-25 CGIs methylated in the genome of ExE. In certain aspects, the genomic sequence comprises 25-50 CGIs methylated in the genome of ExE. In certain aspects, the genomic sequence comprises 50-75 CGIs methylated in the genome of ExE. In certain aspects, the genomic sequence comprises 50-75 CGIs methylated in the genome of ExE. In certain aspects, the genomic sequence comprises 75-100 CGIs methylated in the genome of ExE. In certain aspects, the genomic sequence comprises 100-200 CGIs methylated in the genome of ExE. In certain aspects, the genomic sequence comprises 200-300 CGIs methylated in the genome of ExE. In certain aspects, the genomic sequence comprises 300-400 CGIs methylated in the genome of ExE. In certain aspects, the genomic sequence comprises 400-500 CGIs methylated in the genome of ExE. In certain aspects, the genomic sequence comprises 500-600 CGIs methylated in the genome of ExE. In certain aspects, the genomic sequence comprises 600-700 CGIs methylated in the genome of ExE. In certain aspects, the genomic sequence comprises 700-800 CGIs methylated in the genome of ExE. In certain aspects, the genomic sequence comprises 800-900 CGIs methylated in the genome of ExE. In certain aspects, the genomic sequence comprises 900-1000 CGIs methylated in the genome of ExE. In certain aspects, the genomic sequence comprises 1000-1100 CGIs methylated in the genome of ExE. In certain aspects, the genomic sequence comprises 1100-1200 CGIs methylated in the genome of ExE. In certain aspects, the genomic sequence comprises 1200-1300 CGIs methylated in the genome of ExE.

[0112] As used herein, eradication of the cancer refers to a substantial reduction in cancerous cells as compared to an original sample. In certain embodiments, the substantial reduction means a reduction of 90% or more of cancerous cells. In certain embodiments, the substantial reduction means a reduction of 95% or more of cancerous cells. In certain embodiments, the substantial reduction means a reduction of 98% or more of cancerous cells. In certain embodiments, the substantial reduction means a reduction of 99% or more of cancerous cells. In certain embodiments, the substantial reduction means a reduction of 99.5% or more of cancerous cells. In certain embodiments, the substantial reduction means a reduction of 99.9% or more of cancerous cells. In certain embodiments, the substantial reduction means a reduction of 99.99% or more of cancerous cells. In certain embodiments, the substantial reduction means a reduction of 99.999% or more of cancerous cells. In certain embodiments, the substantial reduction means a reduction of 100% of cancerous cells. In certain embodiments, the substantial reduction means only a trace amount cancerous cells exist.

[0113] **Methods for Determining Probability Distribution**

[0114] In another aspect, the invention is directed to a method of determining a probability distribution of haplotypes comprising the steps of receiving sequencing data comprising reads of methylation sequences for a genomic sequence from the cfDNA sample, wherein the genomic sequence comprises a plurality of CpG Islands (CGI) methylated in the genome of extraembryonic ectoderm (ExE) and not methylated in corresponding epiblast or adult tissue, assigning a training or validation set based on the methylated ExE CGI data applying a machine learning method to estimate the probability distribution of all haplotypes across ExE sites, and determining one or more classifications of tumor versus normal samples based on a prediction score (P-score) as used herein is obtained from the machine learning method.

[0115] In certain aspects, the machine learning method is random forest. In certain aspects, the machine learning method is a support vector machine. In certain aspects, the machine learning method is deep learning.

[0116] In certain aspects, the above methods further include a method of evaluating the performance of the prediction comprising performing an in silico simulation by comparing randomly sampled sequencing reads from epiblast or adult tissue with the ExE reads. In some

embodiments, the method further comprises a step of determining a tissue of origin from the sequencing data.

[0117] **Determining Tissue of Origin**

[0118] Some aspects of the present disclosure are directed to a method of determining a tissue origin comprising receiving targeted bisulfite sequencing data comprising reads of methylation sequences for a genomic sequence from a cfDNA sample, wherein the genomic sequence comprises a plurality of CpG Islands (CGI) methylated in the genome of extraembryonic ectoderm (ExE) and not methylated in corresponding epiblast or adult tissue, and determining a tissue of origin by calculating a relative abundance of haplotypes from the methylated genomic regions by defining a tissue-specific index (TSI) for each haplotype. In some embodiments, the TSI is calculated by the formula:

$$TSI = \frac{\sum_{j=1}^{n} 1 - \frac{10^{PKR(j)}}{10^{PKR(\max)}}}{n-1}$$

wherein n is the number of tissues, PKR(j) is the fraction of a specific haplomer in tissue, and j and PKR max are PKR of the highest methylated tissue. In some embodiments, the sequences comprise one or more sequences provided in Table 2.

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[0119] The description of embodiments of the disclosure is not intended to be exhaustive or to limit the disclosure to the precise form disclosed. While specific embodiments of, and examples for, the disclosure are described herein for illustrative purposes, various equivalent modifications are possible within the scope of the disclosure, as those skilled in the relevant art will recognize. For example, while method steps or functions are presented in a given order, alternative embodiments may perform functions in a different order, or functions may be performed substantially concurrently. The teachings of the disclosure provided herein can be applied to other procedures or methods as appropriate. The various embodiments described herein can be combined to provide further embodiments. Aspects of the disclosure can be modified, if necessary, to employ the compositions, functions and concepts of the above references and

application to provide yet further embodiments of the disclosure. These and other changes can be made to the disclosure in light of the detailed description.

[0120] Specific elements of any of the foregoing embodiments can be combined or substituted for elements in other embodiments. Furthermore, while advantages associated with certain embodiments of the disclosure have been described in the context of these embodiments, other embodiments may also exhibit such advantages, and not all embodiments need necessarily exhibit such advantages to fall within the scope of the disclosure.

[0121] All patents and other publications identified are expressly incorporated herein by reference for the purpose of describing and disclosing, for example, the methodologies described in such publications that might be used in connection with the present invention. These publications are provided solely for their disclosure prior to the filing date of the present application. Nothing in this regard should be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention or prior publication, or for any other reason. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicants and does not constitute any admission as to the correctness of the dates or contents of these documents.

[0122] One skilled in the art readily appreciates that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The details of the description and the examples herein are representative of certain embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Modifications therein and other uses will occur to those skilled in the art. These modifications are encompassed within the spirit of the invention. It will be readily apparent to a person skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

[0123] The articles “a” and “an” as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to include the plural referents. Claims or descriptions that include “or” between one or more members of a group are considered satisfied if one, more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process unless indicated to the contrary or otherwise evident from

the context. The invention includes embodiments in which exactly one member of the group is present in, employed in, or otherwise relevant to a given product or process. The invention also includes embodiments in which more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process. Furthermore, it is to be understood that the invention provides all variations, combinations, and permutations in which one or more limitations, elements, clauses, descriptive terms, etc., from one or more of the listed claims is introduced into another claim dependent on the same base claim (or, as relevant, any other claim) unless otherwise indicated or unless it would be evident to one of ordinary skill in the art that a contradiction or inconsistency would arise. It is contemplated that all embodiments described herein are applicable to all different aspects of the invention where appropriate. It is also contemplated that any of the embodiments or aspects can be freely combined with one or more other such embodiments or aspects whenever appropriate. Where elements are presented as lists, e.g., in Markush group or similar format, it is to be understood that each subgroup of the elements is also disclosed, and any element(s) can be removed from the group. It should be understood that, in general, where the invention, or aspects of the invention, is/are referred to as comprising particular elements, features, etc., certain embodiments of the invention or aspects of the invention consist, or consist essentially of, such elements, features, etc. For purposes of simplicity those embodiments have not in every case been specifically set forth in so many words herein. It should also be understood that any embodiment or aspect of the invention can be explicitly excluded from the claims, regardless of whether the specific exclusion is recited in the specification. For example, any one or more active agents, additives, ingredients, optional agents, types of organism, disorders, subjects, or combinations thereof, can be excluded.

[0124] Where the claims or description relate to a composition of matter, it is to be understood that methods of making or using the composition of matter according to any of the methods disclosed herein, and methods of using the composition of matter for any of the purposes disclosed herein are aspects of the invention, unless otherwise indicated or unless it would be evident to one of ordinary skill in the art that a contradiction or inconsistency would arise. Where the claims or description relate to a method, e.g., it is to be understood that methods of making compositions useful for performing the method, and products produced according to the method, are aspects of the invention, unless otherwise indicated or unless it would be evident to one of ordinary skill in the art that a contradiction or inconsistency would arise.

[0125] Where ranges are given herein, the invention includes embodiments in which the endpoints are included, embodiments in which both endpoints are excluded, and embodiments in which one endpoint is included and the other is excluded. It should be assumed that both endpoints are included unless indicated otherwise. Furthermore, it is to be understood that unless otherwise indicated or otherwise evident from the context and understanding of one of ordinary skill in the art, values that are expressed as ranges can assume any specific value or subrange within the stated ranges in different embodiments of the invention, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise. It is also understood that where a series of numerical values is stated herein, the invention includes embodiments that relate analogously to any intervening value or range defined by any two values in the series, and that the lowest value may be taken as a minimum and the greatest value may be taken as a maximum. Numerical values, as used herein, include values expressed as percentages. For any embodiment of the invention in which a numerical value is prefaced by "about" or "approximately", the invention includes an embodiment in which the exact value is recited. For any embodiment of the invention in which a numerical value is not prefaced by "about" or "approximately", the invention includes an embodiment in which the value is prefaced by "about" or "approximately".

[0126] "Approximately" or "about" generally includes numbers that fall within a range of 1% or in some embodiments within a range of 5% of a number or in some embodiments within a range of 10% of a number in either direction (greater than or less than the number) unless otherwise stated or otherwise evident from the context (except where such number would impermissibly exceed 100% of a possible value). It should be understood that, unless clearly indicated to the contrary, in any methods claimed herein that include more than one act, the order of the acts of the method is not necessarily limited to the order in which the acts of the method are recited, but the invention includes embodiments in which the order is so limited. It should also be understood that unless otherwise indicated or evident from the context, any product or composition described herein may be considered "isolated".

[0127] **EXAMPLES**

[0128] **Introduction**

[0129] Recently, a new generation of biomarkers have been established with the discovery of genetic alterations that are responsible for the initiation and progression of human cancers. These alterations include single-base substitutions, insertions, deletions and translocations. These somatic mutations can also be detected in cell-free circulating tumor DNA (cfDNA) [6]. The development of non-invasive liquid biopsy methods based on the analysis of ctDNA provides an opportunity for a new generation of diagnostic approaches. A recently developed blood test was able to detect eight common cancer types through the assessment of the levels of circulating proteins and mutations in cfDNA, with a sensitivity ranging from 69-98% and specificity higher than 99% [7]. However, mutation-based liquid biopsy tests suffer from low sensitivity due to intra- and inter-tumor heterogeneity [8] since not all samples of one cancer type contain the same genetic driver alterations. For instance, analysis of lung adenocarcinoma samples has led to the identification of 22 drivers [9] but up to 25% of patients contain no genetic alterations in any of those genes [10, 11]. Furthermore, the existence of low frequency sub-clones renders mutation-based diagnostics even more complicated: in stage I disease, the fraction of cfDNA is around 0.1% [12] and thus, detection of sub-clonal mutations with a frequency of 5% in early stage disease challenges the detection limit of current sequencing technologies [13].

[0130] In recent years, DNA methylation profiling has been adopted as a promising approach for liquid biopsies [14]. Aberrant DNA methylation is ubiquitous in human cancer and has been shown to occur early during carcinogenesis, thus providing attractive potential biomarkers for the early detection of cancer [15]. Compared to a normal genome, cancer genomes are globally hypomethylated and locally hypermethylated in CpG Islands (CGI) [16, 17]. Markers associated with these two features have been extensively used for methylation-based ctDNA detection [18, 19]. For instance, FBN1, FBN2, HLTF, PHACTR3, SEPT9, SNCA, SST, TAC1, VIM have been used individually for colorectal cancer (CRC) detection [20]. However, single gene-based diagnosis suffers from low accuracy due to tumor heterogeneity. Genome-wide assays such as whole- genome bisulfite sequencing (WGBS) and reduced representation bisulfite sequencing (RRBS) thus have been tested to improve prediction performance. For instance, plasma hypomethylation gave a sensitivity and specificity of 74% and 94%, respectively, for the detection of nonmetastatic cancer cases, when a mean of 93 million WGBS reads per case were obtained [18]. Recently, methylated DNA immunoprecipitation sequencing (MeDIP–seq), a genome-wide assay, was demonstrated for sensitive tumour detection and classification using

plasma cell-free DNA methylomes [21]. In terms of analytical methods, since CpG mean methylation-based methods are not sufficiently sensitive for early cancer detection, methylation haplotype blocks (MHB; i.e. co-methylated stretches of DNA) have been used instead and are able to detect 2% tumor DNA [22]. This approach has led to the development of a novel methylation haplotype analysis tool, CancerDetector, which is able to detect 0.1% tumor DNA as demonstrated by spike-in experiments [23]. Genome-wide assays are promising in terms of both sensitive early cancer detection and cancer type classification, but in general suffer from higher cost and longer turnaround-time. Targeted assays which only interrogate a set of predefined genomic regions represent a solution that balances information obtain and cost. For instance, padlock-based targeted sequencing [24] have been evaluated for noninvasive detection of hepatocellular carcinoma (HCC) with a sensitivity of 83.3% and specificity of 90.5% using as few as 10 markers [25]. Detection HCC is relatively easy compared to other cancer types since up to 20% of cfDNA derives from liver tissue even in normal controls [26]. Recently, a marker with 4 consecutive CpG sites were characterized with amplicon-based bisulfite sequencing in breast cancer and a fully methylated pattern was identified for early identification of metastasis [27]. Though with sensitivity as low as 25%, this method represents a novel way for joint analysis of multiple CpG sites in a single locus. The published studies that use targeted sequencing were mainly to address the detection of single cancer type, thus ultrasensitive methods for non-invasive detection of multiple cancer types remain to be developed. Epigenetic restriction of extraembryonic lineages mirrors the somatic transition to cancer [28]. An extraembryonic methylation signature was discovered to distinguish cancer samples from matched normal tissues for almost all cancer types tested. Based on these findings, the extraembryonic signature, coupled with DNA methylation haplotype analysis, represents a universal framework for ultra-sensitive non-invasive early cancer diagnosis.

[0131] **Results**

[0132] **Extraembryonically hyper-methylated CGIs provide a universal cancer signature**

[0133] Placenta has long been considered to be a tissue of pseudo-malignancy [29], with several phenotypes, such as its angiogenic, immune suppressive and invasive abilities, reminiscent of human cancer. The DNA methylation landscape of extraembryonic ectoderm (ExE), the

progenitor of placenta, was compared with that of the epiblast of a mouse E6.5 conceptus [28] (FIG. 1A). Using this data, ExE hyper-methylated CGIs (ExE Hyper CGIs) was identified as a DNA methylation signature able to distinguish these two tissue types. Interestingly, ExE Hyper CGIs are more conserved on the sequence level than the genomic background (FIG. 1B), and the majority of ExE Hyper CGIs in mouse have a human ortholog that is localized near a CGI (FIG. 1C). Strikingly, it was found that the ExE Hyper CGI signature is hypermethylated in 14 cancer type profiled within The Cancer Genome Atlas (TCGA) project that contain matched normal tissues [28]. The only exception is thyroid cancer, which could potential be explained by the observation that FGF and WNT pathways are shared during tissue specification of ExE and normal thyroid epithelia [30]. Performance of ExE hyper CGIs was next tested in cancer prediction using TCGA pan-cancer data sets. When TCGA samples were randomly assigned into a training and validation set, ExE hyper CGIs were able to classify tumor versus normal samples with high sensitivity and specificity using support vector machine (SVM) classification method (Methods, AUC = 0.98, FIG. 1D). Similar result was obtained when an independent method random forest was applied on the same data sets (AUC = 0.98, Methods and FIG. 6). This observation suggests that a vast majority of cases of each tumor type can be correctly identified when using ExE hyper CGIs, and that human cancer types are significantly more homogenous when analyzed for their methylation status of ExE hyper CGIs than when profiled for the mutation status of any driver genes (FIG. 1E). For example, although somatic mutations in TP53 represent the most frequent genetic alterations in human cancer, many cancer types such as kidney renal papillary cell carcinoma (KIRP) and kidney renal clear cell carcinoma (KIRC) demonstrate low mutation frequencies in TP53 (FIG. 1E). ExE hyper CGIs thus represent a novel DNA methylation signature for pan-cancer diagnosis and the basis for developing the instant non-invasive liquid biopsy platform.f.

[0134] **DNA methylation haplotypes improve detection sensitivity**

[0135] The development of non-invasive liquid biopsy methods based on the DNA methylation of ctDNA has revolutionized cancer diagnosis [21]; however, several challenges remain. First, disordered methylation is frequently observed in cancer [31], which is one of the reasons why single CpG-based diagnostic platforms suffer from low sensitivity. The overall sensitivity of SEPT9, for example, is only 60% for colorectal cancer (CRC) detection [32]. Second, the

fraction of ctDNA among cell-free DNA is as low as 0.01% in early stage diseases [33], which requires nearly zero background contributed by normal cells to make tumor cell detection possible. However, normal cells acquire low-level methylation (~ 1%) when measured at single CpG sites due to noise, aging [34] and other stochastic processes [35]. To overcome these issues, a novel approach was developed based on the observation that DNA methylation haplotypes, measured in phase on the same molecule, provide a better choice for diagnostic purposes. Even when measured from bulk data, DNA methylation information obtained from a single sequenced fragment is guaranteed to stem from a single chromosome and a single cell. Thus, the methylation pattern of CpGs of each fragment represents a discrete DNA methylation haplotype (FIG. 2A). In normal somatic tissues, fully methylated reads are very rare when analyzing ExE Hyper CGIs. Thus, the proportion of fully methylated reads (PMR) calculated from sequencing data represents a novel way to quantify the extent of DNA methylation (FIGS. 7 and 8). This approach significantly reduces background noise as compared to standard approaches. For example, OTX2 is a developmental regulator and hypermethylated in ExE and placenta, and also serves as one of the ExE hyper CGI markers. When its mean methylation level was used, a considerable extent of background noise was observed in normal samples. In contrast, PMR-based quantification at this locus significantly reduced background noise (FIG. 2B).

[0136] To evaluate the performance of PMR, silico simulations were performed by randomly sampling sequencing reads from normal-like tissue epiblast as well as tumor-like tissue ExE as a spike-in. The fraction of spike-in ranged from 0.01% to 1%, which matches the fraction of ctDNA in cell- free DNA (Methods). Besides mean methylation and PMR, DNA methylation haplotype load (MHL), which quantifies level of co-methylation [22], was also included for comparison (FIGS. 9, 10 and 11). Using this approach, all three methods had significant predictive power in both the 1% and 0.1% spike-in groups; however, when the fraction of spike-in decreased to 0.01%, only the PMR-based prediction reached significance when the mean coverage of the spike-in was 5x or higher (FIG. 2C). It is noted that PMR is a *k*-mer-based approach, and highest sensitivity was achieved with a *k* of 5 when it was tested on simulated 0.01% spike-in group (FIG. 12).

[0137] **An efficient workflow to enrich for DNA methylation haplotypes**

[0138] Several recent studies have adopted either reduced-representation bisulfite sequencing (RRBS) [22], whole-genome bisulfite sequencing (WGBS) [23] or methylated DNA immunoprecipitation sequencing (MeDIP-seq) [21] approaches to profile cell-free DNA, all of which suffer from poor coverage in regions of interest in exchange for the availability of genome-wide information. Instead of these approaches, targeted bisulfite sequencing (targeted-BS) were used since this assay produces data with a stronger signal from regions of interest, associated with a lower cost as compared to the other methods. To this end, a highly specific target- capture pipeline was established using the SeqCap Epi technology [36], which is able to enrich ExE hyper CGIs (6.2 Mb in total; **Methods**) with an on-target rate of ~80%. Given the low fraction of tumor-derived DNA in plasma, most sequencing reads obtained from plasma samples stem from normal DNA, which is largely unmethylated in the target regions. Methylated DNA fragments were further specifically enriched using the MBD2 protein, followed by targeted-BS, to analyze tumor-derived DNA (**FIG. 3A**). The customized probe set exhibits similar performance as commercial probe sets in terms of enrichment uniformity; specifically, 80% of loci have a coverage higher than 60% of the median coverage (**FIG. 3B**). When tested on biopsy samples of both tumor and normal tissues, the targeted-BS approach achieved >400 fold enrichment compared to WGBS; even on challenging samples such as cell- free DNA, >100 fold enrichment was observed (**FIG. 3C**). When coupling this workflow with MBD enrichment before bisulfite conversion, high specificity was achieved with on average more than 90% of reads partially or fully methylated (**FIG. 3D**).

[0139] **Unbiased measurement of DNA methylation across assays**

[0140] By definition, PMR is the number of fully methylated *k*-mer haplotypes divided by the total number of *k*-mers in each genomic feature such as a CpG island, where it was set to 5 to maximize sensitivity (**FIG. 12**). Similarly, MHL is the normalized PMR at different *k*-mer lengths (**Methods**, $k = 1$ to 10). Thus both PMR and MHL are haplotype-based methods that are locally normalized, but neither of them can be applied without bias across assays: when the same sample was profiled by targeted-BS with or without MBD enrichment, neither PMR nor MHL were comparable between these two assays (**FIGS. 13 and 14**). An alternative method of global normalization normalizes the number of haplotypes in a region by total number of haplotypes across all regions. For a given haplotype width *k* (i.e. $k = 5$), the globally normalized coverage of

each type of DNA methylation haplotype was compared for the same samples that were profiled by both assays -- with or without MBD enrichment. Two cell lines (HuES64 and HCT116) and two primary tissues (normal uterus and uterine cancer) were profiled using this approach. The highest Pearson correlation coefficient (PCC) was observed between these two approaches when using the number of fully methylated DNA methylation haplotypes (mean PCC = 0.998) (**FIG. 4A**). For example, when the normalized coverage of fully methylated reads (NMR) was assessed for normal uterus and uterine cancer, nearly perfect correlation was observed between assays with or without MBD enrichment (PCC > 0.99, p-value < 10^{-16}) (**FIG. 4B and FIG. 15**). As expected, unbiased measurements were also observed when comparing targeted-BS and WGBS, though there were larger variations due to lower sequencing depth in WGBS-assayed samples (PCC = 0.958 for uterine cancer and PCC = 0.979 for normal uterus, p-value < 10^{-16}) (**FIG. 4C**). Taking together, NMR is an unbiased metric to quantify haplotype-level DNA methylation across WGBS and targeted-BS approaches with or without MBD enrichment. This methodological improvement led to the development of markers from existing data and validate them on new data.

[0141] **Ultra-sensitive cancer detection using DNA methylation haplotypes**

[0142] Since ctDNA levels are very low in most early-stage and many advanced stage cancer patients [6], a major challenge is how to identify a trace amount of ctDNAs out of total cfDNAs. To test the sensitivity of the MBD enrichment-based workflow, experiments mixing DNA from ES cells (HuES64) were first performed with DNA from a colon cancer cell line, HCT116, as spike-in. The NMR-based method confidently predicted 0.01% spike-in when at least 1 µg of total input DNA was used (**FIG. 16A**). However, when 50ng of total input DNA was analyzed, the prediction limit dropped to 0.1% (**FIG. 16B**). Novel analytical approaches such as NMR could improve sensitivity on targeted-BS data even without MBD enrichment which performs well with lower input DNA. When testing the targeted-BS workflow without MBD enrichment with 50 ng DNA as input, conditions with 0.01% spike-in were correctly identified with as few as 50 CGIs (**FIG. 5A and FIG. 17**). In contrast, mean methylation and MHL-based methods were only able to correctly identify the tumor signature when the fraction of spike-in DNA was larger than 0.1% (**FIG. 20A**). Detection of HCT116 DNA is easier than that of other samples, since its genome is almost fully methylated, next performed were similar dilution experiments

with primary colon cancer tissue as the spike-in. Again, NMR-based method confidently detected cancer DNA spiked-in at 0.01% (**FIGS. 5B and 18**), while mean methylation and MHL-based methods only detected 1% cancer DNA spiked-in (**FIG. 20B**). Note that the detection sensitivity depends on the background noise stemming from normal cells; for example, when uterine cancer DNA was spiked-in with normal uterus DNA, the NMR-based method was able to detect 0.1% cancer DNA (**FIG. 5C**), while both mean methylation and MHL-based methods only detected 1% cancer DNA (**FIG. 20C**). Detection sensitivity also depends on choices of parameters; for example, highest sensitivity was achieved when *k*-mer length was set to 5 for NMR method (**FIG. 19).**

[0143] Finally, the experimental and computational pipeline on plasma samples obtained from patients with colon adenocarcinoma were tested using age-matched normal individuals as negative controls. Included were two samples each from stage I, II and III patients, respectively. The platform was capable of detecting all cancers, including those in stage I, with high confidence (FDR < 1%), and no false positives were observed (**Table 1A**). To further assess the sensitivity of the method, the fraction of reads predicted to stem from tumor cells were estimated. In the colon cancer cohort, the estimated fraction of cancer DNA ranged from 0.05% to 20% (**Methods; FIG. 21**), suggesting a prediction resolution of 0.05% for colon cancer. A breast cancer patient cohort (Infiltrating ductal carcinoma) was next tested, including two cases each for stages I, II and III. The NMR-based method detected 5 of 6 cancer samples, with one false negative of a stage II sample, CDX171 (FDR < 1%, **Table 1B**), while mean methylation and MHL- based methods correctly identified only one sample each. The false negative can likely be attributed to a low tumor DNA fraction, as the estimated tumor fraction for CDX171 was around 0.03%, which is similar to background noise (**Methods and FIG. 22**).

[0144] **Machine learning methods**

[0145] Extensive prediction models using machine learning approaches (random forest, support vector machines, and deep learning) was developed to estimate the full probability distribution of all haplotypes across ExE sites with regard to each tumor type. These methods will improve the prediction accuracy of the cell type of origin based on cfDNA samples.

[0146] Pan-cancer associated methylation sites are provided in Table 3.

[0147] **Discussion**

[0148] DNA methylation haplotypes have been used for many years, but only recently was shown to be useful for cancer diagnosis; for instance, Guo et. al. demonstrated that a DNA methylation haplotype-based metric, MHL, combined with methylation haplotype blocks (MHB). An experimental and computational framework for ultra-sensitive, non-invasive early cancer detection using fully methylated DNA methylation haplotypes was proposed. As demonstrated by dilution experiments, this framework outperformed mean methylation and MHL-based methods and was able to detect 0.01% colon cancer spike-in with as few as 50 CGIs. When tested on human plasma samples, both colon and breast cancer samples were correctly detected at early stages, with a detection limit of 0.05%; this threshold is sufficiently sensitive to detect most stage I tumors. This is the first study that utilizes a universal cancer signature for non-invasive pan-cancer diagnosis, which is potentially cost effective compared to genome- wide assays [21].

[0149] **Cohort**

[0150] As described below, tumor and normal samples from 12 cancer types, with the exception of bladder and prostate cancer, in which only normal samples were included. For cancer types, different major subtypes were included whenever possible, featured by breast invasive carcinoma. All samples were processed uniformly in Broad Institute and profiled by targeted bisulfite sequencing with customized probe design that covers 8M of genomic regions which are mainly hyper-methylated in human cancer.

Tissue	Status	Subtype	# Samples
AML	Tumor		8
Bladder	Tumor		0
	Normal		2
Breast	Tumor	breast (TN)	8
		breast PR-ER-her2+	3
		breast PR-ER+her2-	1
		breast PR-ER+her2+	3
		breast PR+ER-her2-	1
		breast PR+ER-her2+	2
		breast PR+ER+her2-	8

		breast PR+ER+her2+	6
	Normal		4
Colon	Tumor	Colon adenocarcinoma	10
	Normal		2
Esophagus	Tumor	Esophageal carcinoma	10
	Normal		2
Kidney	Tumor	Kidney carcinoma (renal clear cell, renal papillary cell)	14
	Normal		2
Liver	Tumor	Liver hepatocellular carcinoma	0
	Normal		2
Lung	Tumor	adenocarcinoma	13
		squamous	6
	Normal		2
Ovary	Tumor	Adenocarcinoma of ovary	15
	Normal		2
Pancreas	Tumor	Pancreatic adenocarcinoma	11
	Normal		2
Prostate	Tumor		0
	Normal		2
Stomach	Tumor	Adenocarcinoma of stomach	5
	Normal		2

[0151] **Tissue of Origin**

[0152] An ultra-sensitive method was developed based on DNA methylation haplotypes of extraembryonically methylated CpG islands. This method could detect 0.05% of tumor DNA from cell-free DNA of patient plasma. To further develop this method and predict tissue of origin with high sensitivity, the method includes identifying cancer specific DNA methylation haplotypes. For each CpG position in designed regions, the relative abundance of all possible *k*-mer haplotypes ($k = 5$) were calculated across all tissue samples, which includes tumor and normal samples. Then a tissue-specific index (TSI) was defined for each *k*-mer as:

[0153] $$TSI = \frac{\sum_{j=1}^{n} 1 - \frac{10^{PKR(j)}}{10^{PKR(\max)}}}{n-1}$$

[0154] Where n indicate the number tissues, PKR(j) denotes fraction of a specific k-mer in tissue j and PKR max denotes PKR of the highest methylation tissue. Cancer specific DNA methylation haplotypes were selected by TSI with a cutoff of 0.6. The addition of cancer-specific DNA methylation haplotypes to the original signature enables the prediction of tissue of origin with high sensitivity.

[0155] Identified regions of cancer-specific DNA methylation are provided in Table 2.

[0156] **Methods**

[0157] **Targeted-BS and MBD enrichment**

[0158] Genomic DNA from cultured cells was extracted using Genomic DNA Clean & Concentrator kit (Zymo Research). Human tumor DNA was purchased from OriGene Technologies or BioChain Institute. Genomic DNA was sheared to average fragment size of 180 – 220 bp in 130 µl microTUBE using S2 focused-ultrasonicator (Covaris) for 300 sec at intensity 5, duty cycle 10 and 200 cycles per burst. The sheared DNA was concentrated with 1.8 volumes of Agencourt AMPure XP beads (Beckman Coulter) prior to bisulfite conversion. Purified human cell-free DNA and frozen human plasma from cancer patients were obtained from the BioChain Institute. Free circulating DNA was isolated from 4ml human plasma using QIAamp MinElute ccfDNA Mini Kit (Qiagen) scaling up the reactions as described in manufacturer's manual. In order to enrich for methylated DNA, selected samples were processed with MethylMiner Methylated DNA Enrichment Kit (Thermo Fisher Scientific). DNA bound to MBD2 protein coupled to streptavidin beads was eluted with provided high-salt buffer in a single elution step and DNA was ethanol- precipitated. Pellets were dissolved in 20 µl water. Sheared genomic DNA, cfDNA and MBD- enriched DNA was bisulfite-converted using EpiTect Fast bisulfite conversion kit (Qiagen) following kit's instructions and extending the two 60 °C cycles to 20 min. Illumina library construction was performed post-bisulfite conversion using Accel-NGS Methyl-Seq kit (Swift Biosciences) following the manufacturer's recommendations for NimbleGen SepCap Epi Hybridization Capture (Appendix Section A). Libraries were amplified by 8-14 cycles of PCR using Accel-NGS Methyl-Seq Unique Dual Indexing primers (Swift Biosciences). SeqCap Epi hybridization reactions contained a total of 1 µg of a pool of 3–4 PCR-amplified pre-capture libraries, 2 µl of xGen Universal BlockersTS Mix (Integrated DNA

Technologies) blocking oligonucleotides, and the custom SeqCap probe pool. After hybridization at 47 °C (typically ~70 h), streptavidin pull-down and washes, the entire bead-bound captured material was amplified by 9-10 cycles of PCR. Hybrid-selected libraries were sequenced on an Illumina HiSeq 2500 instrument in rapid mode together with a 10% spike-in of a non-indexed PhiX174 library.

[0159] **Probe set design for targeted-BS**

[0160] 1,265 CGIs were selected which are hypermethylated in extraembryonic tissues [28] for targeted bisulfite-sequencing. Specifically, 473 CGIs are hypermethylated in mouse extraembryonic ectoderm and were lifted over to human genome; the rest is hypermethylated in 8 out of 14 TCGA cancer types and also human placenta. To cover loci with multiple hypermethylated CGIs, such as the OTX2 locus, CGIs that are 20k bp apart were merged. The resulting regions were extended 2k upstream and downstream, respectively, to cover CpG shores. Probes were designed by NimbleDesign with default parameters (design.nimblegen.com). The resulting design covers 6.1 Mbps with an estimated coverage of 98.2%.

[0161] **Data processing**

[0162] Raw sequencing reads were pre-processed by 'trim_galore (v0.4.4)', with the following parameters: '--clip_R1 5 --three_prime_clip_R1 2 --clip_R2 10 --three_prime_clip_R2 2'. Low-quality base calls and adapters were trimmed off from the 3' end of the reads by default. Trimmed reads were aligned to human reference genome GRCh37 using Bismark (v0.19.0) [37] with default parameters. Duplicate reads were identified and removed using tools in Bismark. DNA methylation haplotypes were extracted using an in-house tool called mHaplotype (github.com/JiantaoShi/mHaplotype). Reads with methylated cytosines in a non-CpG context (CHG, CHH) were removed to eliminate potential bias caused by incomplete bisulfite conversion.

[0163] **In silico simulation**

[0164] ExE and Epiblast represent typical tumor-like and normal-like genomes, respectively, in terms of DNA methylation landscapes. To evaluate the performance of different cancer

prediction methods, in silico simulations were performed by randomly sampling sequencing reads from ExE and epiblast samples. Briefly, ExE and epiblast RRBS data were obtained from the public data set GSE98963, which contains 4 biological replicates for each tissue. DNA methylation haplotypes were extracted by the in-house tool 'mHaplotype' and biological replicates were pooled. Sequencing reads were randomly sampled from epiblast as well as ExE as spike-in, representing 1%, 0.1% and 0.01% of total reads, in three groups of simulations, respectively. In each group, the mean coverages of spike-in DNA ranged from 1 to 20, each with 10 replicates. Negative controls were also included, in which spike-in reads were sampled from epiblast.

[0165] **Estimating methylation levels**

[0166] Mean methylation levels were estimated as the number of sites reporting a C, divided by the total number of sites reporting a C or T. The methylation pattern of CpGs on each fragment represents a discrete DNA methylation haplotype. Methylation haplotype load (MHL), the normalized fraction of methylated haplotypes at different lengths, was calculate as previously described [22]:

$$MHL = \frac{\sum_{k=1}^{10} w_k * PMR_k}{\sum_{k=1}^{10} w_k}$$

$$w_k = k$$

[0167] Where k is the length of haplotypes, and for a haplotype of length L, all substrings with length from 1 to a maximum of 10 in this calculation was considered. w_k is the weight for k-mer haplotype. In the present study, $w_k = k$ was applied. PMR_k is the fraction of fully successive methylated CpGs for haplotypes of length k (k-mer) (FIG. 8). In this study, k was set to 5 to maximize detection sensitivity (FIG. 12). To calculate the normalized coverage of fully methylated reads (NMR), the number of fully methylated k-mers was determined in each CGI which is then divided by the total number of fully methylated k-mers in all designed regions, followed by a mean scaling. Again, k was set to 5 to maximize detection sensitivity (FIG. 19).

[0168] **Prediction the presence of cancer DNA**

[0169] Presence of cancer-specific DNA methylation suggests presence of cancer DNA in a mixture. As described above, four metrics, mean methylation, MHL, PMR and NMR, were used for DNA methylation quantification and cancer prediction. Four types of samples were used for prediction: tumor tissue samples, normal tissue samples, normal cfDNA samples and patient cfDNA samples. For a given CGI, the DNA methylation in these groups were represented as $Me_{(t)}$, $Me_{(n)}$, $Me_{(f)}$, $Me_{(p)}$, respectively. Regardless of metrics used, the general steps for cancer prediction are quite similar.

[0170] **Marker identification**

[0171] ExE hyper CGIs are largely hyper-methylated in cancer vs. normal. Markers were redefined for each cancer type and metric used to maximize detection sensitivity. Specifically, tumor tissue samples were compared to normal tissue samples to define markers that are hypermethylated in tumors with a threshold of 0.1 ($Me_{(t)} - Me_{(n)} > 0.1$).

[0172] **Marker refinement**

[0173] Selected markers were then ranked in descending order based on the difference of methylation between tumor samples and normal cfDNA (Me(t) – Me(f)). The top 200 regions were chosen as markers for cancer prediction.

[0174] **Significance test**

[0175] The test samples were compared to normal cfDNA samples using cancer markers defined above, the resulting difference of methylation was defined as $\Delta Me = Me_{(p)} - Me_{(f)}$. Instead of using actual values of methylation difference, the number of markers with increased methylation ($\Delta Me > 0$) and decreased methylation ($\Delta Me < 0$) were counted. The higher the number of markers with increased methylation, the more likely a cancer sample is detected. P- value is computed by one-sided binomial tested and corrected for multiple testing using Benjamini–Hochberg procedure.

[0176] **Predicting the fraction of tumor DNA**

[0177] The fraction of tumor DNA was predicted by comparing the observed data to simulated normal cfDNA data with tumor DNA as spike-in, the fractions of which ranged from 0.01% to 100%. NMR was compared between observed (NMP_o) and simulated samples (NMP_s) using pre-defined markers for each cancer type, the resulting difference was denoted as $\Delta NMR = NMR_s - NMR_o$. Then a distance metric was calculated as follows:

$$d = abs(log2\left(\frac{sum(\Delta NMR > 0)}{sum(\Delta NMR < 0)}\right))$$

[0178] The predicted tumor fraction was defined as the value that minimized the distance d.

[0179] **Cancer prediction using TCGA 450K array data**

[0180] In order to evaluate performance of ExE hyper CGIs in cancer prediction, 14 TCGA cancer types were tested that contain matched normal tissues in TCGA. Samples from thyroid cancer data set were removed, since thyroid cancer and normal thyroid tissue cannot be distinguished by ExE hyper CGIs [28]. This pan-cancer cohort consists of 685 tumor samples and 710 normal samples.

[0181] Half of the samples were randomly chosen as a training set, and the remainder were used for validation. Support vector machine (SVM) with a Gaussian kernel from the R package kernlab was used for classification. To resolve dependence between ExE hyper CGIs, 50 CGIs were randomly chosen for classification and this process was repeated 200 times, the resulting prediction scores were averaged as final concensus scores. Receiver operating characteristic (ROC) curves were generated by R package ROCR.

[0182] Similary, random forest (RF) was implemented using the 'randomForest' function of the 'randomForest' R package, using default parameter settings. Classification accuracy was calculated as the proportion of samples in the validation set that the trained model correctly classified. False positive rate and true positive rate were calculated using the 'roc' function of the 'pROC' R package, based on the 'out-of-bag' votes for the training data. Area under the ROC

curve (AUC) was calculated based on these values using the 'auc' function, also from the 'pROC' package.

[0183] **Data availability**

[0184] All datasets have been deposited in the Gene Expression Omnibus and are accessible under GSE84236. Additional data include: TCGA DNA methylation, mutation data, and the full name of tumor types from the Broad Firehose (gdac.broadinstitute.org).

Disease Name	**Cohort**	**Cases**
Adrenocortical carcinoma	ACC	92
Bladder urothelial carcinoma	BLCA	412
Breast invasive carcinoma	BRCA	1098
Cervical and endocervical cancers	CESC	307
Cholangiocarcinoma	CHOL	51
Colon adenocarcinoma	COAD	460
Colorectal adenocarcinoma	COADREAD	631
Lymphoid Neoplasm Diffuse Large B-cell Lymphoma	DLBC	58
Esophageal carcinoma	ESCA	185
FFPE Pilot Phase II	FPPP	38
Glioblastoma multiforme	GBM	613
Glioma	GBMLGG	1129
Head and Neck squamous cell carcinoma	HNSC	528
Kidney Chromophobe	KICH	113
Pan-kidney cohort (KICH+KIRC+KIRP)	KIPAN	973
Kidney renal clear cell carcinoma	KIRC	537
Kidney renal papillary cell carcinoma	KIRP	323
Acute Myeloid Leukemia	LAML	200
Brain Lower Grade Glioma	LGG	516
Liver hepatocellular carcinoma	LIHC	377
Lung adenocarcinoma	LUAD	585
Lung squamous cell carcinoma	LUSC	504
Mesothelioma	MESO	87
Ovarian serous cystadenocarcinoma	OV	602
Pancreatic adenocarcinoma	PAAD	185
Pheochromocytoma and Paraganglioma	PCPG	179
Prostate adenocarcinoma	PRAD	499
Rectum adenocarcinoma	READ	171
Sarcoma	SARC	261

Skin Cutaneous Melanoma	SKCM	470
Stomach adenocarcinoma	STAD	443
Stomach and Esophageal carcinoma	STES	628
Testicular Germ Cell Tumors	TGCT	150
Thyroid carcinoma	THCA	503
Thymoma	THYM	124
Uterine Corpus Endometrial Carcinoma	UCEC	560
Uterine Carcinosarcoma	UCS	57
Uveal Melanoma	UVM	80

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[0224] Table 1A:

ID	Stages	Fraction	# > 0	# < 0	# = 0	P-value	FDR
CDX173	I	0.08%	124	61	15	2.11E-06	8.42E-06
CDX174	I	20%	190	10	0	1.47E-44	1.03E-43
CDX175	II	1%	162	31	7	6.65E-23	3.33E-22
CDX176	II	0.05%	117	74	9	1.14E-03	3.43E-03
CDX109	III	2%	176	22	2	2.53E-31	1.52E-30
CDX110	III	10%	192	8	0	3.58E-47	2.86E-46
CDX182	Normal	0.01%	85	102	13	9.06E-01	1.00E+00
CDX181	Normal	0.01%	63	120	17	1.00E+00	1.00E+00

[0225] Table 1B:

ID	Stages	Fraction	# > 0	# < 0	# = 0	P-value	FDR
CDX169	I	1%	150	45	5	1.07E-14	6.42E-14
CDX170	I	0.4%	122	72	6	2.03E-04	8.14E-04
CDX171	II	0.03%	77	119	4	9.99E-01	1.00E+00
CDX172	II	2%	162	36	2	1.38E-20	9.66E-20
CDX107	III	0.7%	125	72	3	9.75E-05	4.88E-04
CDX108	III	2%	169	29	2	1.57E-25	1.26E-24
CDX182	Normal	0.01%	75	116	9	9.99E-01	1.00E+00
CDX181	Normal	0.03%	65	127	8	1.00E+00	1.00E+00

[0226] Table 2: TOO Methylation Sites

chr1:1072370-1072847	chr11:65190825-65191058	chr16:72821141-72821592
chr1:10895896-10896117	chr11:65222491-65222750	chr16:73099813-73100791
chr1:109203594-109204378	chr11:65341621-65342501	chr16:743925-745943
chr1:1093212-1093476	chr11:65343330-65343849	chr16:78079753-78080166
chr1:110185962-110186164	chr11:65553750-65555573	chr16:80574742-80575090
chr1:110626529-110627484	chr11:65779312-65779767	chr16:80965953-80966478
chr1:110880395-110880624	chr11:66034752-66035054	chr16:84029457-84029710
chr1:111505882-111507007	chr11:66035217-66035447	chr16:84328520-84328720

chr1:111746338-111747303	chr11:66049751-66050229	chr16:84346477-84346931
chr1:113044411-113044992	chr11:66314208-66314455	chr16:84401958-84402497
chr1:113392143-113392807	chr11:66335576-66336151	chr16:85171020-85171323
chr1:113497987-113498206	chr11:67232299-67232558	chr16:85783863-85785131
chr1:1141671-1142150	chr11:67770427-67771629	chr16:85863382-85863601
chr1:11538670-11540342	chr11:67806252-67806611	chr16:85932122-85932942
chr1:116694665-116694983	chr11:68611251-68611807	chr16:86546360-86546632
chr1:116710838-116711260	chr11:69258150-69258544	chr16:87902455-87903460
chr1:11710460-11710788	chr11:69924339-69925197	chr16:88292764-88293010
chr1:11779567-11780016	chr11:705795-706534	chr16:88716990-88717606
chr1:118727817-118728097	chr11:70962174-70964161	chr16:88803803-88804112
chr1:120835962-120839391	chr11:71954817-71955659	chr16:88850205-88850537
chr1:12655927-12656248	chr11:720562-721369	chr16:89070647-89070904
chr1:1362955-1363299	chr11:72301303-72301746	chr16:89267824-89268087
chr1:1370768-1371449	chr11:72463093-72463717	chr16:89268493-89268865
chr1:13839506-13840613	chr11:72492282-72492644	chr16:89323281-89323661
chr1:13909607-13909842	chr11:74022429-74022703	chr16:89632593-89632799
chr1:14026482-14027200	chr11:75236190-75237781	chr16:90014251-90014613
chr1:14219351-14219737	chr11:75917272-75917926	chr17:10632790-10633490
chr1:146556313-146556676	chr11:77122737-77123088	chr17:11501632-11502328
chr1:14924611-14925993	chr11:78673008-78673213	chr17:1163342-1163773
chr1:149605515-149605903	chr11:789872-790133	chr17:12692738-12693690
chr1:150254366-150254637	chr11:8102359-8102913	chr17:1390457-1390786
chr1:150266477-150266689	chr11:826942-827625	chr17:1395120-1395372
chr1:151300523-151300724	chr11:8284103-8285032	chr17:14212364-14212788
chr1:151445872-151446142	chr11:86382696-86383586	chr17:15244706-15245126
chr1:151693992-151694282	chr11:87908244-87908614	chr17:15466360-15466843
chr1:151812254-151812525	chr11:9025096-9026315	chr17:1546743-1547324
chr1:151966633-151966893	chr11:93583375-93583717	chr17:1551731-1553249
chr1:152079998-152081705	chr11:94473536-94474338	chr17:15847758-15849513
chr1:154298206-154298544	chr11:94501367-94502696	chr17:16283928-16284768
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chr1:155043413-155043922	chr11:98891544-98891821	chr17:2627241-2628302
chr1:155830196-155830489	chr12:103350090-103350422	chr17:26578273-26578682
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chr1:156616554-156616946	chr12:103359249-103359629	chr17:26698360-26699557
chr1:156646293-156647260	chr12:104850254-104852395	chr17:26711384-26712311
chr1:156814882-156815792	chr12:105478090-105478517	chr17:27038085-27038919
chr1:156893520-156894232	chr12:106532107-106533696	chr17:27332269-27333188
chr1:157963541-157963947	chr12:107711604-107714107	chr17:27503599-27504014

chr1:158119489-158119704	chr12:108297427-108297743	chr17:27942533-27945388
chr1:159141203-159141718	chr12:109162409-109162722	chr17:27949430-27950277
chr1:15929824-15930289	chr12:109729573-109729826	chr17:29298047-29298606
chr1:160040129-160040668	chr12:110150048-110150262	chr17:29718231-29719291
chr1:16085148-16085862	chr12:110156268-110156496	chr17:29814615-29815662
chr1:161228478-161229028	chr12:111471961-111473546	chr17:30593199-30594033
chr1:162760251-162760722	chr12:112204499-112204979	chr17:30845904-30846702
chr1:162792177-162792574	chr12:115104849-115105548	chr17:32953154-32953801
chr1:16543684-16544307	chr12:115120775-115122945	chr17:33787402-33787845
chr1:166134259-166136448	chr12:115135926-115136350	chr17:33814235-33814947
chr1:167789397-167789647	chr12:115889598-115889995	chr17:34091137-34091919
chr1:17033769-17034728	chr12:116354788-116355187	chr17:3438842-3439046
chr1:171810468-171811325	chr12:116946196-116946607	chr17:35060323-35060692
chr1:179555402-179555770	chr12:117316390-117317611	chr17:35303285-35303572
chr1:180881317-180882592	chr12:117536291-117537421	chr17:36102034-36104766
chr1:182584178-182584545	chr12:120031495-120033212	chr17:36105335-36105583
chr1:184633224-184633663	chr12:120799373-120799912	chr17:36575500-36575782
chr1:1875618-1875877	chr12:122277302-122277539	chr17:36584421-36585453
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[0227] Table 3: Pan Cancer Methylation Sites

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CLAIMS

What is claimed is:

1. A method of characterizing a cell-free DNA (cfDNA) sample from a subject, comprising:
    a) receiving sequencing data comprising reads of methylation sequences for a genomic sequence from the cfDNA sample, wherein the genomic sequence comprises a plurality of CpG Islands (CGI) methylated in the genome of extraembryonic ectoderm (ExE) and not methylated in corresponding epiblast or adult tissue,
    b) determining a proportion of haplotypes of the genomic sequence that are fully methylated, and
    c) characterizing the cfDNA sample as comprising fully methylated cfCDNA if the proportion of haplotypes is greater than a significance threshold.

2. The method of claim 1, wherein each haplotype comprises five CGI methylated in the genome of ExE and not methylated in corresponding epiblast or adult tissue.

3. The method of claims 1-2, wherein the cfDNA sample comprises between 0.01% and 0.1% tumor DNA.

4. The method of claims 1-3, wherein the sequencing data comprises sequence information for less than 0.3% of the genome of the subject.

5. The method of claims 1-4, wherein the sequencing data comprises sequence information substantially limited to one or more regions of the subject's genome having a plurality of CGI methylated in the genome of ExE and not methylated in corresponding epiblast or adult tissue.

6. The method of claims 1-5, wherein fully methylated haplotypes determined in step b) are compared to one or more pre-established fully methylated haplotype signatures and the cfDNA sample is further characterized as corresponding or not corresponding to the pre-established fully methylated haplotype signature.

7. The method of claim 6, wherein the pre-established fully methylated haplotype signature has been identified via a method comprising random forest, support vector machine, or deep learning analysis.

8. The method of claims 1-7, wherein the sequencing data comprising reads of methylation sequences for a genomic sequence from the cfDNA sample has been enriched for sequences comprising methylation.

9. The method of claim 8, wherein the enrichment comprises an MBD2 protein-based enrichment method.

10. The method of claims 1-9, wherein the cfDNA sample was obtained from plasma, urine, stool, menstrual fluid, or lymph fluid.

11. The method of claims 1-10, wherein the genomic sequence comprises a contiguous sequence of about 8 megabases of the human genome comprising a plurality of CpG Islands (CGI) methylated in the genome of extraembryonic ectoderm (ExE) and/or one or more regions identified in Table 3.

12. The method of claims 1-10, wherein the genomic sequence comprises 50-75 CpG Islands (CGI) methylated in the genome of extraembryonic ectoderm (ExE).

13. The method of claims 1-12, further comprising a step of determining a tissue of origin from the sequencing data.

14. A method for detecting cancer in a subject, comprising
    a) receiving sequencing data comprising reads of methylation sequences for a genomic sequence from a cfDNA sample from the subject, wherein the genomic sequence comprises a plurality of CpG Islands (CGI) methylated in the genome of extraembryonic ectoderm (ExE) and not methylated in corresponding epiblast or adult tissue,
    b) determining a proportion of haplotypes of the genomic sequence that are fully methylated, and
    c) detecting cancer in the subject if the proportion of fully methylated haplotypes is greater than a significance threshold.

15. The method of claim 14, wherein each haplotype comprises five CGI methylated in the genome of ExE and not methylated in corresponding epiblast or adult tissue.

16. The method of claims 14-15, wherein the cfDNA sample comprises between 0.01% and 0.1% tumor DNA.

17. The method of claims 14-16, wherein the sequencing data comprises sequence information for less than 0.3% of the genome of the subject.

18. The method of claims 14-17, wherein the sequencing data comprises sequence information substantially limited to one or more regions of the subject's genome having a plurality of CGI methylated in the genome of ExE and not methylated in corresponding epiblast or adult tissue.

19. The method of claims 14-18, wherein fully methylated haplotypes determined in step b) are compared to one or more pre-established fully methylated haplotype signatures corresponding to one or more tumor types, and the presence or absence of the one or more tumor types are detected in the subject.

20. The method of claim 19, wherein the one or more tumor types comprise one or more of acute myeloid leukemia, bladder cancer, breast cancer, colon cancer, esophageal cancer, kidney cancer, liver cancer, lung cancer, ovarian cancer, pancreatic cancer, prostate cancer, or stomach cancer.

21. The method of claims 19-20, wherein the pre-established fully methylated haplotype signatures corresponding to one or more tumor types have been identified via a method comprising random forest, support vector machine, or deep learning analysis.

22. The method of claims 14-21, wherein the sequencing data comprising reads of methylation sequences for a genomic sequence from the cfDNA sample has been enriched for sequences comprising methylation.

23. The method of claim 22, wherein the enrichment comprises an MBD2 protein-based enrichment method.

24. The method of claims 14-23, wherein the cfDNA sample was obtained from plasma, urine, stool, menstrual fluid, or lymph fluid.

25. The method of claims 14-24, wherein the presence of cancer is detected in the sample with 100% sensitivity and 95% specificity.

26. The method of claims 14-25, wherein the cancer is stage I or stage III.

27. The method of claims 14-26, wherein the cancer is selected from the group comprising adenocarcinoma, acute myeloid leukemia, bladder cancer, breast cancer, colon cancer, esophageal cancer, kidney cancer, liver cancer, lung cancer, ovarian cancer, pancreatic cancer, prostate cancer, stomach cancer, and uterine cancer.

28. The method of claims 14-27, further comprising a step of treating the subject for cancer when cancer is detected in the subject.

29. The method of claims 14-28, further comprising a step of determining a tissue of origin from the sequencing data.

30. A method of detecting eradication of cancer from a subject, comprising
    a) receiving sequencing data comprising reads of methylation sequences for a genomic sequence from a cfDNA sample from a subject after a cancer treatment, wherein the genomic sequence comprises a plurality of CpG Islands (CGI) methylated in the genome of extraembryonic ectoderm (ExE) and not methylated in corresponding epiblast or adult tissue,
    b) determining a proportion of haplotypes of the genomic sequence that are fully methylated, and
    c) detecting cancer in the subject if the proportion of fully methylated haplotypes is greater than a significance threshold,
    d) wherein if cancer is not detected in the subject then the cancer has been eradicated from the subject.

31. The method of claims 14-29, wherein the genomic sequence comprises a contiguous sequence of about 8 megabases of the human genome comprising a plurality of CpG Islands (CGI) methylated in the genome of extraembryonic ectoderm (ExE).

32. The method of claims 14-29, wherein the genomic sequence comprises 50-75 CpG Islands (CGI) methylated in the genome of extraembryonic ectoderm (ExE).

33. A method of determining a probability distribution of haplotypes comprising
    a) receiving sequencing data comprising reads of methylation sequences for a genomic sequence from a cfDNA sample, wherein the genomic sequence comprises a plurality of CpG Islands (CGI) methylated in the genome of extraembryonic ectoderm (ExE) and not methylated in corresponding epiblast or adult tissue,
    b) assigning a training or validation set based on the methylated ExE CGI data,
    c) applying a machine learning method to estimate the probability distribution of all haplotypes across ExE sites, and
    d) determining one or more classifications of tumor versus normal samples based on a prediction score obtained from the machine learning method.

34. The method of claim 33, wherein the machine learning method is random forest.

35. The method of claim 33, wherein the machine learning method is a support vector machine.

36. The method of claim 33, wherein the machine learning method is deep learning.

37. The method of claims 33-36, further comprising the method step of evaluating the performance of the prediction comprising performing an in silico simulation by comparing randomly sampled sequencing reads from epiblast or adult tissue with the ExE reads.

38. The method of claims 33-37, further comprising the step of determining a tissue of origin from the sequencing data.

39. A method of determining a tissue origin comprising
    a) receiving targeted bisulfite sequencing data comprising reads of methylation sequences for a genomic sequence from a cfDNA sample, and

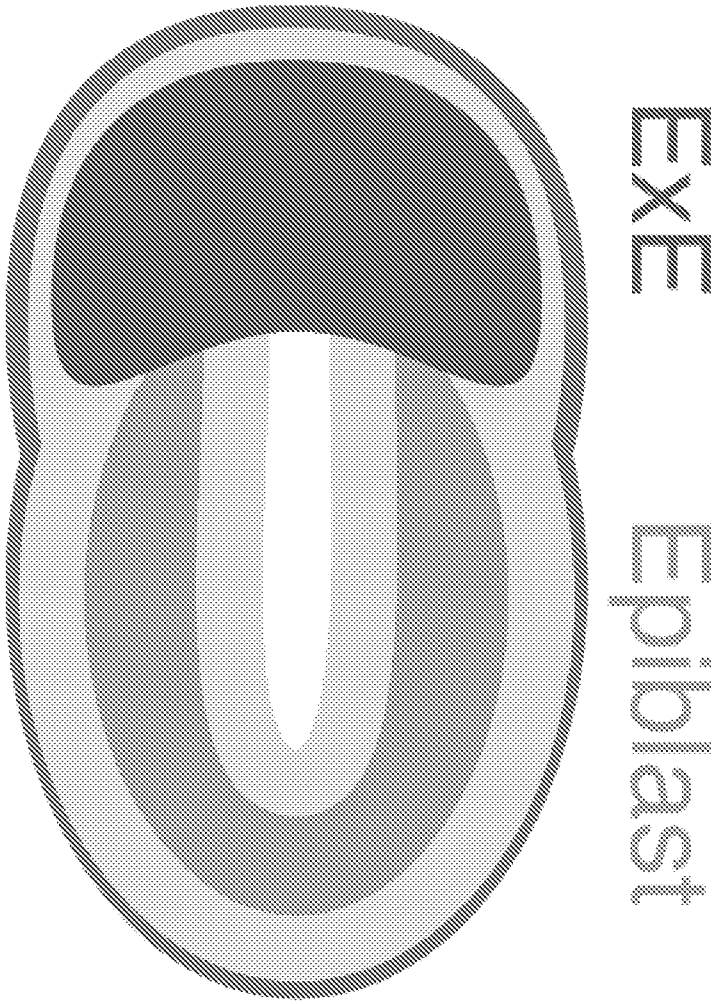
b) determining a tissue of origin by calculating a relative abundance of haplotypes from the methylated genomic regions by defining a tissue-specific index (TSI) for each haplotype.

40. The method of claim 39, wherein the TSI is calculated by the formula:

$$TSI = \frac{\sum_{j=1}^{n} 1 - \frac{10^{PKR(j)}}{10^{PKR(\max)}}}{n-1}$$

wherein n is the number of tissues, PKR(j) is the fraction of a specific haplomer in tissue, and j and PKR max are PKR of the highest methylated tissue.

41. The method of claims 39-40, wherein methylation in one or more of the regions identified in Table 2 are measured.



Mouse E6.5 Conceptus

FIG. 1A

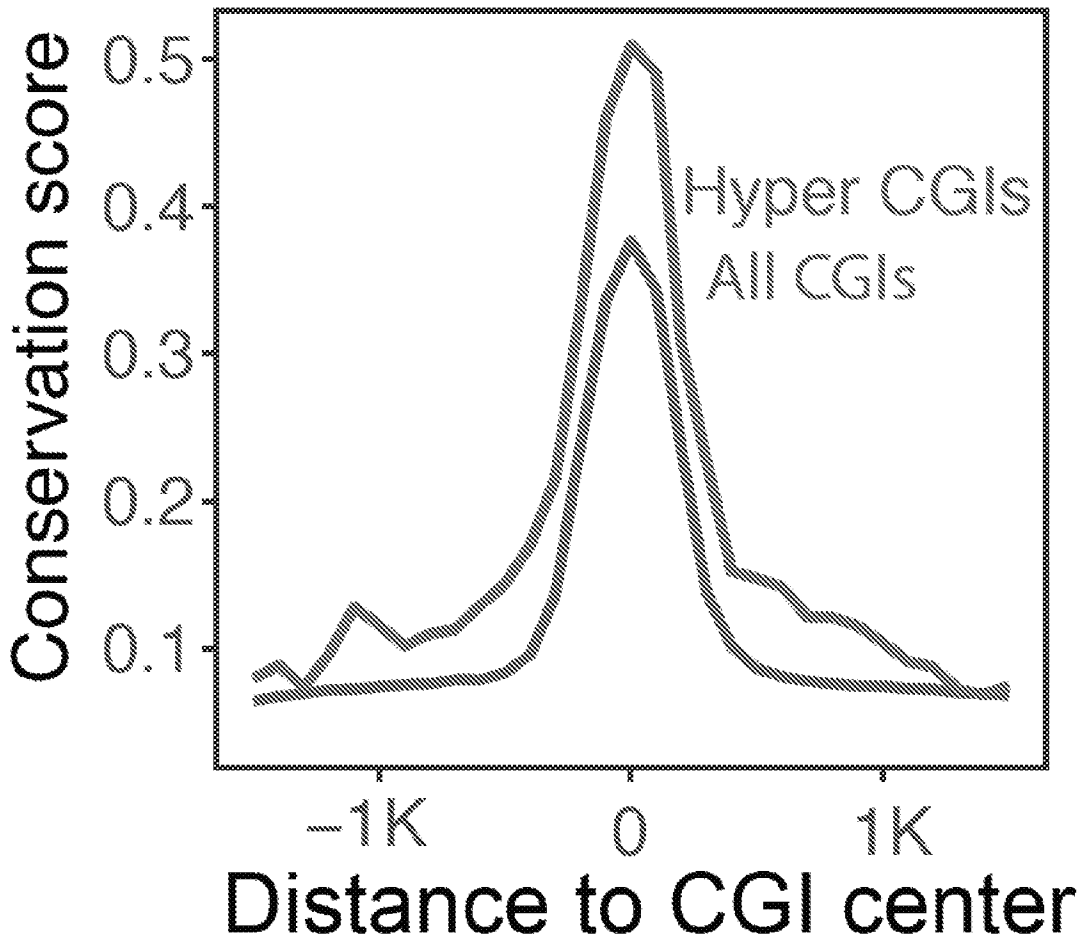

Conservation score
0.5
0.4
0.3
0.2
0.1
Hyper CGIs
All CGIs
−1K
0
1K
Distance to CGI center

FIG. 1B

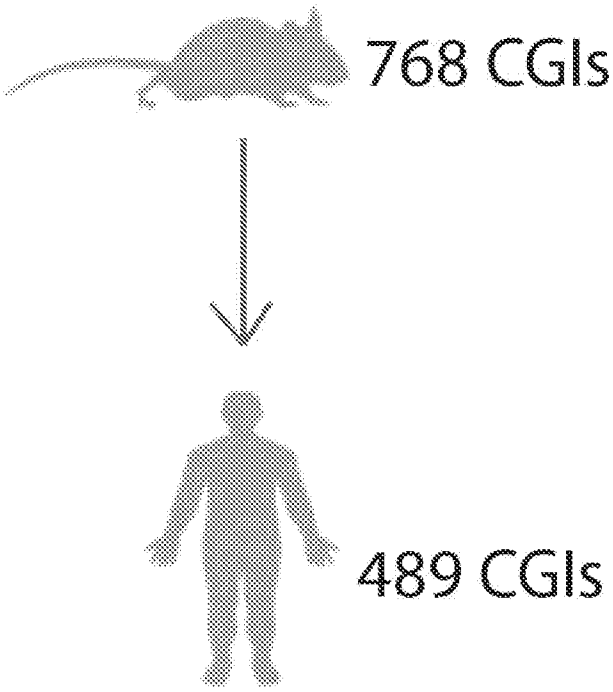

768 CGIs
489 CGIs

FIG. 1C

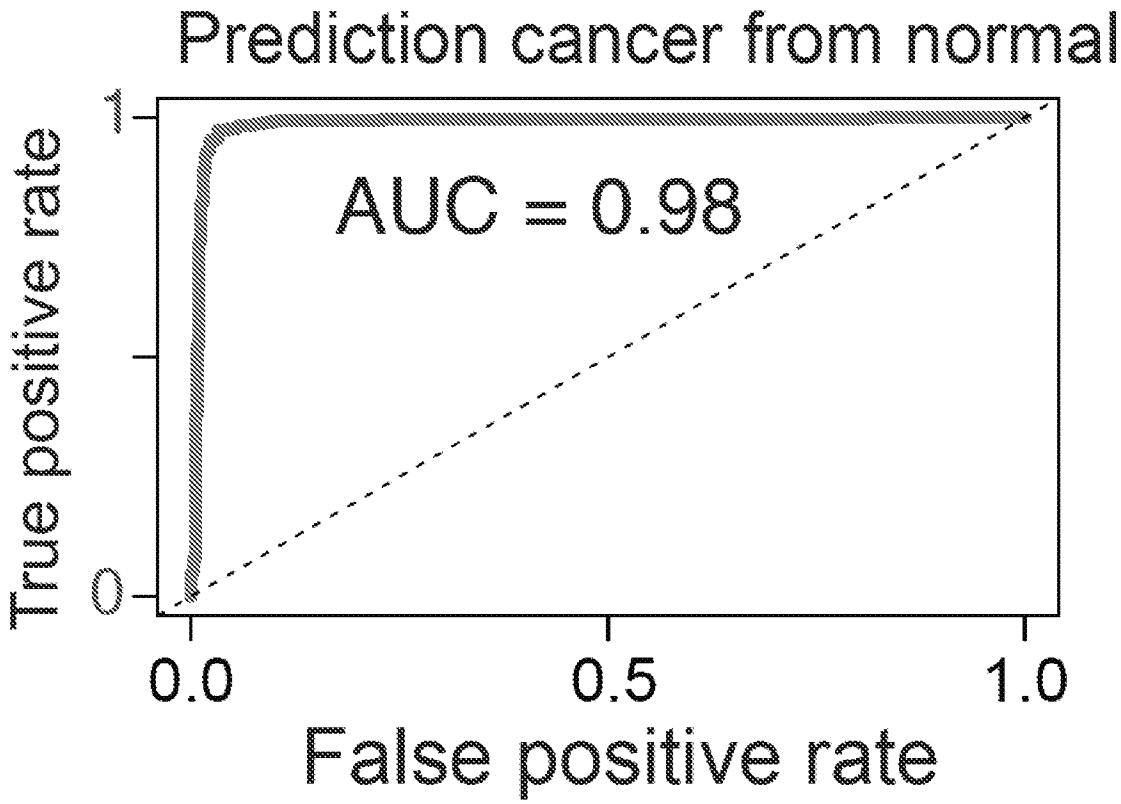

Prediction cancer from normal
True positive rate
1
0
AUC = 0.98
0.0
0.5
1.0
False positive rate

FIG. 1D

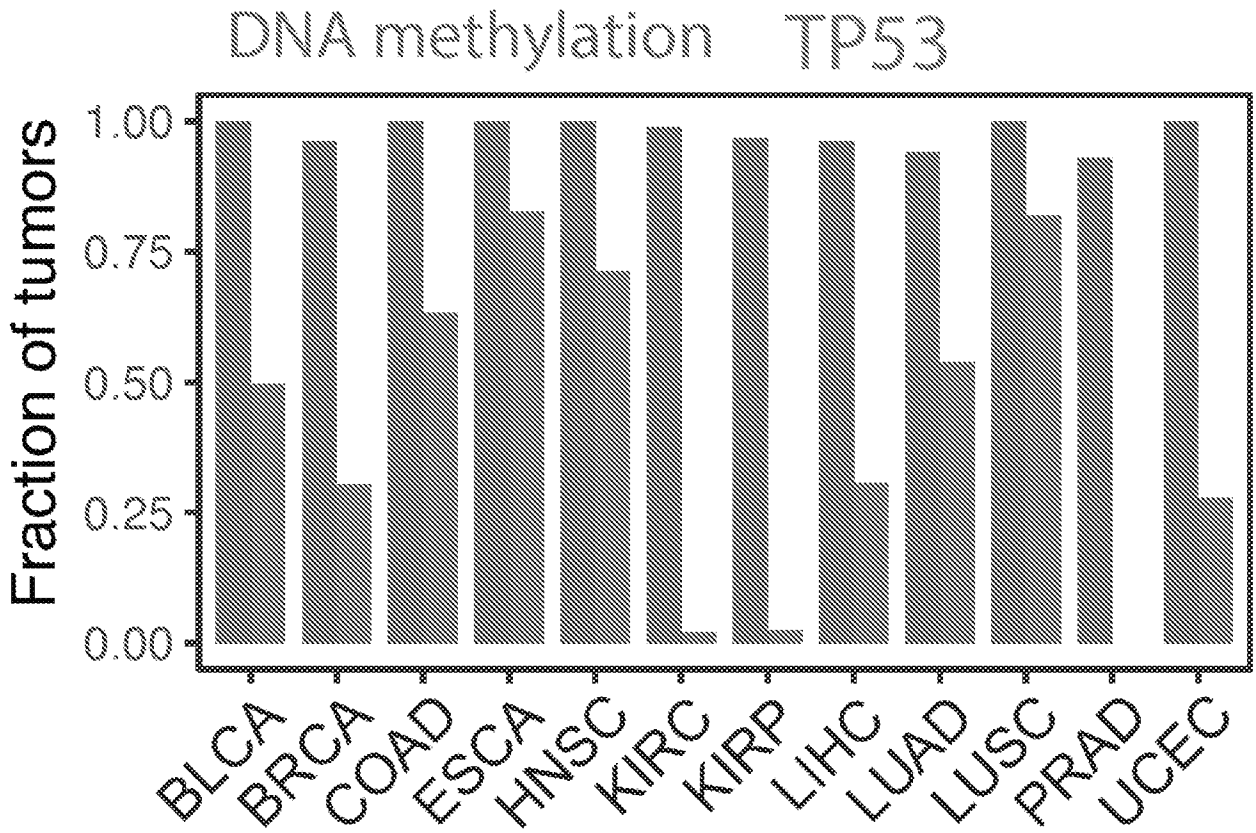
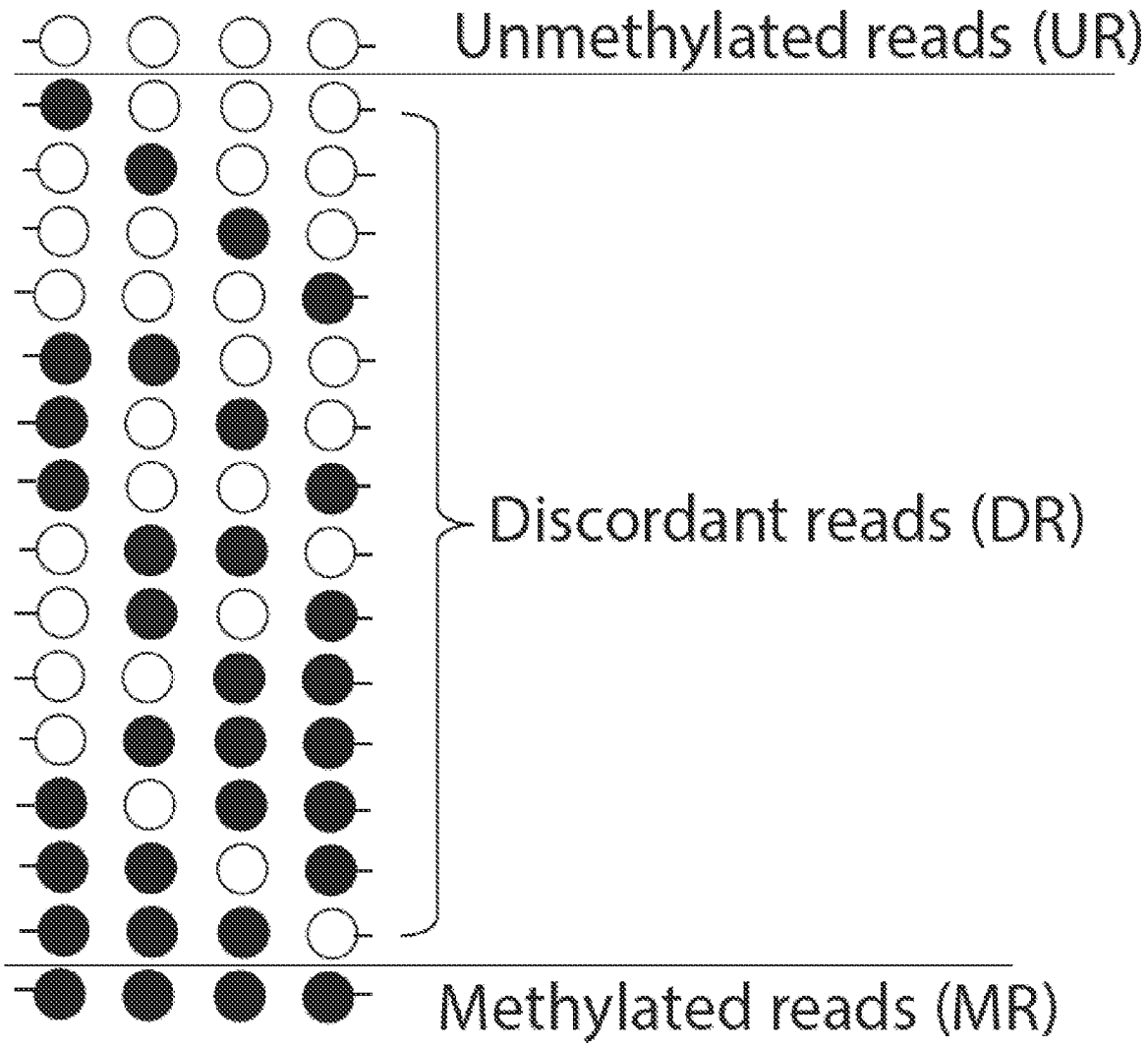

DNA methylation TP53
Fraction of tumors
1.00
0.75
0.50
0.25
0.00
BLCA
BRCA
COAD
ESCA
HNSC
KIRC
KIRP
LIHC
LUAD
LUSC
PRAD
UCEC

FIG. 1E



$$PMR = \frac{\text{no. of MR}}{\text{Total number of reads}}$$

FIG. 2A

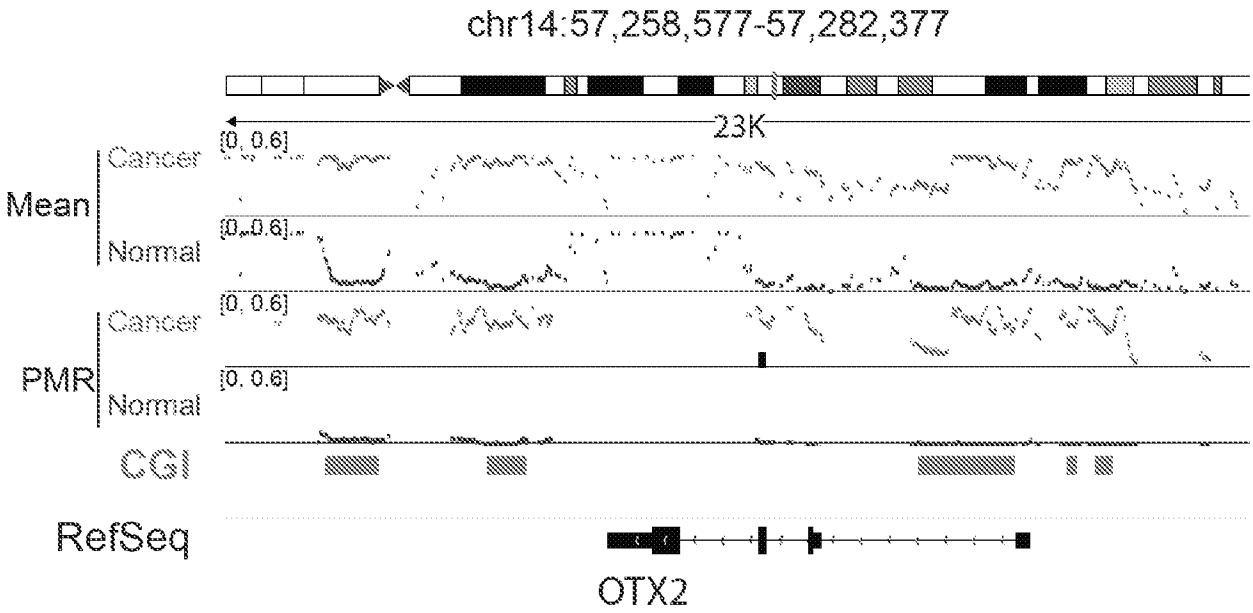

chr14:57,258,577-57,282,377
23K
Mean
Cancer
Normal
PMR
Cancer
Normal
[0, 0.6]
CGI
RefSeq
OTX2

FIG. 2B

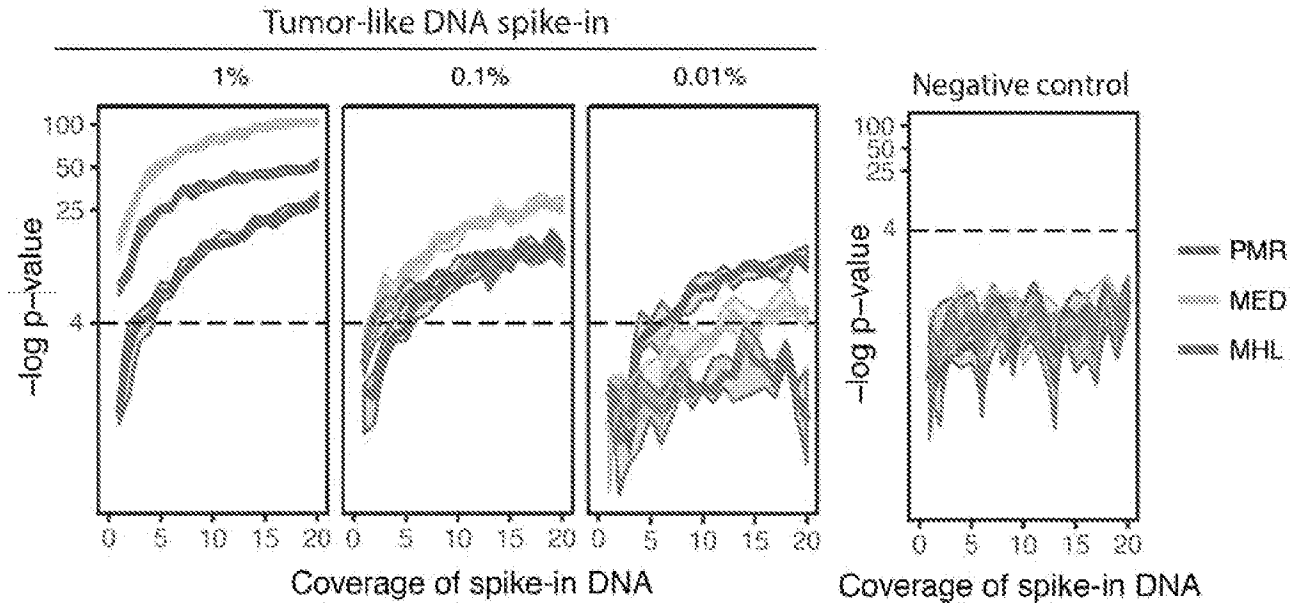

Tumor-like DNA spike-in
1%
0.1%
0.01%
Negative control
−log p−value
Coverage of spike-in DNA
PMR
MED
MHL

FIG. 2C

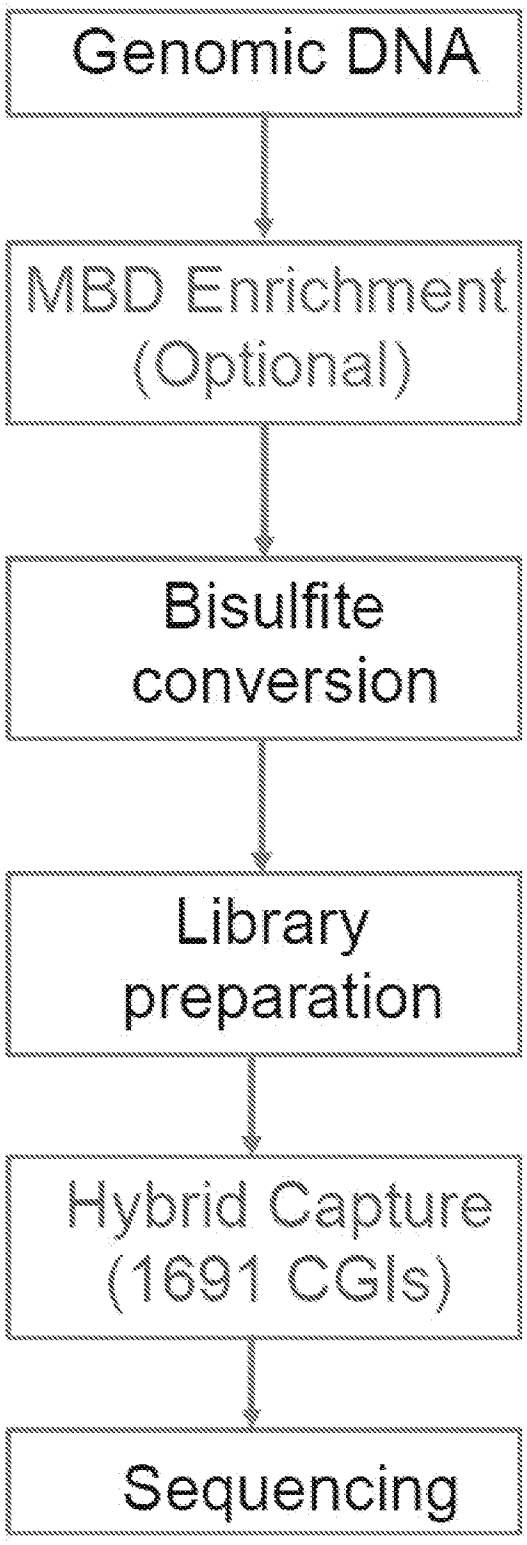

Genomic DNA
MBD Enrichment (Optional)
Bisulfite conversion
Library preparation
Hybrid Capture (1691 CGIs)
Sequencing

FIG. 3A

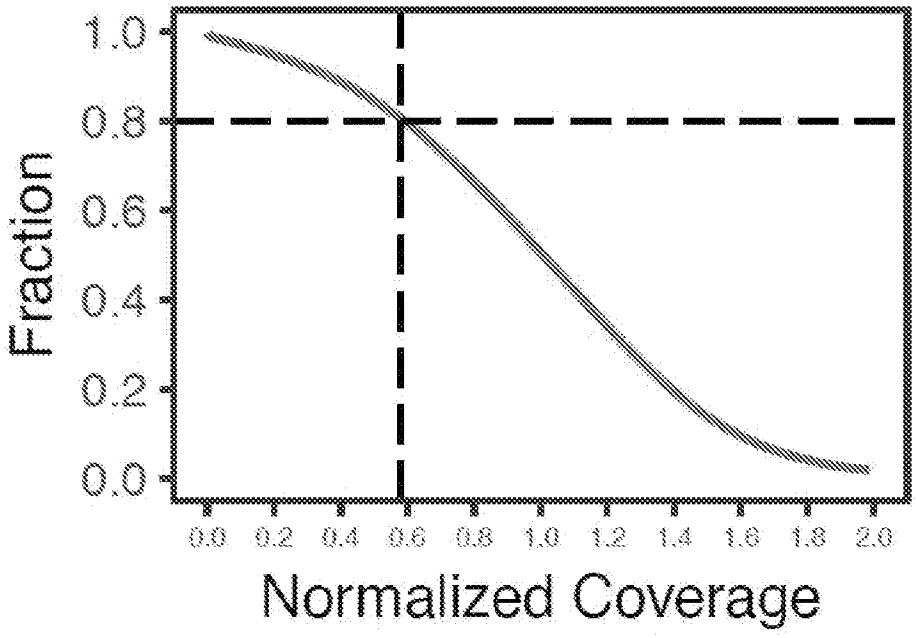

Fraction
1.0
0.8
0.6
0.4
0.2
0.0
0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0
Normalized Coverage

FIG. 3B

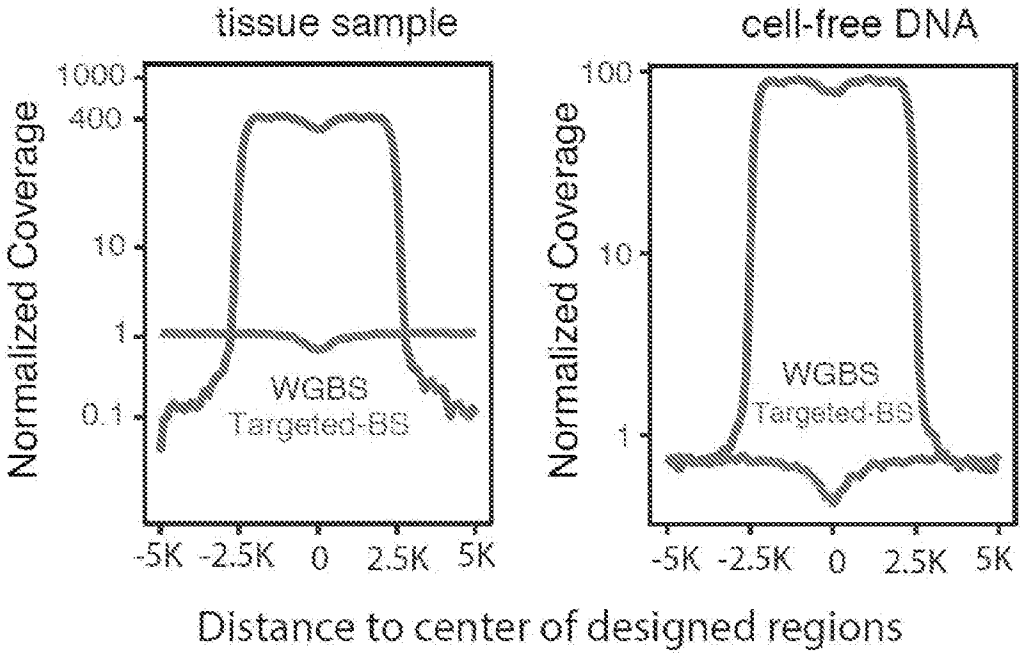

tissue sample
cell-free DNA
Normalized Coverage
1000
400
10
1
0.1
WGBS
Targeted-BS
-5K -2.5K 0 2.5K 5K
Normalized Coverage
100
10
1
WGBS
Targeted-BS
-5K -2.5K 0 2.5K 5K
Distance to center of designed regions

FIG. 3C

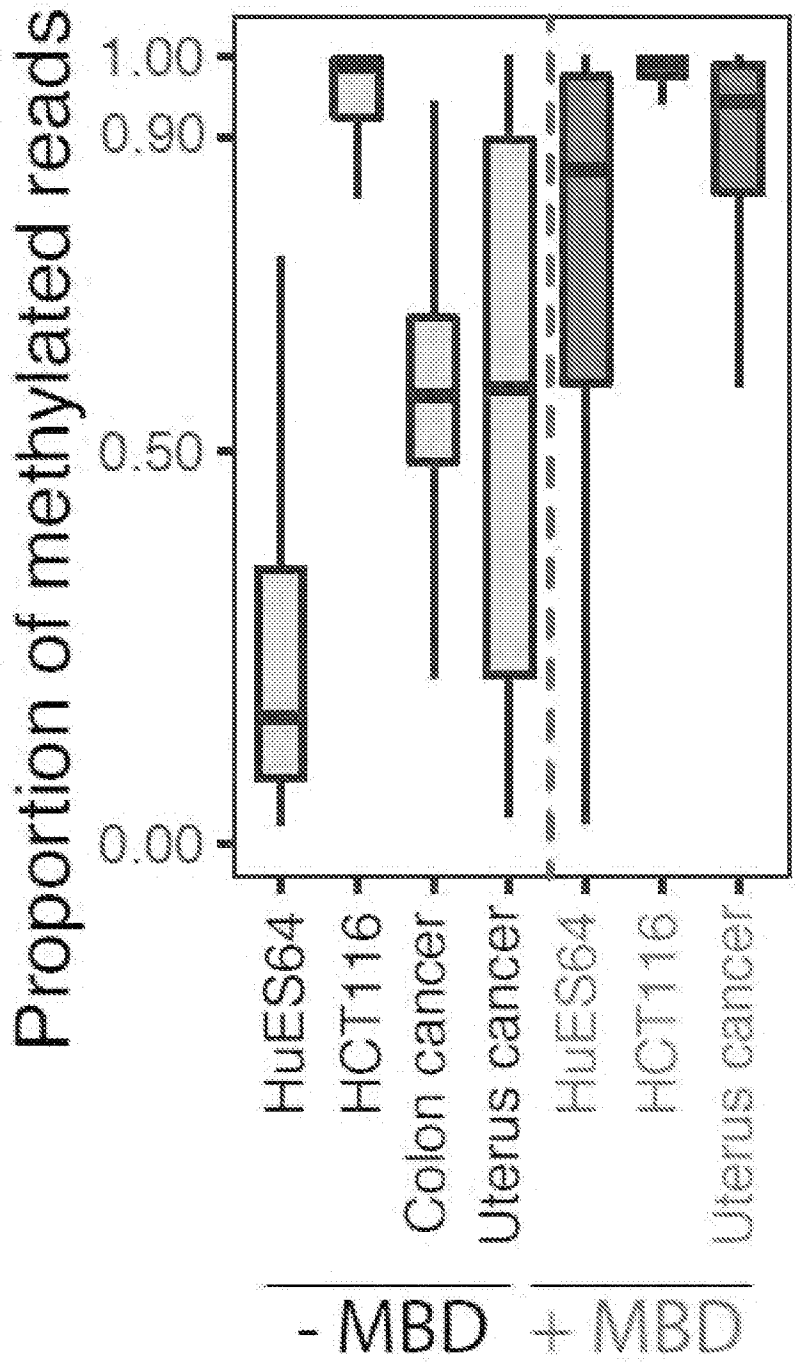

Proportion of methylated reads
1.00
0.90
0.50
0.00
HuES64
HCT116
Colon cancer
Uterus cancer
HuES64
HCT116
Uterus cancer
- MBD
+ MBD

FIG. 3D

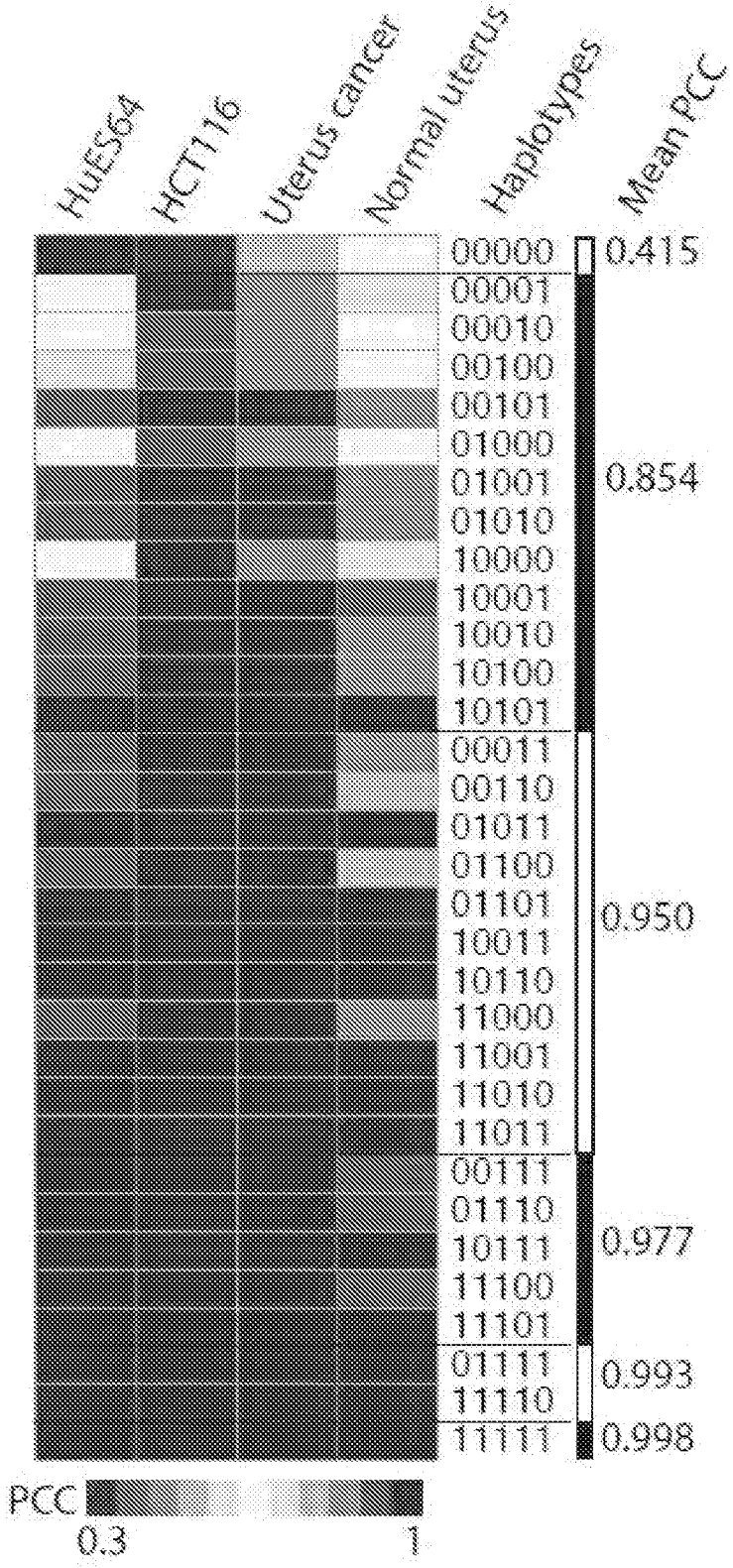

HuES64
HCT116
Uterus cancer
Normal uterus
Haplotypes
Mean PCC
00000
00001
00010
00100
00101
01000
01001
01010
10000
10001
10010
10100
10101
00011
00110
01011
01100
01101
10011
10110
11000
11001
11010
11011
00111
01110
10111
11100
11101
01111
11110
11111
0.415
0.854
0.950
0.977
0.993
0.998
PCC
0.3
1

FIG. 4A

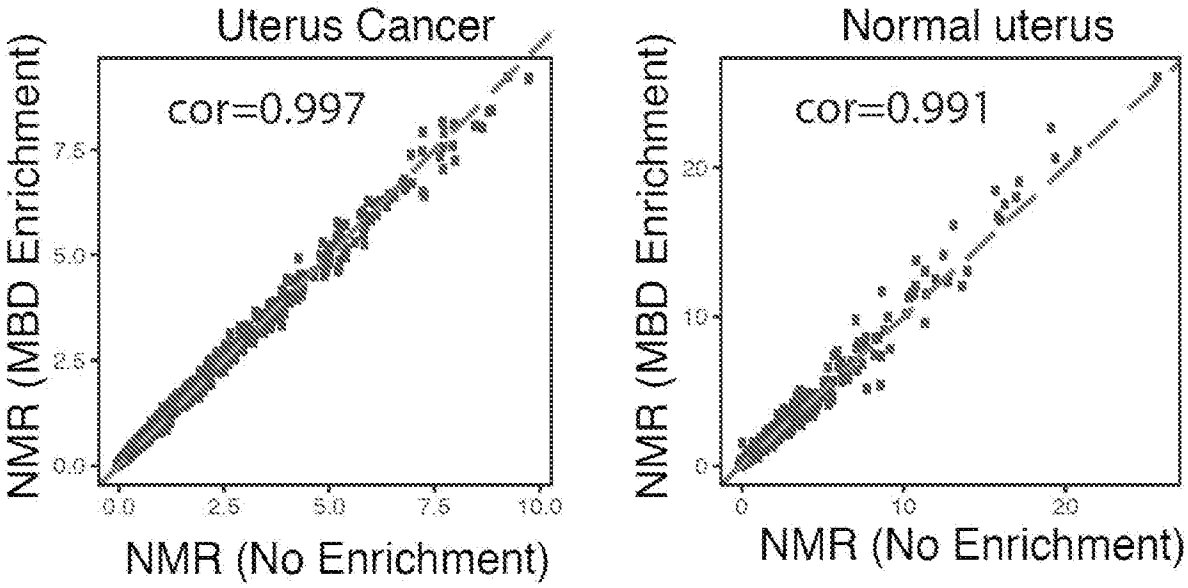

Uterus Cancer
cor=0.997
NMR (MBD Enrichment)
NMR (No Enrichment)
0.0
2.5
5.0
7.5
10.0
Normal uterus
cor=0.991
NMR (MBD Enrichment)
NMR (No Enrichment)
0
10
20

FIG. 4B

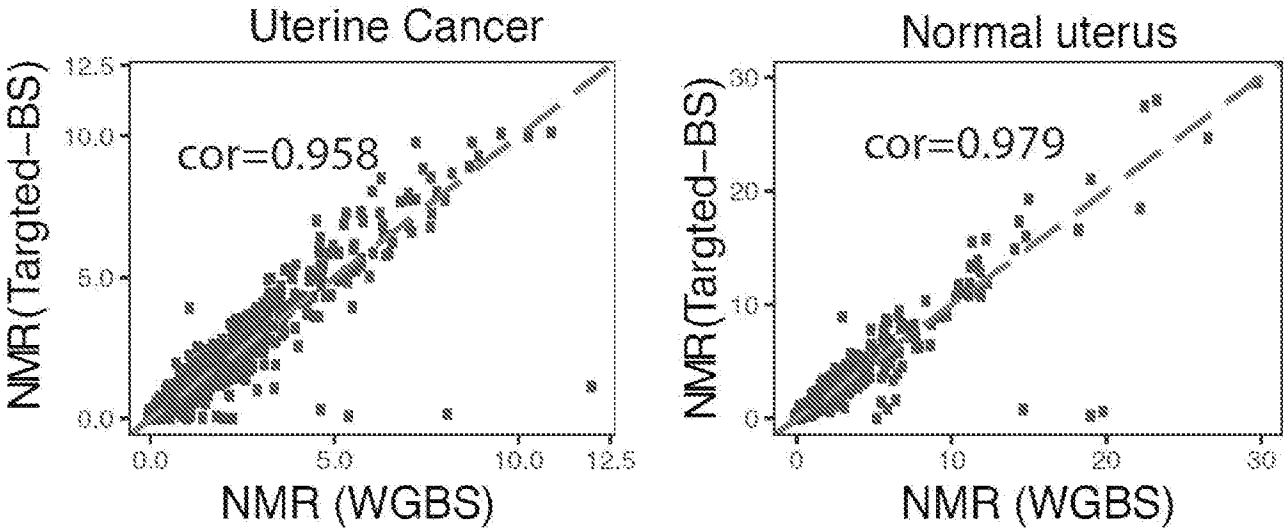

Uterine Cancer
cor=0.958
NMR(Targted-BS)
NMR (WGBS)
0.0
5.0
10.0
12.5
Normal uterus
cor=0.979
NMR(Targted-BS)
NMR (WGBS)
0
10
20
30

FIG. 4C

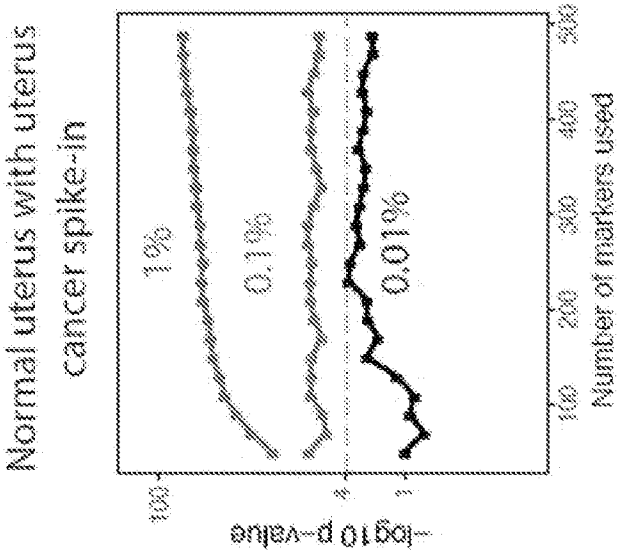


FIG. 5C

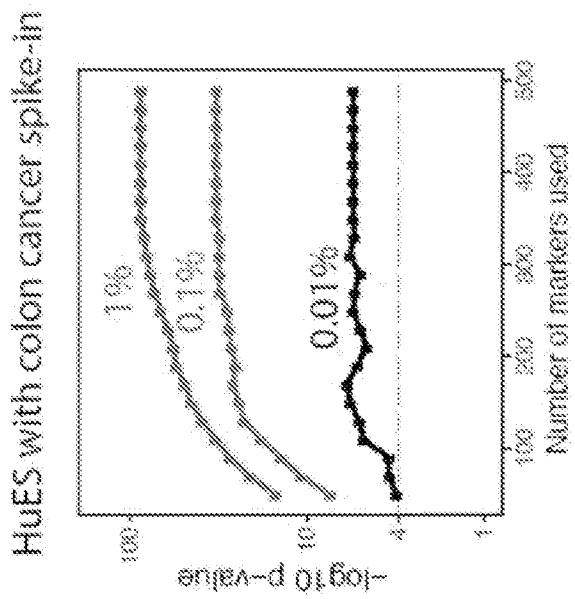


FIG. 5B

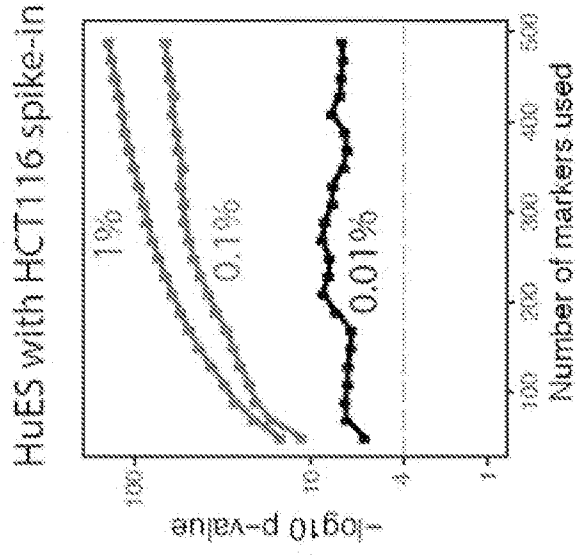


FIG. 5A

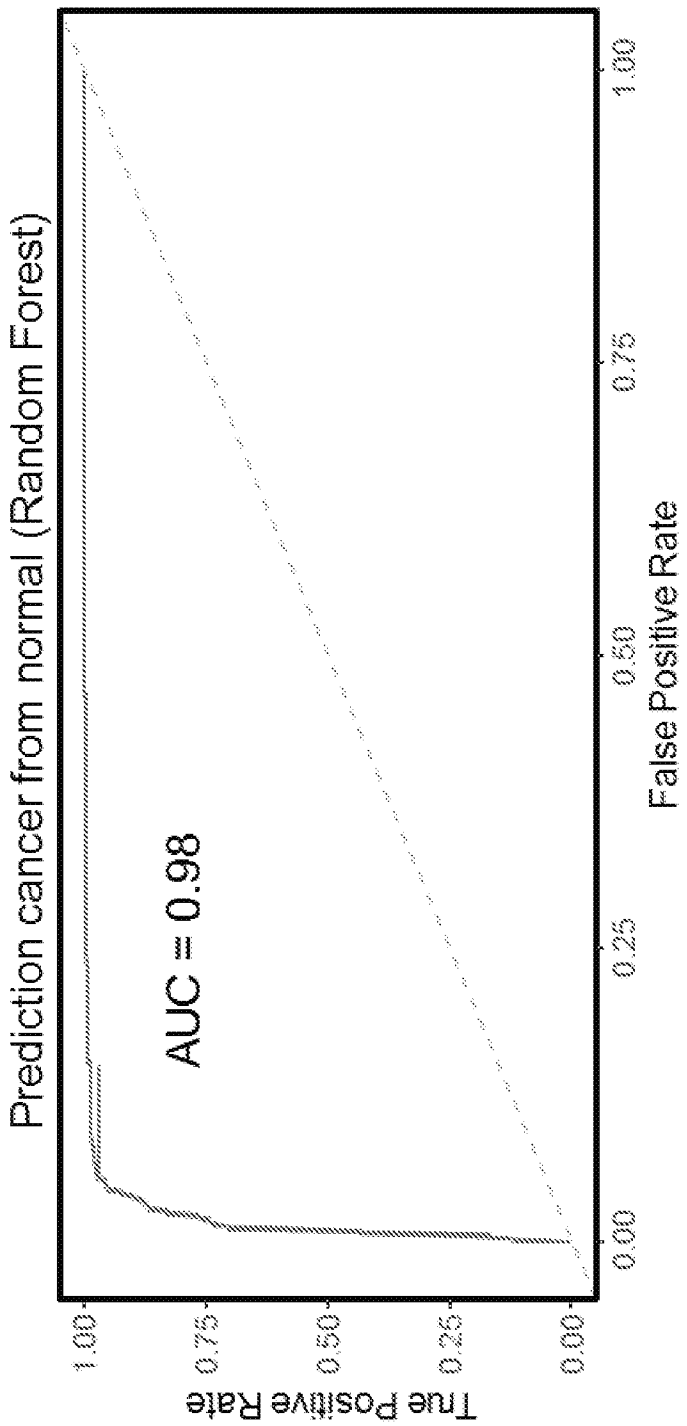

Prediction cancer from normal (Random Forest)
AUC = 0.98
True Positive Rate
False Positive Rate
0.00
0.25
0.50
0.75
1.00

FIG. 6

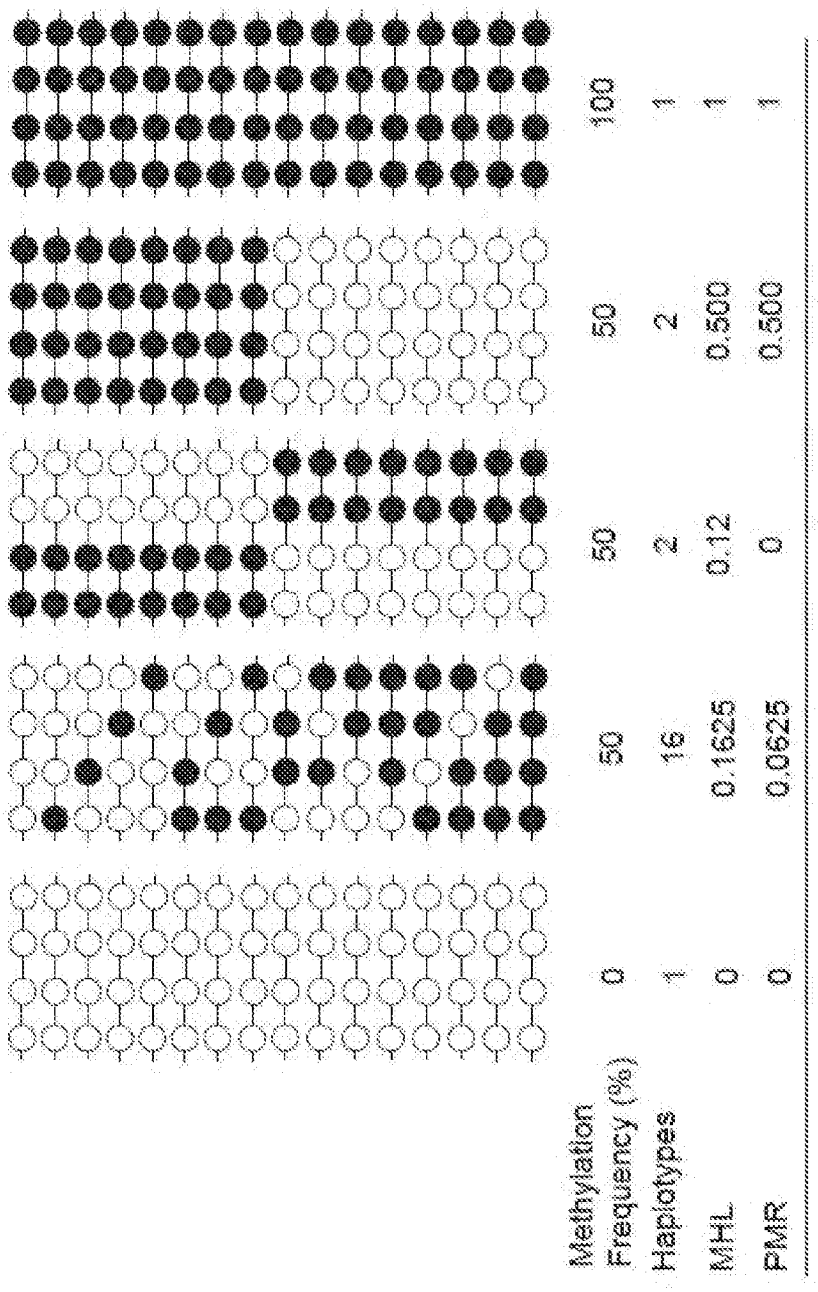

Methylation Frequency (%)
0
50
50
50
100
Haplotypes
1
16
2
2
1
MHL
0
0.1625
0.12
0.500
1
PMR
0
0.0625
0
0.500
1

FIG. 7

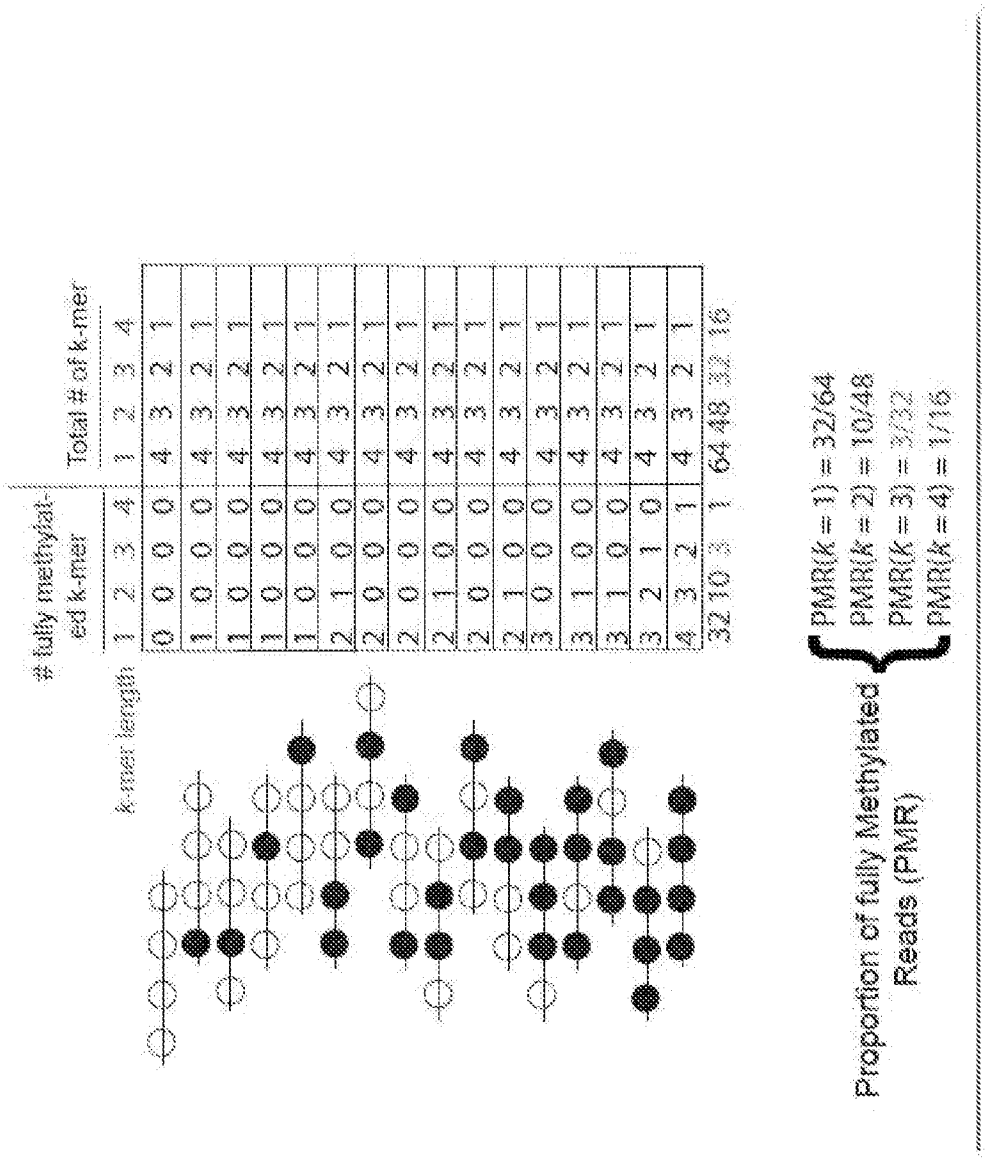

k-mer length
fully methylated k-mer
Total # of k-mer
1 2 3 4
0 0 0 0 4 3 2 1
1 0 0 0 4 3 2 1
1 0 0 0 4 3 2 1
1 0 0 0 4 3 2 1
1 0 0 0 4 3 2 1
2 1 0 0 4 3 2 1
2 0 0 0 4 3 2 1
2 1 0 0 4 3 2 1
2 0 0 0 4 3 2 1
2 1 0 0 4 3 2 1
3 0 0 0 4 3 2 1
3 1 0 0 4 3 2 1
3 1 0 0 4 3 2 1
3 2 1 0 4 3 2 1
4 3 2 1 4 3 2 1
32 10 3 1 64 48 32 16
Proportion of fully Methylated Reads (PMR)
PMR(k = 1) = 32/64
PMR(k = 2) = 10/48
PMR(k = 3) = 3/32
PMR(k = 4) = 1/16

FIG. 8

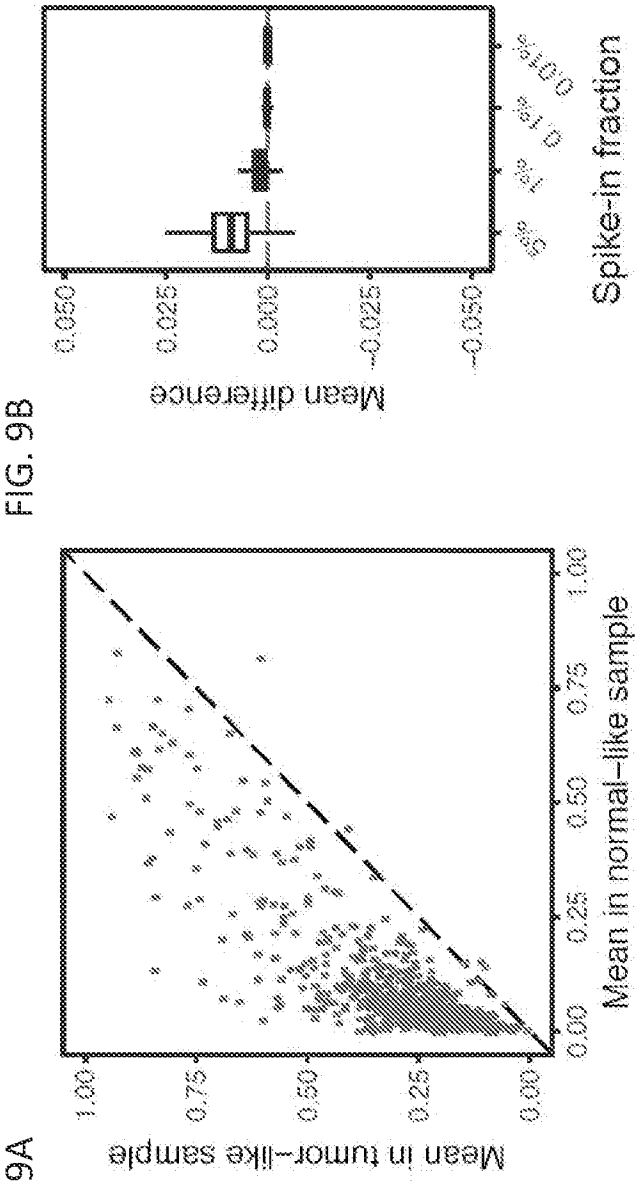


FIG. 9A

FIG. 9B

Cancer-like Spike-in	# > 0	# < 0	# = 0	P-value
5%	673	45	4	5.3×10^{-145}
1%	599	119	4	3.9×10^{-78}
0.1%	477	242	3	6.5×10^{-19}
0.01%	400	313	9	6.3×10^{-4}

FIG. 9C

FIG. 10A

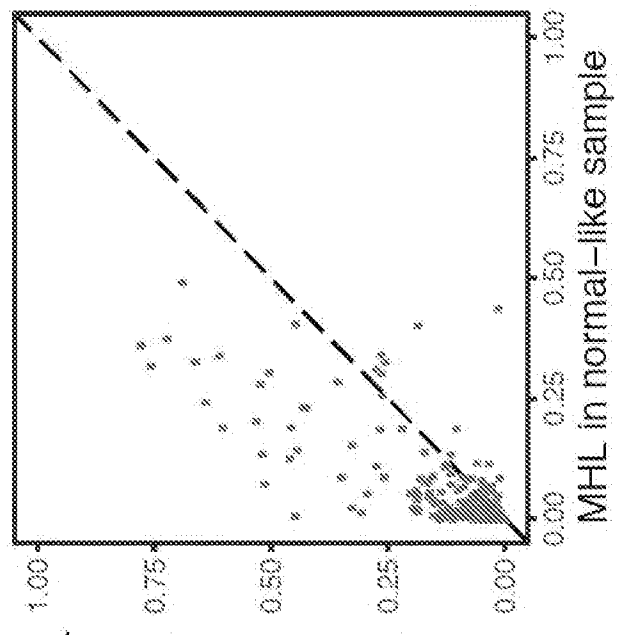


FIG. 10B

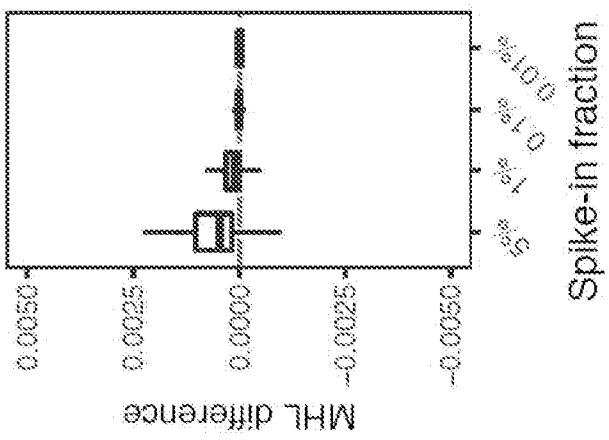


FIG. 10C

Cancer-like Spike-in	# > 0	# < 0	# = 0	P-value
5%	545	94	0	1.9×10^{-78}
1%	469	169	1	7.4×10^{-34}
0.1%	391	237	11	4.2×10^{-10}
0.01%	316	270	53	3.1×10^{-2}

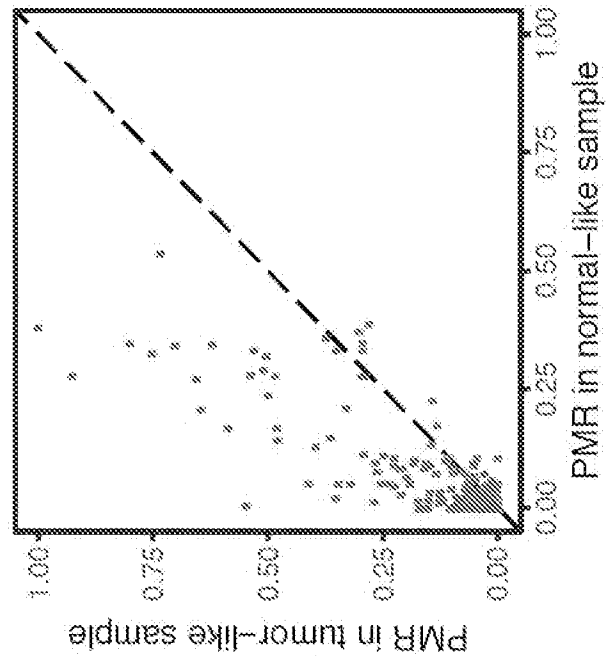


FIG. 11A

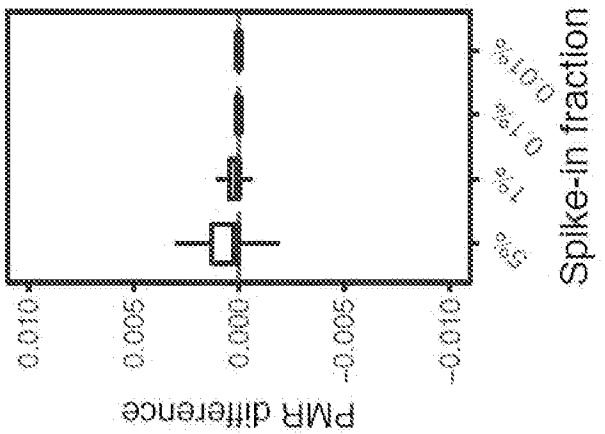


FIG. 11B

FIG. 11C

Cancer-like Spike-in	# > 0	# < 0	# = 0	P-value
5%	289	89	121	4.5×10^{-26}
1%	266	115	118	3.4×10^{-15}
0.1%	247	137	115	1.1×10^{-8}
0.01%	228	142	129	4.5×10^{-6}

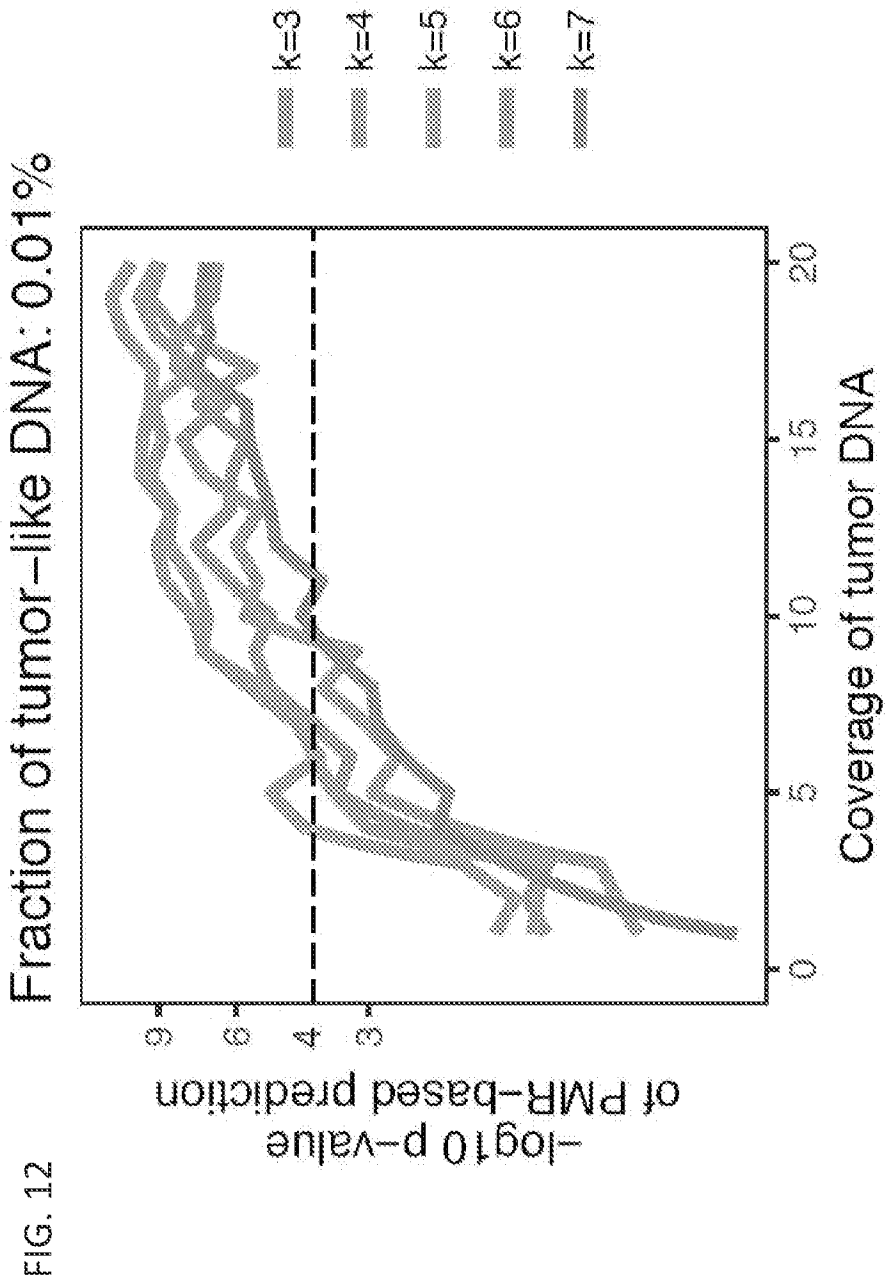

Fraction of tumor−like DNA: 0.01%
k=3
k=4
k=5
k=6
k=7
−log10 p−value
of PMR−based prediction
8
6
4
3
0
5
10
15
20
Coverage of tumor DNA

FIG. 12

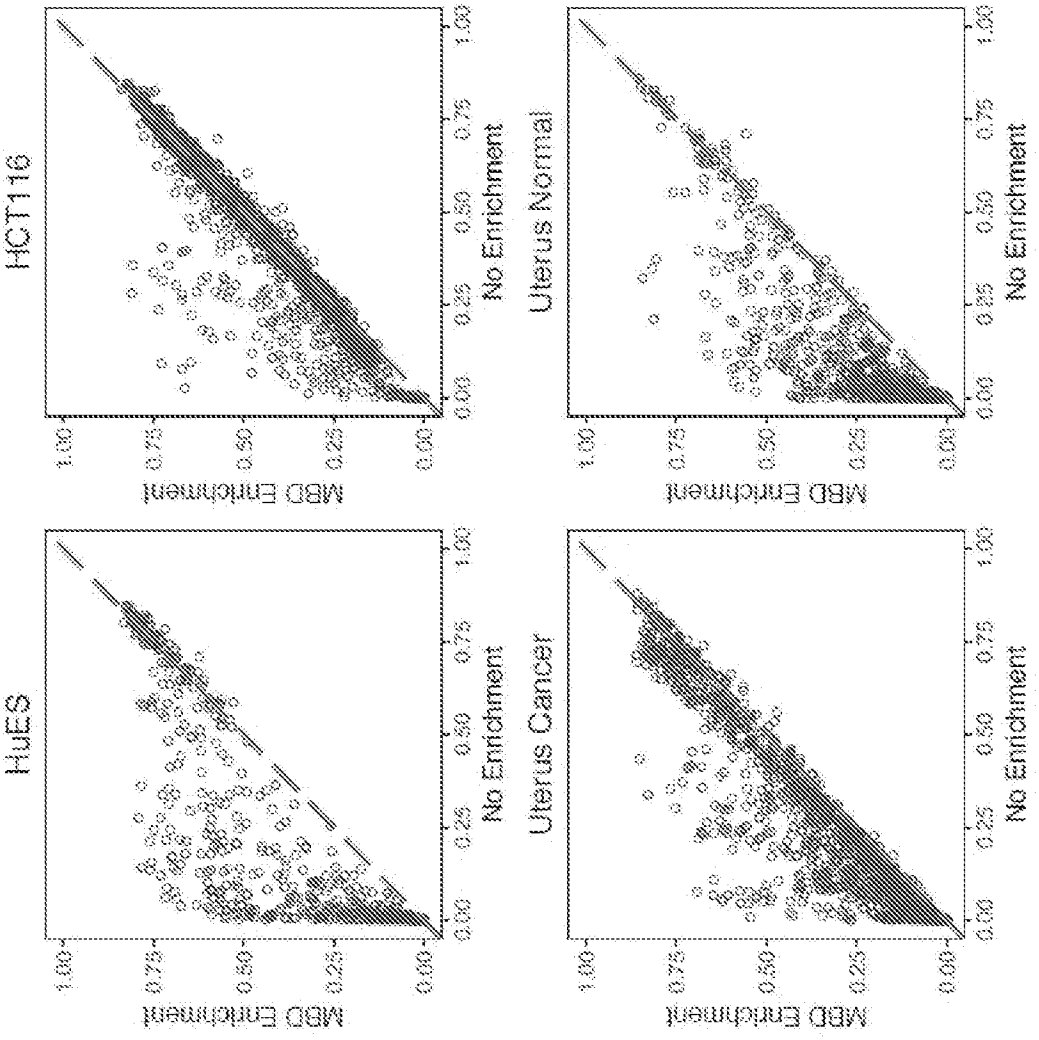

HCT116
HuES
Uterus Normal
Uterus Cancer
MBD Enrichment
No Enrichment
0.00
0.25
0.50
0.75
1.00

FIG. 13

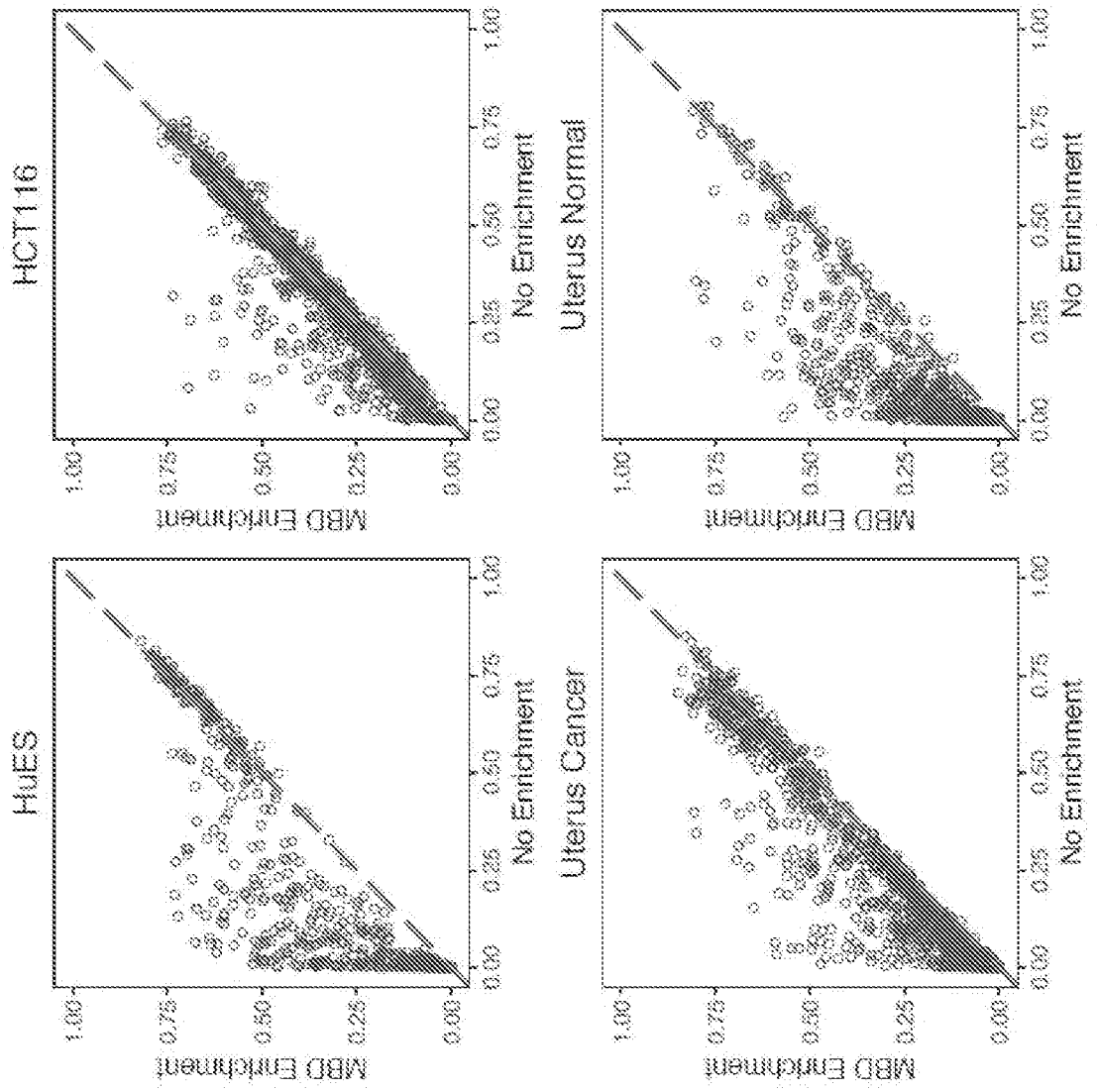

HCT116
HuES
Uterus Normal
Uterus Cancer
MBD Enrichment
No Enrichment
0.00
0.25
0.50
0.75
1.00

FIG. 14

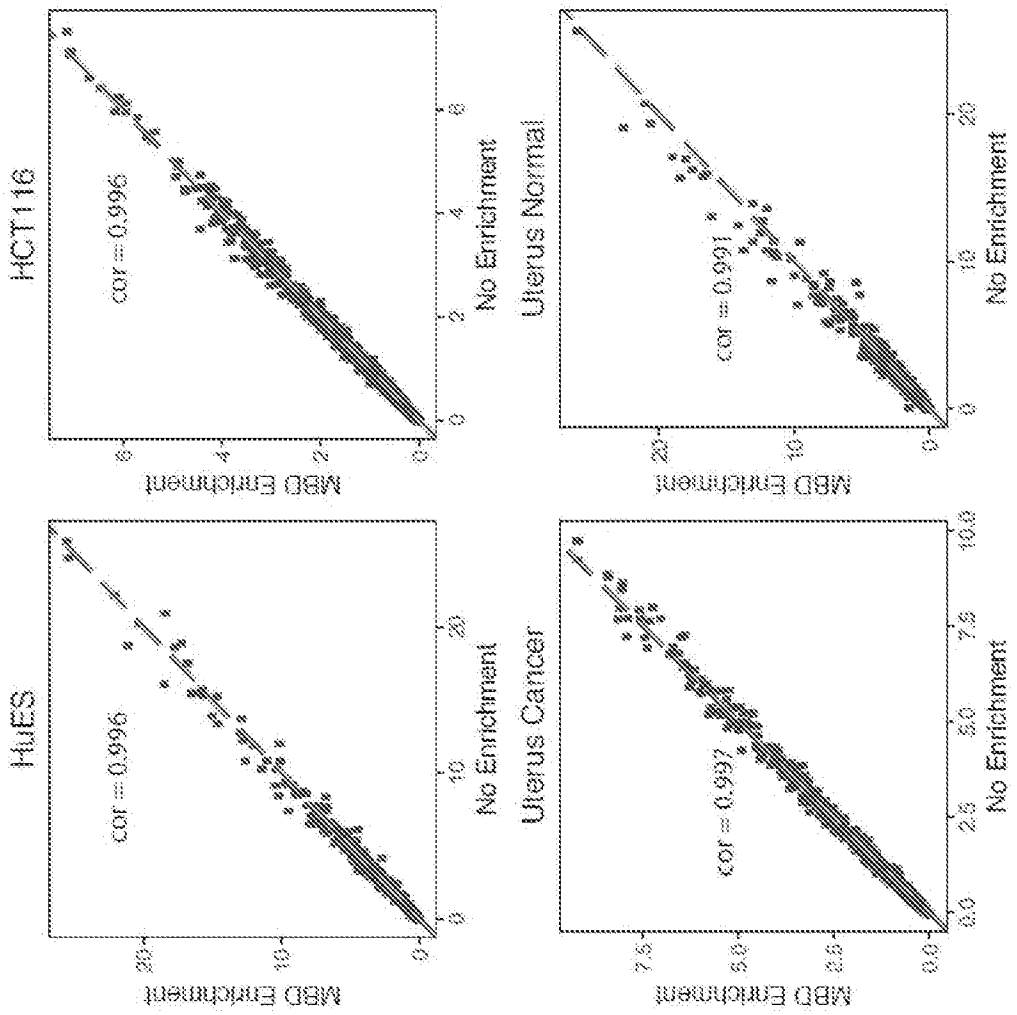

HCT116
cor = 0.996
MBD Enrichment
No Enrichment
HuES
cor = 0.996
MBD Enrichment
No Enrichment
Uterus Normal
cor = 0.991
MBD Enrichment
No Enrichment
Uterus Cancer
cor = 0.992
MBD Enrichment
No Enrichment

FIG. 15

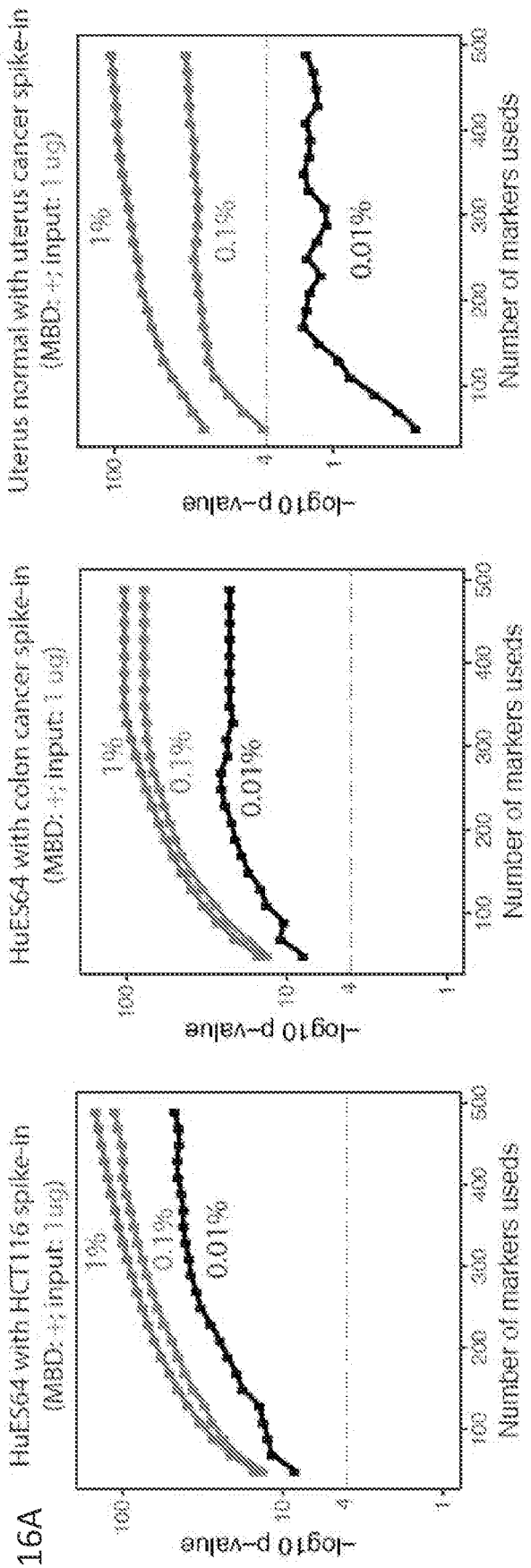

HuES64 with HCT116 spike-in
(MBD: +; input: 1 ug)
HuES64 with colon cancer spike-in
(MBD: +; input: 1 ug)
Uterus normal with uterus cancer spike-in
(MBD: +; input: 1 ug)
1%
0.1%
0.01%
-log10 p-value
Number of markers useds

FIG. 16A

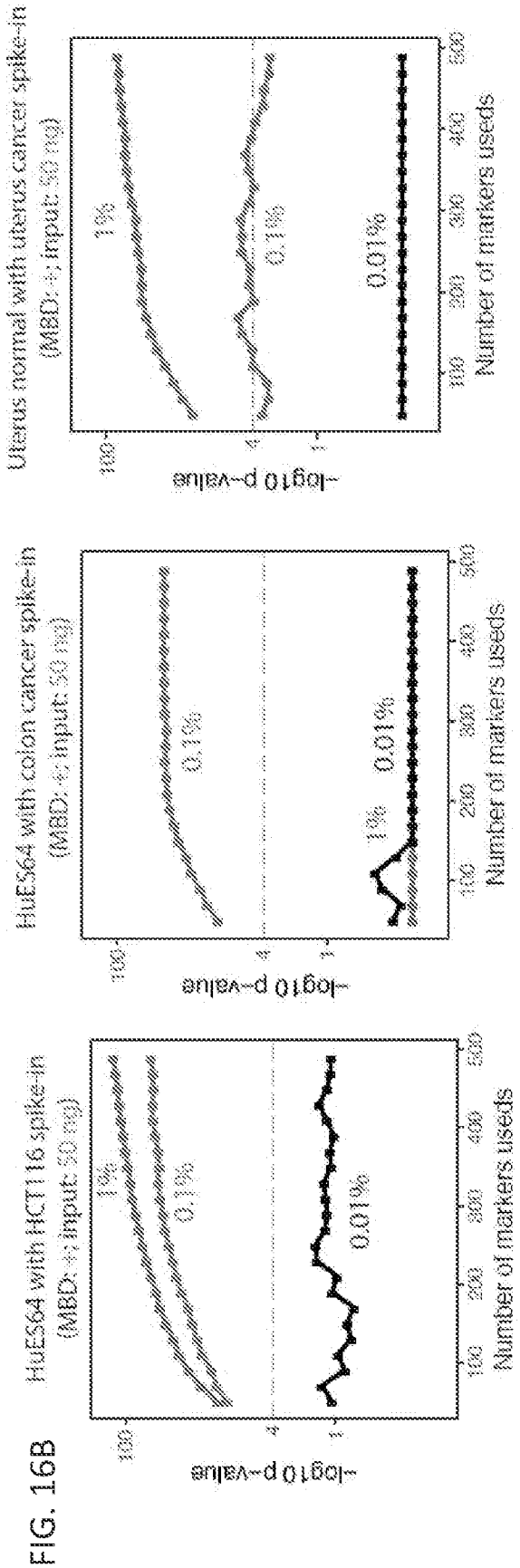

HuES64 with HCT116 spike-in
(MBD: +; input: 50 ng)
1%
0.1%
0.01%
−log10 p−value
100
4
1
100
200
300
400
500
Number of markers useds
HuES64 with colon cancer spike-in
(MBD: +; input: 50 ng)
0.1%
1%
0.01%
−log10 p−value
100
4
1
100
200
300
400
500
Number of markers useds
Uterus normal with uterus cancer spike-in
(MBD: +; input: 50 ng)
1%
0.1%
0.01%
−log10 p−value
100
4
1
100
200
300
400
500
Number of markers useds

FIG. 16B

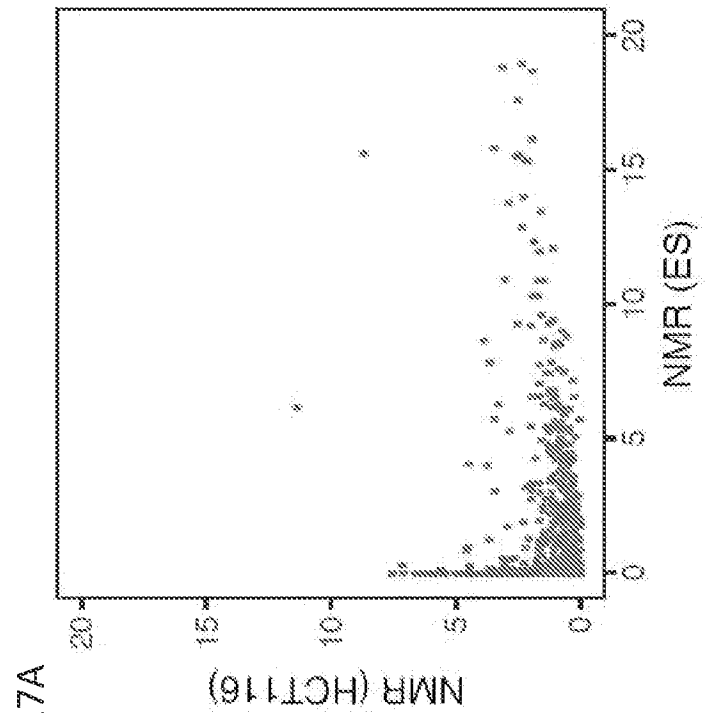


FIG. 17A

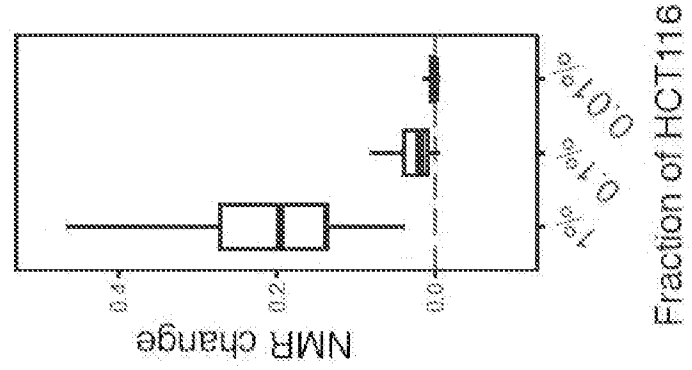


FIG. 17B

FIG. 17C

HCT116 Spike-in	# > 0	# < 0	# = 0	P-value
1%	199	1	0	1.3×10^{-58}
0.1%	174	13	13	2.0×10^{-37}
0.01%	85	25	90	3.9×10^{-9}

FIG. 18A

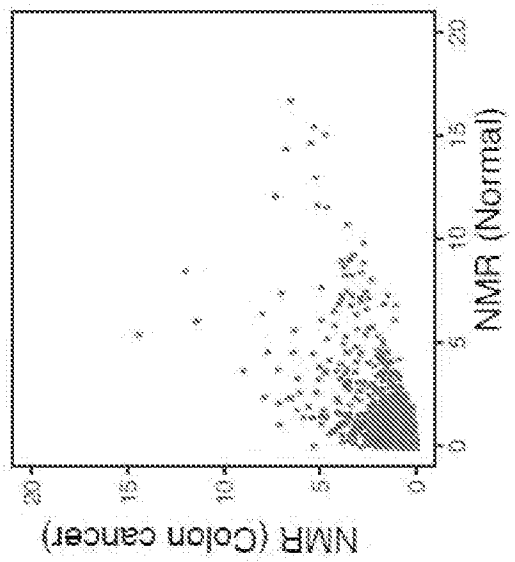


FIG. 18B

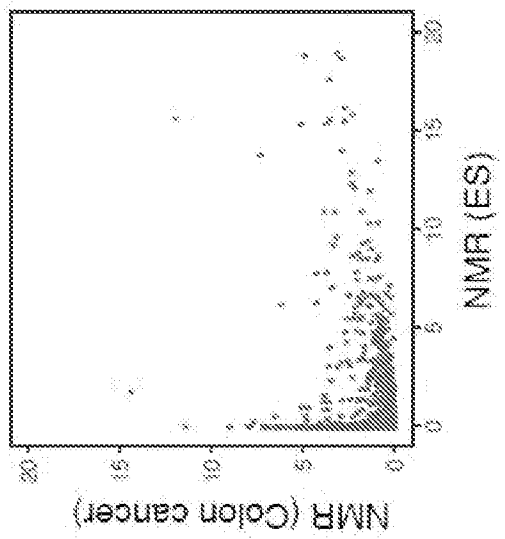


FIG. 18C

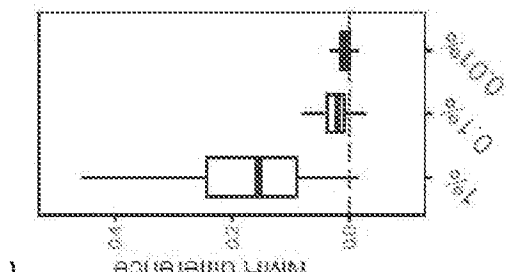


FIG. 18D

Colon cancer Spike-in	# > 0	# < 0	# = 0	P-value
1%	196	3	1	1.6×10^{-54}
0.1%	140	16	44	3.3×10^{-26}
0.01%	61	22	117	1.1×10^{-5}

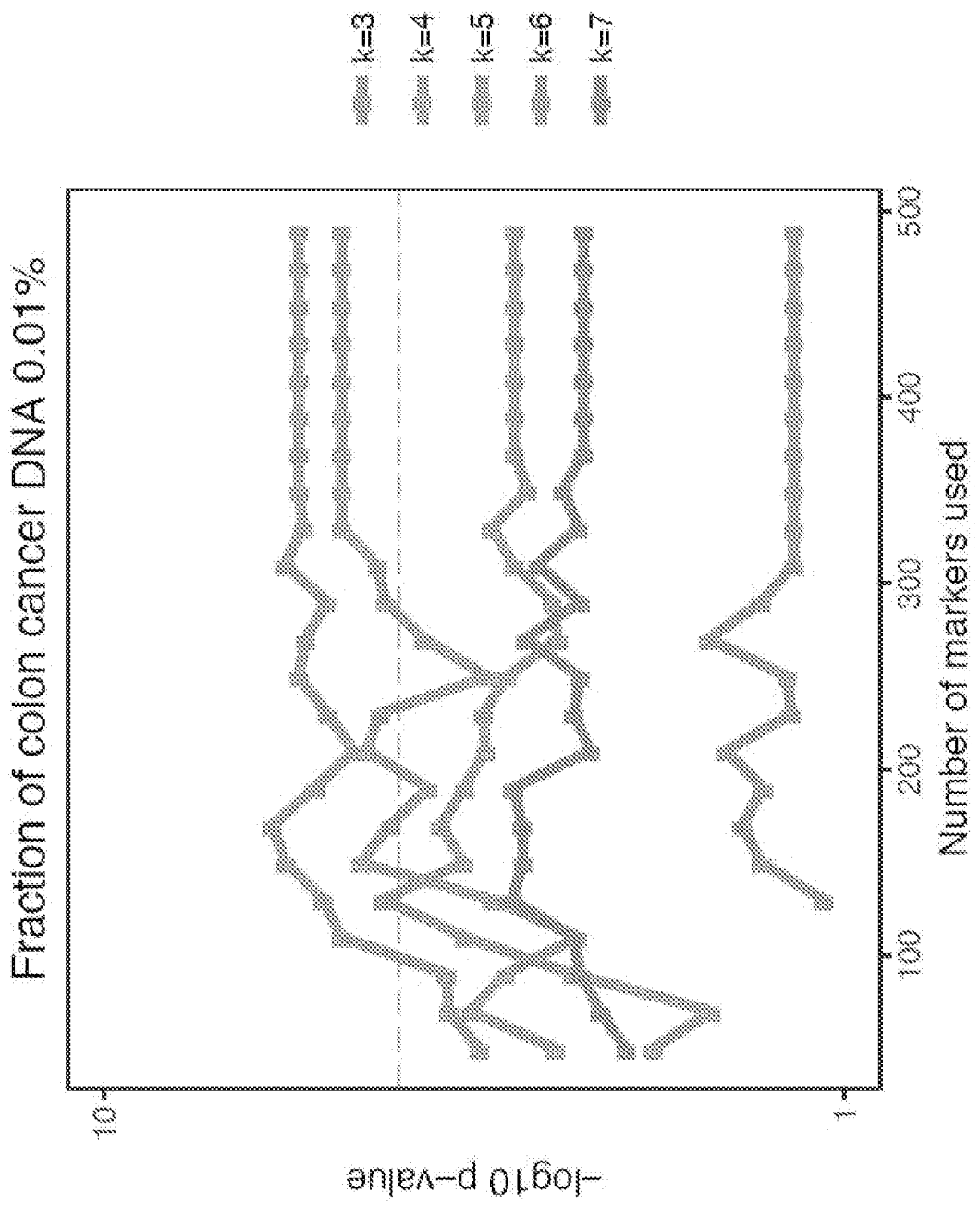

Fraction of colon cancer DNA 0.01%
k=3
k=4
k=5
k=6
k=7
10
1
−log10 p−value
100
200
300
400
500
Number of markers used

FIG. 19

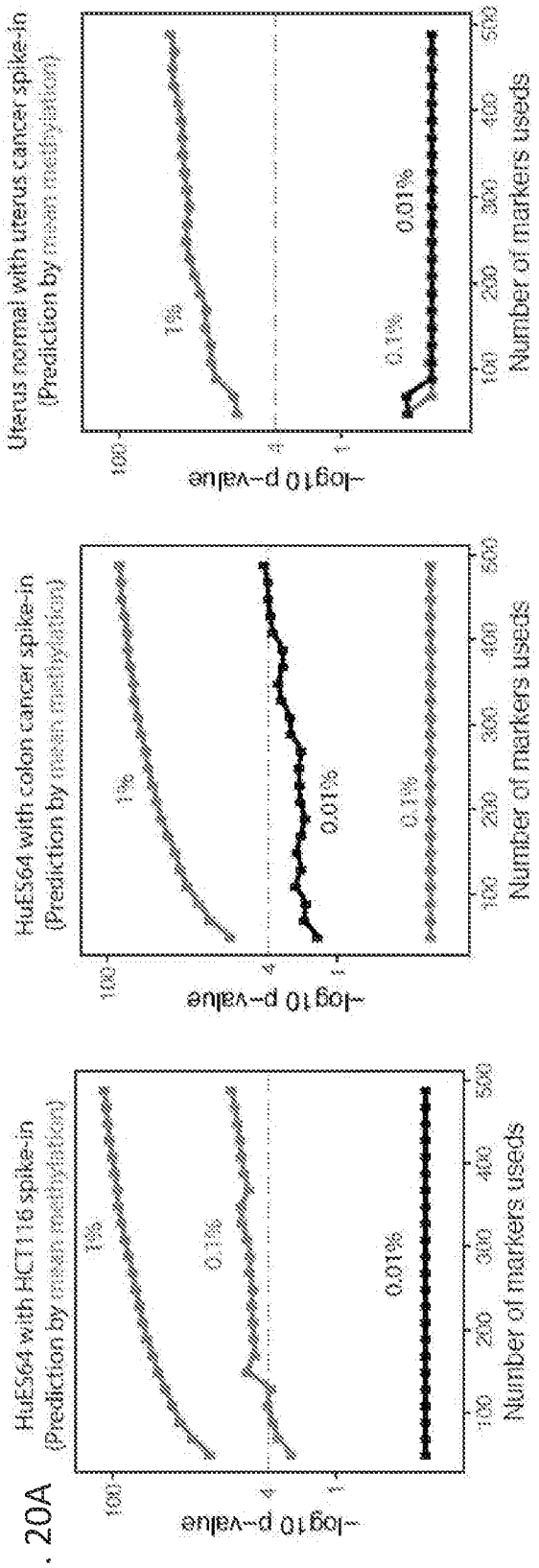

HuES64 with HCT116 spike-in
(Prediction by mean methylation)
1%
0.1%
0.01%
−log10 p−value
100
4
1
100
200
300
400
500
Number of markers useds
HuES64 with colon cancer spike-in
(Prediction by mean methylation)
1%
0.01%
0.1%
−log10 p−value
100
4
1
100
200
300
400
500
Number of markers useds
Uterus normal with uterus cancer spike-in
(Prediction by mean methylation)
1%
0.1%
0.01%
−log10 p−value
100
4
1
100
200
300
400
500
Number of markers useds

FIG. 20A

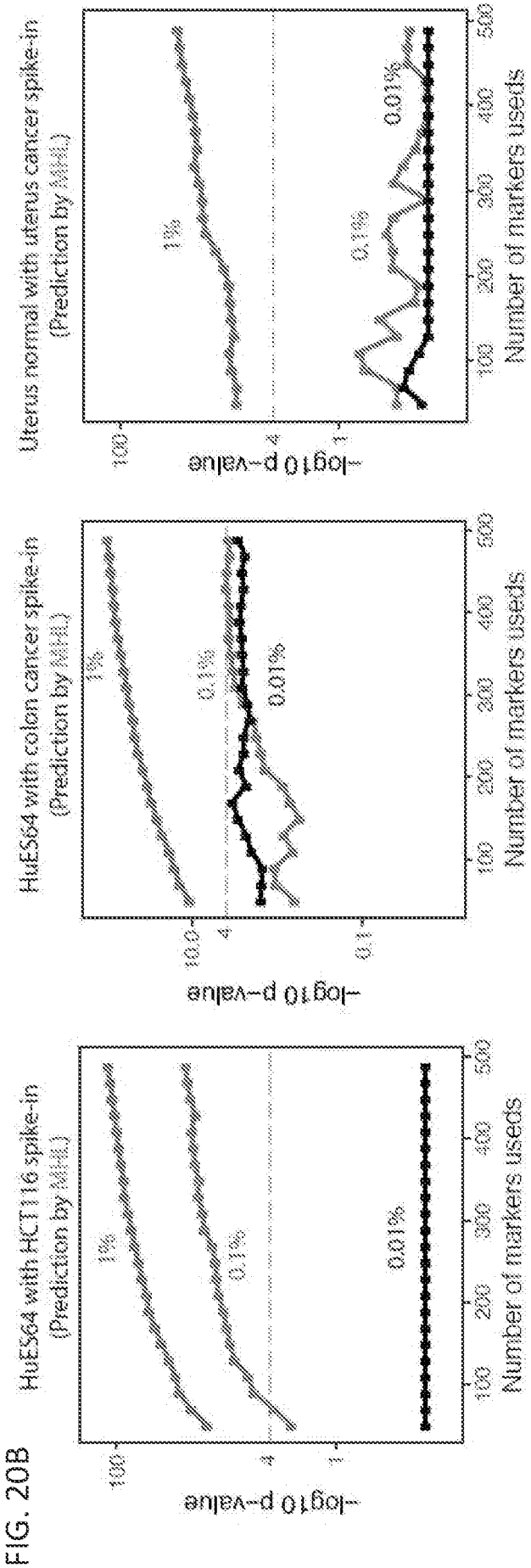

HuES64 with HCT116 spike-in
(Prediction by MHL)
HuES64 with colon cancer spike-in
(Prediction by MHL)
Uterus normal with utenus cancer spike-in
(Prediction by MHL)
1%
0.1%
0.01%
−log10 p−value
Number of markers useds

FIG. 20B

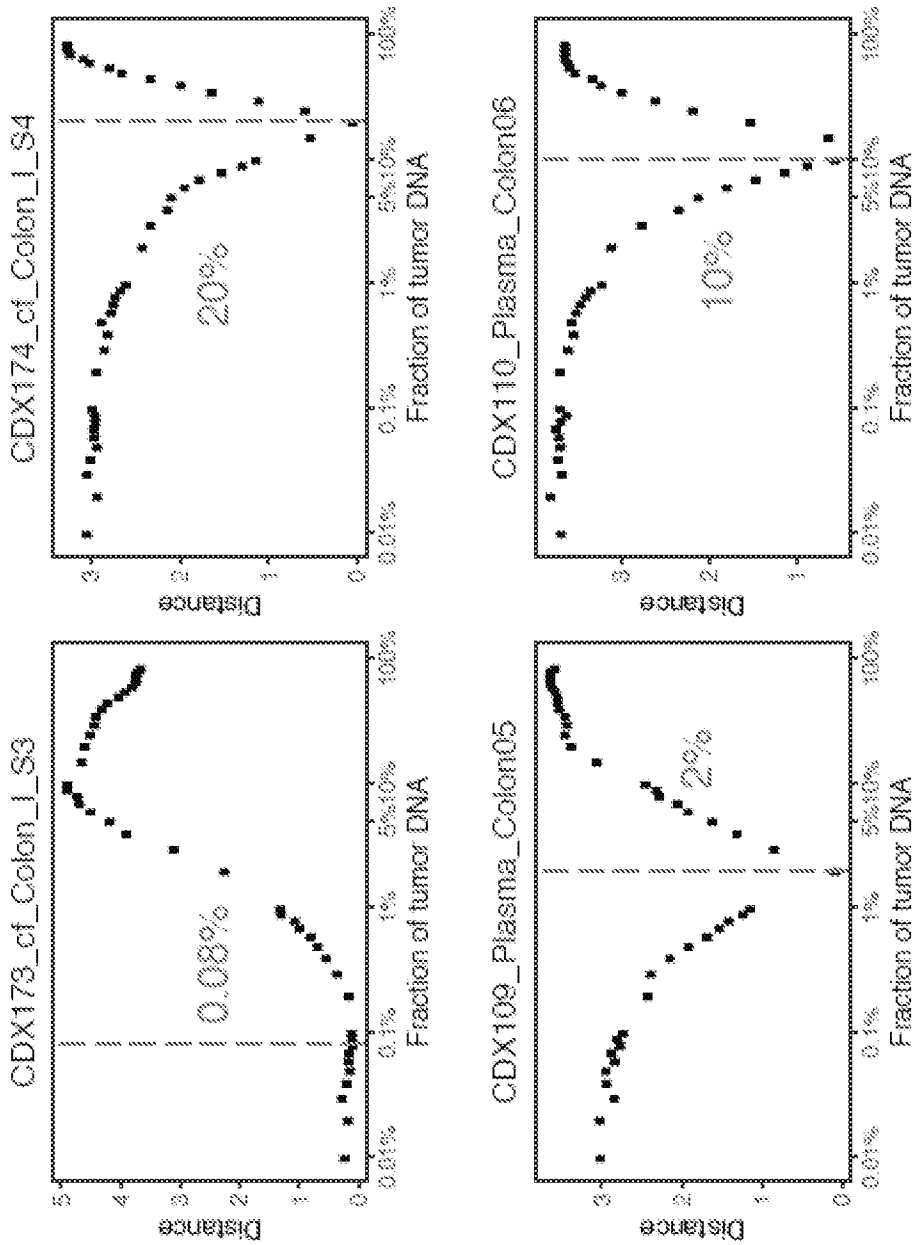

CDX173_cf_Colon_1_S3
0.08%
CDX174_cf_Colon_1_S4
20%
CDX109_Plasma_Colon05
2%
CDX110_Plasma_Colon06
10%
Distance
Fraction of tumor DNA
0.01%
0.1%
1%
5%
10%
100%

FIG. 21

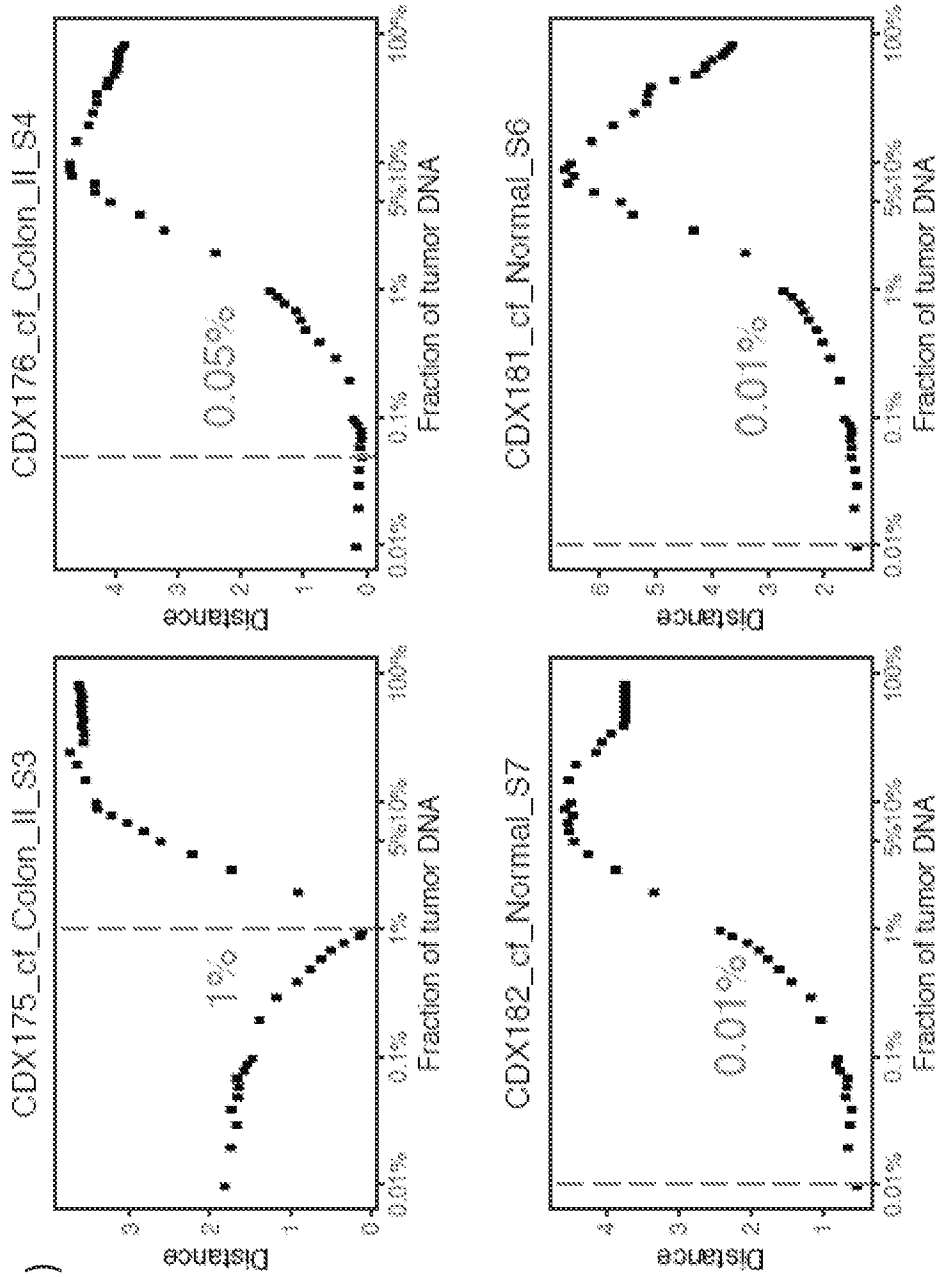

CDX176_ct_Colon_II_S4
CDX175_ct_Colon_II_S3
CDX181_ct_Normal_S6
CDX182_ct_Normal_S7
0.05%
1%
0.01%
0.01%
Distance
Fraction of tumor DNA
0.01%
0.1%
1%
5%
10%
100%

FIG. 21 (Cont.)

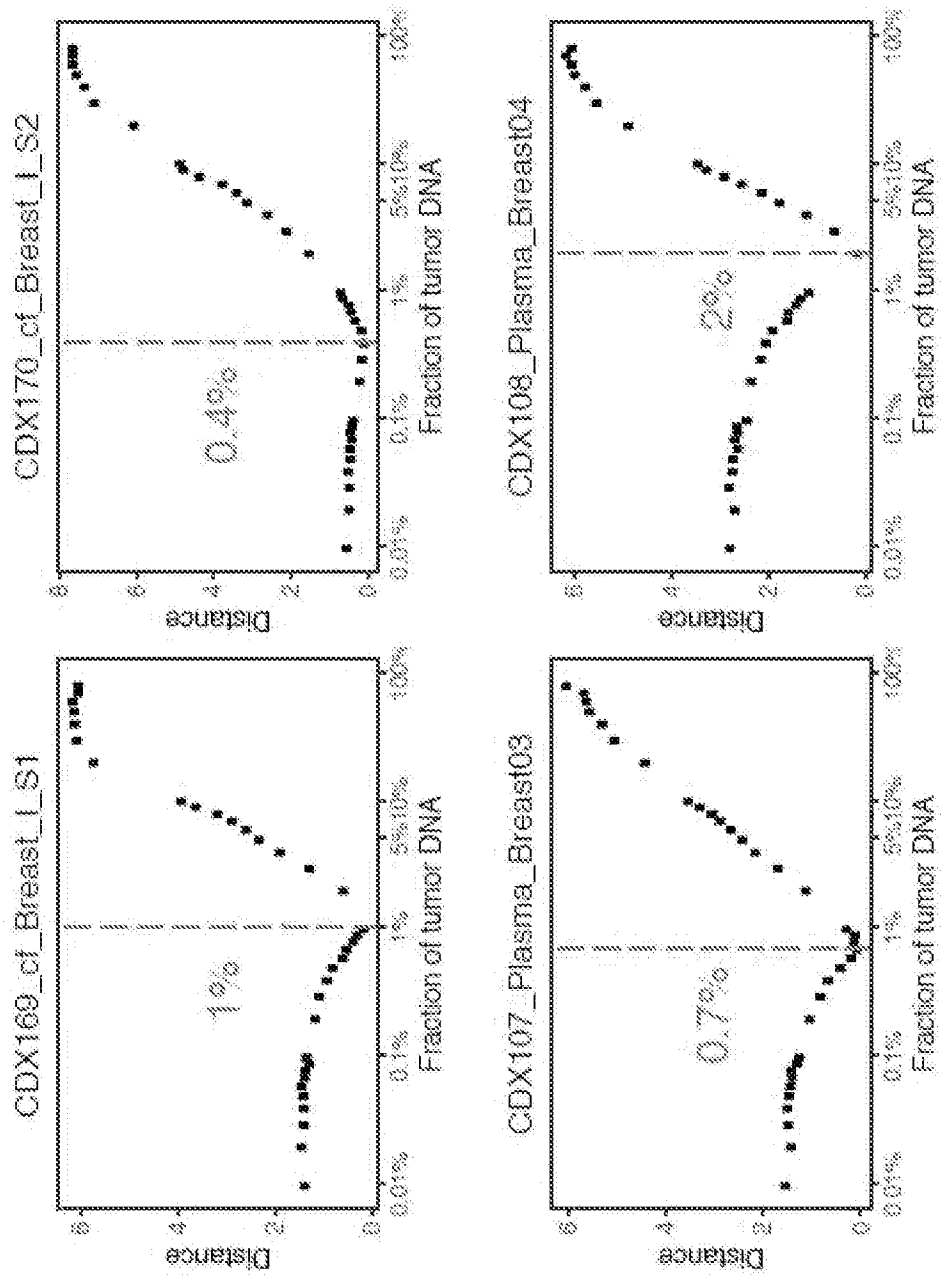

CDX169_ct_Breast_I_S1
CDX170_ct_Breast_I_S2
CDX107_Plasma_Breast03
CDX108_Plasma_Breast04
Distance
Fraction of tumor DNA
0.01%
0.1%
1%
5%
10%
100%
1%
0.4%
0.7%
2%

FIG. 22

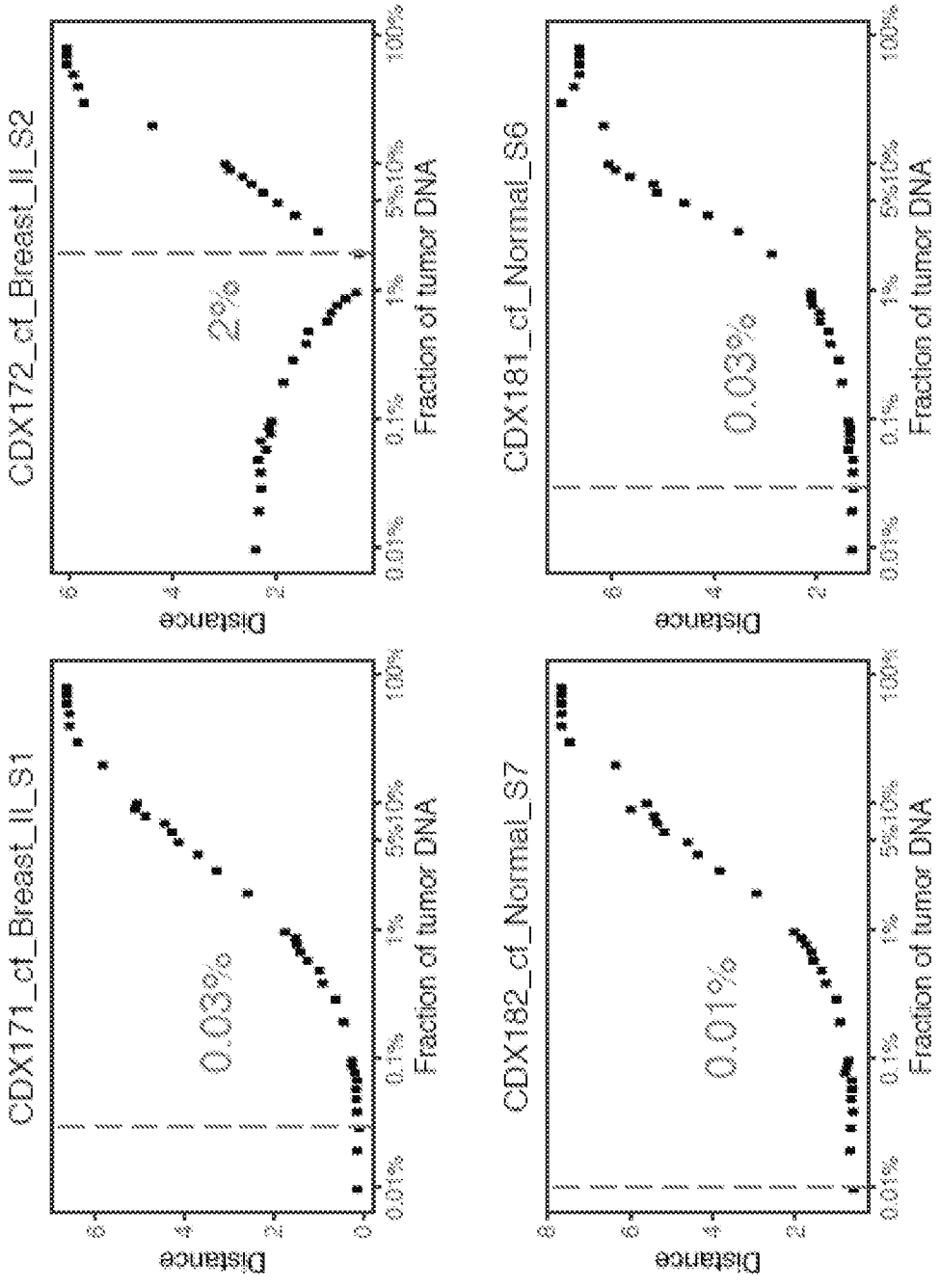

CDX171_ct_Breast_IL_S1
0.03%
CDX172_ct_Breast_IL_S2
2%
CDX181_cf_Normal_S6
0.03%
CDX182_cf_Normal_S7
0.01%
Distance
Fraction of tumor DNA
0.01%
0.1%
1%
5%
10%
100%

FIG. 22 (Cont.)

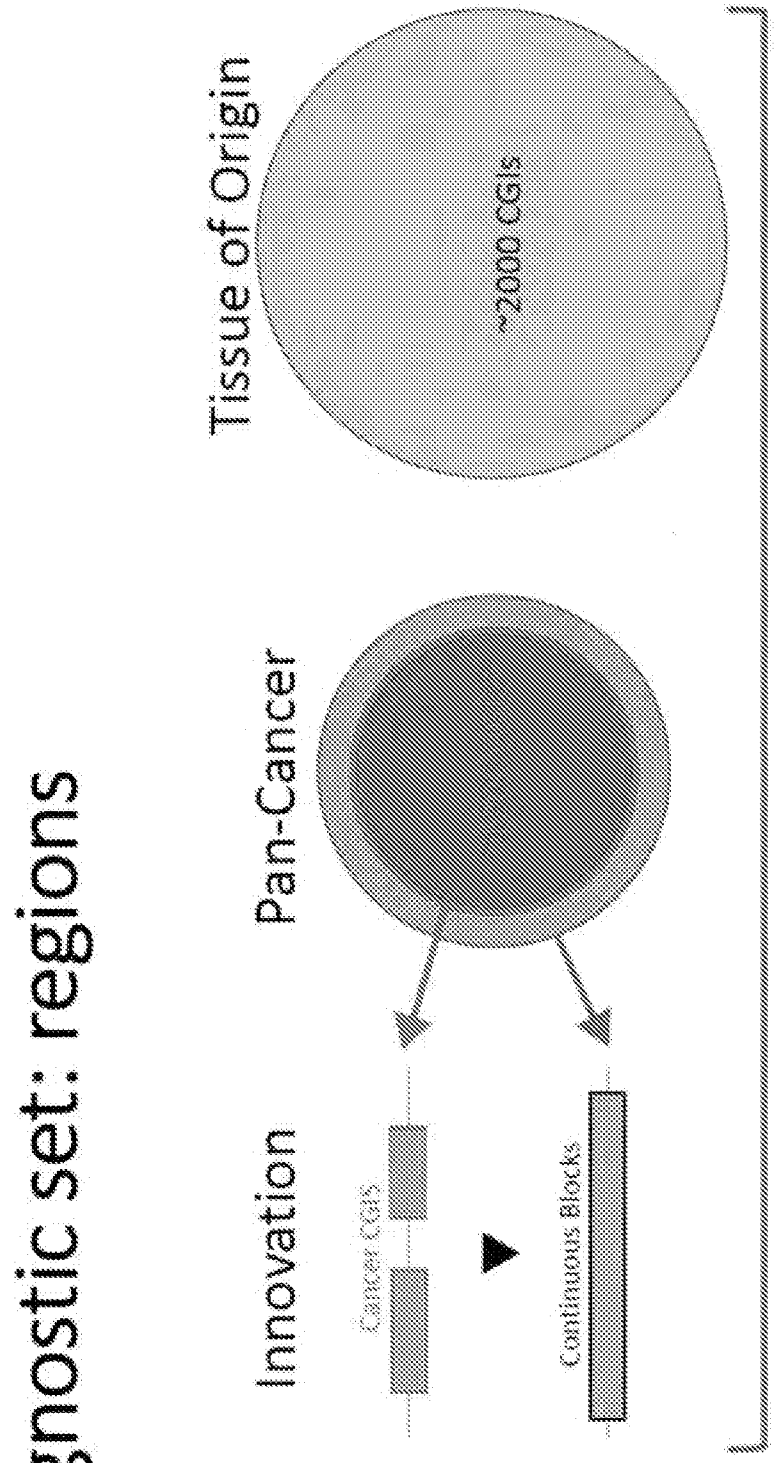

Diagnostic set: regions
Innovation
Pan-Cancer
Tissue of Origin
~2000 CGIs
Cancer CGIs
Continuous Blocks

FIG. 23

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2021/064210

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12Q 1/6886; G16B 20/10; G16B 20/20 (2022.01)

CPC - C12Q 1/6886; C12Q 2600/106; C12Q 2600/154; C12Q 2600/172; G16B 20/10; G16B 20/20 (2022.02)

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)

see Search History document

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

see Search History document

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

see Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2020/0109456 A1 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE et al) 09 April 2020 (09.04.2020) *entire document*	1, 2, 14, 15, 30 --- 33-36
X	US 2020/0087731 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 19 March 2020 (19.03.2020) entire document	39, 40
Y	WO 2019/200410 A1 (FREENOME HOLDINGS INC.) 17 October 2019 (17.10.2019) entire document	33-36
A	US 2020/0131582 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA et al) 30 April 2020 (30.04.2020) entire document	1, 2, 14, 15, 30, 33-36, 39, 40

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 February 2022	MAR 0 8 2022
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Harry Kim Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2021/064210

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 3-13, 16-29, 31, 32, 37, 38, 41
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.