

## SUMMARY

[0007a] In one aspect, there is provided a method of analyzing a biological sample of an organism, the biological sample including nucleic acid molecules originating from normal cells and potentially from cells associated with cancer, wherein at least some of the nucleic acid molecules are cell-free in the biological sample, the method comprising: analyzing a plurality of DNA molecules from the biological sample, wherein analyzing a DNA molecule includes: determining a location of the DNA molecule in a genome of the organism; determining whether the DNA molecule is methylated at one or more sites; for each of a plurality of sites: determining a respective number of DNA molecules that are methylated at the site; calculating a first methylation level based on the respective numbers of DNA molecules methylated at the plurality of sites; comparing the first methylation level to a first cutoff value; determining a first classification of a level of cancer based on the comparison.

[0007b] In another aspect, there is provided a method of determining a first methylation profile from a biological sample of an organism, the biological sample including cell-free DNA comprising a mixture of cell-free DNA molecules originating from a first tissue and from a second tissue, the method comprising: obtaining a second methylation profile corresponding to DNA molecules of the second tissue, the second methylation profile providing a second methylation density at each of a plurality of loci in a genome of the organism, the second methylation density at a particular locus corresponding to a proportion of DNA molecules of the second tissue that are methylated; determining a cell-free methylation profile from the cell-free DNA molecules of the mixture, the cell-free methylation profile providing a mixture methylation density at each of the plurality of loci; determining a percentage of the cell-free DNA molecules in the mixture that are from the first tissue; and determining the first methylation profile of the first tissue by: for each of the plurality of loci: calculating a differential parameter that includes a difference between the second methylation density of the second methylation profile and the mixture methylation density of the cell-free methylation profile, the difference being scaled by the percentage.

[0007c] In another aspect, there is provided a method of determining a first methylation profile from a biological sample of an organism, the biological sample including cell-free DNA comprising a mixture of cell-free DNA originating from a first tissue and from a second tissue, the method comprising: analyzing a plurality of DNA molecules from the biological sample, wherein analyzing a DNA molecule includes: determining a location of the DNA molecule in a genome of the organism; determining a genotype of the DNA molecule; determining whether the

DNA molecule is methylated at one or more sites; identifying a plurality of first loci at which a first genome of the first tissue is heterozygous for a respective first allele and a respective second allele and a second genome of the second tissue is homozygous for the respective first allele; for each of the first loci: for each of one or more sites associated with the locus: determining a number of DNA molecules that are methylated at the site and correspond to the respective second allele of the locus; calculating a methylation density based on the numbers of DNA molecules methylated at the one or more sites of the locus and corresponding to the respective second allele of the locus; creating the first methylation profile of the first tissue from the methylation densities for the first loci.

[0007d] In another aspect, there is provided a method of detecting a chromosomal abnormality from a biological sample of an organism, the biological sample including cell-free DNA comprising a mixture of cell-free DNA originating from a first tissue and from a second tissue, the method comprising: analyzing a plurality of DNA molecules from the biological sample, wherein analyzing a DNA molecule includes: determining a location of the DNA molecule in a reference genome; determining whether the DNA molecule is methylated at one or more sites; for each of a plurality of sites: determining a respective number of DNA molecules that are methylated at the site; calculating a first methylation level of a first chromosomal region based on the respective numbers of DNA molecules methylated at sites within the first chromosomal region; comparing the first methylation level to a cutoff value; determining a classification of an abnormality in the first tissue for the first chromosomal region based on the comparison.

[0007e] In another aspect, there is provided a computer product comprising a non-transitory computer readable medium storing a plurality of instructions that when executed control a computer system to perform the method of any one of claims 1-111.

[0008] Embodiments provide systems, methods, and apparatuses for determining and using methylation profiles of various tissues and samples. Examples are provided. A methylation profile can be deduced for fetal/tumor tissue based on a comparison of plasma methylation (or other sample with cell-free DNA, e.g., urine, saliva, genital washings) to a methylation profile of the mother/patient. A methylation profile can be determined for fetal/tumor tissue using tissue-specific alleles to identify DNA from the fetus/tumor when the sample has a mixture of DNA. A methylation profile can be used to determine copy number variations in genome of a fetus/tumor. Methylation markers for a fetus have been identified via various techniques. The methylation profile can be determined by determining a size parameter of a size distribution of

DNA fragments, where reference values for the size parameter can be used to determine methylation levels.

[0009] Additionally, a methylation level can be used to determine a level of cancer. In the context of cancer, the measurement of the methylomic changes in plasma can allow one to detect the cancer (e.g. for screening purposes), for monitoring (e.g. to detect response following anti-cancer treatment; and to detect cancer relapse) and for prognostication (e.g. for measuring the load of cancer cells in the body or for staging purposes or for assessing the chance of death from disease or disease progression or metastatic processes).

[0010] A better understanding of the nature and advantages of embodiments of the present invention may be gained with reference to the following detailed description and the accompanying drawings.

#### BRIEF DESCRIPTION OF THE DRAWINGS

[0011] FIG. 1A shows a table 100 of sequencing results for maternal blood, placenta, and maternal plasma according to embodiments of the present invention.

[0012] FIG. 1B shows methylation density in 1-Mb windows of sequenced samples according to embodiments of the present invention.

[0013] FIGS. 2A-2C show plots of the beta-values against the methylation indices: (A) Maternal blood cells, (B) Chorionic villus sample, (C) Term placental tissue.

## WHAT IS CLAIMED IS:

1. A method of analyzing a biological sample of an organism, the biological sample including nucleic acid molecules originating from normal cells and potentially from cells associated with cancer, wherein at least some of the nucleic acid molecules are cell-free in the biological sample, the method comprising:

analyzing a plurality of DNA molecules from the biological sample, wherein analyzing a DNA molecule includes:

determining a location of the DNA molecule in a genome of the organism;

determining whether the DNA molecule is methylated at one or more sites;

for each of a plurality of sites:

determining a respective number of DNA molecules that are methylated at the site;

calculating a first methylation level based on the respective numbers of DNA molecules methylated at the plurality of sites;

comparing the first methylation level to a first cutoff value;

determining a first classification of a level of cancer based on the comparison.

2. The method of claim 1, wherein determining whether the DNA molecule is methylated at one or more sites includes:

performing methylation-aware sequencing.

3. The method of claim 2, wherein performing methylation-aware sequencing includes:

treating the DNA molecules with sodium bisulfite; and

performing sequencing of the treated DNA molecules.

4. The method of claim 3, wherein the treatment of the cell-free DNA molecules with sodium bisulfite is part of Tet-assisted bisulfite conversion or oxidative bisulfite sequencing for the detection of 5-hydroxymethylcytosine.

5. The method of claim 2, wherein the respective numbers of DNA molecules for each region are determined by aligning sequence reads obtained from the methylation-aware sequencing.

6. The method of claim 1, wherein determining whether the DNA molecule is methylated at one or more sites includes using methylation-sensitive restriction enzymes digestion, methylation-specific PCR, methylation-dependent DNA precipitation, methylated DNA binding protein/peptide or single molecule sequencing without sodium bisulfite treatment.

7. The method of claim 1, further comprising:  
for each of a first plurality of regions of the genome:  
determining a respective number of DNA molecules as being from the region;  
calculating a respective normalized value from the respective number;  
comparing the respective normalized value to a reference value to determine whether the respective region exhibits a deletion or an amplification;  
determining a first amount of regions that are determined to exhibit a deletion or an amplification;  
comparing the first amount to a first threshold value to determine a second classification of a level of cancer; and  
using the first classification and the second classification to determine a third classification of a level of cancer.

8. The method of claim 7, wherein the first threshold value is a percentage of the first plurality of regions that are determined to exhibit a deletion or an amplification.

9. The method of claim 7, wherein the third classification is positive for cancer only when both the first classification and the second classification indicate cancer.

10. The method of claim 7, wherein the third classification is positive for cancer when either the first classification or the second classification indicate cancer.

11. The method of claim 1 or 7, wherein the first classification indicates that cancer exists for the organism, the method further comprising:

identifying a type of cancer associated with the organism by comparing the first methylation level to a corresponding value determined from other organisms, wherein at least two of the other organisms are identified as having different types of cancer.

12. The method of claim 11, wherein the third classification indicates that cancer exists for the organism, the method further comprising:

identifying the type of cancer associated with the organism by comparing the first amount of regions to corresponding values determined from the other organisms.

13. The method of claim 1 or 7, wherein calculating the first methylation level includes:

- identifying a second plurality of regions of the genome;
- identifying one or more sites within each of the regions;
- calculating a region methylation level for each region, wherein the first methylation level is for a first region,

the method further comprising:

- comparing each of the region methylation levels to a respective region cutoff value, including comparing the first methylation level to the first cutoff value;
- determining a second amount of regions that are determined to have a region methylation level exceeding the respective region cutoff value; and
- comparing the second amount of regions to a second threshold value to determine the first classification.

14. The method of claim 13, wherein the regions that are determined to have a region methylation level exceeding the respective region cutoff value correspond to a first set of regions, the method further comprising:

- comparing region methylation levels of the first set of regions to corresponding region methylation levels of other organisms for the first set of regions, the other organisms having at least two of: a first type of cancer, absence of cancer, and a second type of cancer; and

- determining whether the organism has the first type of cancer, absence of cancer, or the second type of cancer based on the comparison.

15. The method of claim 14, further comprising:

- clustering the other organisms based on the region methylation levels of the first set of regions, wherein two of the clusters correspond to any two of: the first type of cancer, absence of cancer, and the second type of cancer,

- wherein the comparison of the region methylations levels determines which cluster the organism belongs.

16. The method of claim 15, wherein the clustering of the other organisms uses the region methylation levels of the organism.

17. The method of claim 15, wherein the clusters include a first cluster corresponding to the first type of cancer, a second cluster corresponding to the second type of cancer, and a third cluster corresponding to absence of cancer.

18. The method of claim 15, wherein the clustering of the other organisms is further based on respective normalized values of a second set of regions for the other organisms, the second set of regions correspond to regions that are determined to exhibit a deletion or an amplification, wherein the respective normalized value for a region is determined from a respective number of DNA molecules from the region, the method further comprising:

for each of the second set of regions:

determining a respective number of DNA molecules as being from the region;

calculating a respective normalized value from the respective number; and

comparing the respective normalized values of the second set of regions for the organism to the respective normalized values of the other organisms as part of determining which cluster the organism belongs.

19. The method of claim 18, wherein the clustering of the other organisms is further based on respective methylation densities of hypermethylated CpG islands, the method further comprising:

for each of the hypermethylated CpG islands:

determining a respective methylation density,

comparing the respective methylation densities of the hypermethylated CpG islands for the organism to the methylation densities of the other organisms as part of determining which cluster the organism belongs.

20. The method of claim 13, further comprising:

for each of the second plurality of regions:

computing a respective difference between the region methylation level and the respective region cutoff value; and

calculating a respective probability corresponding to the respective difference;

wherein determining the second amount of regions includes:

calculating a cumulative score including the respective probabilities.

21. The method of claim 20, wherein calculating the cumulative score includes:

taking a logarithm of the respective probability to obtain a respective logarithm result; and

calculating a sum including the respective logarithm results.

22. The method of claim 21, wherein the cumulative score is a negative of the sum of the respective logarithm results.

23. The method of claim 20, wherein each difference is normalized with a standard deviation associated with the respective region cutoff value.

24. The method of claim 20, wherein the respective probability corresponds to a probability for the respective difference according to a statistical distribution.

25. The method of claim 20, wherein the second threshold value corresponds to a highest cumulative score from a reference group.

26. The method of claim 13, further comprising:

for each of the first plurality of regions:

computing a respective difference between the respective normalized value and the reference value; and

calculating a respective probability corresponding to the respective difference; wherein determining the first amount of regions includes:

calculating a first sum including the respective probabilities.

27. The method of claim 13, wherein the respective region cutoff values are a specified amount from a reference methylation level.

28. The method of claim 13, wherein the second threshold value is a percentage, and wherein comparing the second amount of regions to a second threshold value includes:

dividing the second amount of regions by a second number of the second plurality of regions before comparing to the second threshold value.

29. The method of claim 28, wherein the second number corresponds to all of the second plurality of regions.

30. The method of claim 13, wherein the first plurality of regions are the same as the second plurality of regions, and wherein the respective region cutoff values are dependent on whether the respective region exhibits a deletion or an amplification.

31. The method of claim 30, wherein a respective region cutoff value has a larger magnitude when the respective region exhibits an amplification than when no amplification is exhibited, and wherein a respective region cutoff value has a smaller magnitude when the respective region exhibits a deletion than when no deletion is exhibited.

32. The method of claim 31, wherein a respective region cutoff values test for hypomethylation of the second plurality of regions, wherein a respective region cutoff value has a larger negative value when the respective region exhibits an amplification than when no amplification is exhibited, and wherein a respective region cutoff value has a smaller negative value when the respective region exhibits a deletion than when no deletion is exhibited.

33. The method of claim 13, wherein the biological sample is taken prior to treatment, the method further comprising:

repeating the method of claim 13 for another biological sample taken after treatment to obtain:

a subsequent first amount of regions that are determined to exhibit a deletion or an amplification; and

a subsequent second amount of regions that are determined to have a region methylation level exceeding the respective region cutoff value;

comparing the first amount to the subsequent first amount and the second amount to the subsequent second amount to determine a prognosis of the organism.

34. The method of claim 33, wherein comparing the first amount to the subsequent first amount and the second amount to the subsequent second amount to determine the prognosis of the organism includes:

determining a first difference between the first amount and the subsequent first amount;

comparing the first difference to one or more first difference thresholds;

determining a second difference between the second amount and the subsequent second amount; and

comparing the second difference to one or more second difference thresholds.

35. The method of claim 34, wherein the prognosis is predicted to be worse when the first difference is below one first difference threshold than when the first difference is above the one first difference threshold, and wherein the prognosis is predicted to be worse when the second difference is below one second difference threshold than when the second difference is above the one second difference threshold.

36. The method of claim 35, wherein the one first difference threshold and the one second difference threshold are zero.

37. The method of claim 33, wherein the treatment is immunotherapy, surgery, radiotherapy, chemotherapy, antibody-based therapy, epigenetic therapy or targeted therapy.

38. The method of claim 1, wherein the first cutoff value is a specified distance from a reference methylation level established from a biological sample obtained from a healthy organism.

39. The method of claim 38, wherein the specified distance is a specified number of standard deviations from the reference methylation level.

40. The method of claim 1, wherein the first cutoff value is established from a reference methylation level determined from a previous biological sample of the organism obtained previous to the biological sample being tested.

41. The method of claim 1, wherein comparing the first methylation level to the first cutoff value includes:

determining a difference between the first methylation level and a reference methylation level; and

comparing the difference to a threshold corresponding to the first cutoff value.

42. The method of claim 1, further comprising:

determining a fractional concentration of tumor DNA in the biological sample;  
calculating the first cutoff value based on the fractional concentration.

43. The method of claim 1, further comprising:

determining whether a fractional concentration of tumor DNA in the biological sample is greater than a minimum value; and

if the fractional concentration is not greater than the minimum value, flagging the biological sample.

44. The method of claim 43, wherein the minimum value is determined based on an expected difference in methylation levels for a tumor relative to a reference methylation level.

45. The method of claim 1, further comprising:  
measuring a size of DNA molecules at the plurality of sites; and  
before comparing the first methylation level to the first cutoff value, normalizing the first methylation level based on the measured sizes of the DNA molecules.

46. The method of claim 45, wherein normalizing the first methylation level based on the measured sizes includes:  
selecting DNA molecules having a first size;  
using the selected DNA molecules to calculate the first methylation level, the first cutoff value corresponding to the first size.

47. The method of claim 46, wherein the first size is a range of lengths.

48. The method of claim 46, wherein the DNA molecules are selected based on a physical separation that is dependent on size.

49. The method of claim 46, wherein selecting DNA molecules having a first size includes:  
performing paired-end massively parallel sequencing of the plurality of DNA molecules to obtain pairs of sequences for each of the DNA molecules;  
determining a size of a DNA molecule by comparing the pair of sequences to a reference genome; and  
selecting DNA molecules having the first size.

50. The method of claim 45, wherein normalizing the first methylation level based on the measured sizes includes:  
obtaining a functional relationship between size and methylation levels; and  
using the functional relationship to normalize the first methylation level.

51. The method of claim 50, wherein the functional relationship provides scaling values corresponding to respective sizes.

52. The method of claim 51, further comprising:  
computing an average size corresponding to DNA molecules used to calculate the first methylation level; and  
multiplying the first methylation level by the corresponding scaling value.

53. The method of claim 51, further comprising:  
for each of the plurality of sites:  
for each of the DNA molecules located at the site:  
obtaining a respective size of the DNA molecule at the site; and  
using the scaling value corresponding to the respective size to  
normalize a contribution of the DNA molecule to the respective number of DNA molecules that are methylated at the site.

54. The method of claim 1, wherein the plurality of sites includes CpG sites, wherein the CpG sites are organized into a plurality of CpG islands, each CpG island including one or more CpG sites, wherein the first methylation level corresponds to a first CpG island.

55. The method of claim 54, wherein the CpG islands have a mean methylation density of less than a first percentage in a reference group, and wherein the coefficient of variation for the methylation density in the reference group is less than a second percentage.

56. The method of claim 54, further comprising:  
for each of the CpG islands:  
determining whether the CpG island is hypermethylated relative to a reference group of samples of other organisms by comparing a methylation level of the CpG island to a respective cutoff value;  
for each of the hypermethylated CpG islands:  
determining a respective methylation density;  
calculating a cumulative score from the respective methylation densities; and  
comparing the cumulative score to a cumulative cutoff value to determine the first classification.

57. The method of claim 56, wherein calculating the cumulative score from the respective methylation densities includes:

for each of the hypermethylated CpG islands:

calculating a respective difference between the respective methylation density and a reference density; and

calculating a respective probability corresponding to the respective difference;

and

using the respective probabilities to determine the cumulative score.

58. The method of claim 56, wherein the cumulative score is determined by: taking a logarithm of the respective probability to obtain a respective logarithm result; and

calculating a sum including the respective logarithm results, the cumulative score is a negative of the sum.

59. The method of claim 56, wherein each respective difference is normalized with a standard deviation associated with the reference density.

60. The method of claim 56, wherein the cumulative cutoff value corresponds to a highest cumulative score from the reference group.

61. The method of claim 56, wherein determining whether the first CpG island is hypermethylated includes:

comparing the first methylation level to the first cutoff value and to a third cutoff value,

wherein the first cutoff value corresponds to a mean of methylation densities for the reference group plus a specified percentage, and wherein the third cutoff value corresponds to a specified number of standard deviations plus the mean of methylation densities for the reference group.

62. The method of claim 61, wherein the specified percentage is 2%.

63. The method of claim 61, wherein the specified number of standard deviations is three.

64. The method of claim 1, further comprising:

for each of a first plurality of regions of the genome:

determining a respective number of DNA molecules as being from the region;

calculating a respective normalized value from the respective number;  
comparing the respective normalized value to a reference value to determine whether the respective region exhibits a deletion or an amplification;

determining a first set of regions that are determined to all exhibit one of: a deletion, an amplification, or normal representation, wherein the first methylation level corresponds to the first set of regions;

determining a second set of regions that are determined to all exhibit a second of: a deletion, an amplification, or normal representation;

calculating a second methylation level based on the respective numbers of DNA molecules methylated at sites in the second set of regions,

wherein comparing the first methylation level to the first cutoff value includes:

computing a parameter between the first methylation level and the second methylation; and

comparing the parameter to the first cutoff value.

65. The method of claim 64, wherein the first methylation level is a statistical value of region methylation levels calculated for each region of the first set of regions, and wherein the second methylation level is a statistical value of region methylation levels calculated for each region of the second set of regions.

66. The method of claim 65, wherein the statistical values are determined using Student's t-test, analysis of variance (ANOVA) test, or Kruskal-Wallis test.

67. The method of claim 64, wherein the parameter includes a ratio or a difference.

68. The method of claim 67, wherein computing the parameter includes applying a probability distribution to the ratio or the difference.

69. A method of determining a first methylation profile from a biological sample of an organism, the biological sample including cell-free DNA comprising a mixture of cell-free DNA molecules originating from a first tissue and from a second tissue, the method comprising:

obtaining a second methylation profile corresponding to DNA molecules of the second tissue, the second methylation profile providing a second methylation density at each of a plurality of loci in a genome of the organism, the second methylation density at a particular locus corresponding to a proportion of DNA molecules of the second tissue that are methylated;

determining a cell-free methylation profile from the cell-free DNA molecules of the mixture, the cell-free methylation profile providing a mixture a methylation density at each of the plurality of loci;

determining a percentage of the cell-free DNA molecules in the mixture that are from the first tissue; and

determining the first methylation profile of the first tissue by:

for each of the plurality of loci:

calculating a differential parameter that includes a difference between the second methylation density of the second methylation profile and the mixture methylation density of the cell-free methylation profile, the difference being scaled by the percentage.

70. The method of claim 69, further comprising:

determining a first methylation level from the first methylation profile; and

comparing the first methylation level to a first cutoff value to determine a classification of a level of cancer based on the comparison.

71. The method of claim 69, further comprising:

transforming the first methylation profile to obtain a corrected first methylation profile.

72. The method of claim 71, wherein the transformation is a linear

transformation.

73. The method of claim 69, wherein the differential parameter  $D$  for a locus

is defined as  $D = mbc - \frac{(mbc - mp)}{f * CN}$ , where  $mbc$  denotes the second methylation density of the second methylation profile at the locus,  $mp$  denotes the mixture methylation density of the cell-free methylation profile at the locus,  $f$  is the percentage of cell-free DNA molecules from the first tissue in the biological sample, and  $CN$  represents a copy number at the locus.

74. The method of claim 73, wherein  $CN$  is one for the copy number of the

first tissue being disomic at the locus.

75. The method of claim 73, further comprising:

identifying regions where  $D$  exceeds a threshold.

76. The method of claim 69, further comprising:

identifying the plurality of loci by selecting loci that have any one or more of the following criteria:

- a GC content of greater than 50%;
- a second methylation density of the second methylation profile of less than a first threshold or greater than a second threshold; and
- a minimum of five CpG sites in a region defined by the locus.

77. The method of claim 69, wherein the biological sample is from a female subject pregnant with a fetus, and wherein the first tissue is from the fetus or a placenta and the second tissue is from the female subject.

78. The method of claim 77, further comprising:  
determining a first methylation level from the first methylation profile; and  
comparing the first methylation level to a first cutoff value to determine a classification of a medical condition.

79. The method of claim 78, wherein the medical condition is preeclampsia or a chromosomal abnormality in the first tissue.

80. The method of claim 69, wherein the first tissue is from a tumor within the organism and the second tissue is from a non-malignant tissue of the organism.

81. The method of claim 69, wherein the biological sample also includes DNA molecules from the second tissue, and wherein obtaining the second methylation profile includes:

analyzing the DNA molecules from the second tissue to determine the second methylation profile.

82. The method of claim 69, wherein the obtained second methylation profile corresponds to an average methylation profiles obtained from a group of control samples.

83. The method of claim 69, wherein determining a mixture methylation density of the cell-free methylation profile for a first locus of the plurality of loci includes:

determining a first number of the cell-free DNA molecules that are from the first locus;

determining whether each DNA molecule of the first number is methylated at one or more sites to obtain a second number of methylated DNA molecules; and

calculating the mixture methylation density from the first number and the second number.

84. The method of claim 83, wherein the one or more sites are CpG sites.

85. The method of claim 69, wherein determining the cell-free methylation profile includes:

treating the cell-free DNA molecules with sodium bisulfite; and  
performing sequencing of the treated DNA molecules.

86. The method of claim 85, wherein the treatment of the cell-free DNA molecules is part of Tet-assisted bisulfite conversion or includes treating the cell-free DNA molecules with potassium perruthenate (KRuO<sub>4</sub>).

87. The method of claim 69, wherein determining the cell-free methylation profile includes:

performing single molecule sequencing of the DNA, wherein the sequencing of a single molecule includes determining whether at least one site of a DNA molecule is methylated.

88. A method of determining a first methylation profile from a biological sample of an organism, the biological sample including cell-free DNA comprising a mixture of cell-free DNA originating from a first tissue and from a second tissue, the method comprising:

analyzing a plurality of DNA molecules from the biological sample, wherein analyzing a DNA molecule includes:

determining a location of the DNA molecule in a genome of the organism;  
determining a genotype of the DNA molecule;

determining whether the DNA molecule is methylated at one or more sites;  
identifying a plurality of first loci at which a first genome of the first tissue is heterozygous for a respective first allele and a respective second allele and a second genome of the second tissue is homozygous for the respective first allele;

for each of the first loci:

for each of one or more sites associated with the locus:

determining a number of DNA molecules that are methylated at the site  
and correspond to the respective second allele of the locus;

calculating a methylation density based on the numbers of DNA molecules methylated at the one or more sites of the locus and corresponding to the respective second allele of the locus;

creating the first methylation profile of the first tissue from the methylation densities for the first loci.

89. The method of claim 88, wherein the biological sample is from a female subject pregnant with a fetus, and wherein the first tissue is from the fetus or a placenta and the second tissue corresponds to the female subject.

90. The method of claim 88, wherein the sites correspond to CpG sites.

91. The method of claim 88, wherein each locus includes at least one CpG site.

92. The method of claim 88, wherein each locus is adjacent to at least one CpG site.

93. A method of detecting a chromosomal abnormality from a biological sample of an organism, the biological sample including cell-free DNA comprising a mixture of cell-free DNA originating from a first tissue and from a second tissue, the method comprising:

analyzing a plurality of DNA molecules from the biological sample, wherein analyzing a DNA molecule includes:

determining a location of the DNA molecule in a reference genome;

determining whether the DNA molecule is methylated at one or more sites;

for each of a plurality of sites:

determining a respective number of DNA molecules that are methylated at the site;

calculating a first methylation level of a first chromosomal region based on the respective numbers of DNA molecules methylated at sites within the first chromosomal region;

comparing the first methylation level to a cutoff value;

determining a classification of an abnormality in the first tissue for the first chromosomal region based on the comparison.

94. The method of claim 93, wherein comparing the first methylation level to a cutoff value includes:

normalizing the first methylation level and comparing the normalized first methylation level to the cutoff value.

95. The method of claim 94, wherein the normalization uses a second methylation level of a second chromosomal region.

96. The method of claim 94, wherein the normalization uses a fractional concentration of cell-free DNA from the first tissue.

97. The method of claim 93, wherein calculating a first methylation level includes:

- identifying a plurality of regions of a genome of the organism;
- identifying one or more sites within each of the regions;
- calculating a region methylation level for each region, wherein the first methylation level is for a first region, and
- wherein comparing the first methylation level to a first cutoff value includes
  - comparing each of the region methylation levels to a respective region cutoff value;
  - determining a first number of regions whose region methylation level exceeds the respective region cutoff value; and
  - comparing the first number to a threshold value to determine the classification.

98. The method of claim 97, wherein the threshold value is a percentage, and wherein comparing the first number to a threshold value includes:

- dividing the first number of regions by a second number of regions before comparing to the threshold value.

99. The method of claim 98, wherein the second number of regions is all of the identified plurality of regions.

100. The method of claim 97, wherein the respective region cutoff values are a specified amount from a reference methylation level.

101. The method of claim 93, wherein the biological sample is from a female subject pregnant with a fetus, wherein the first tissue is from the fetus or a placenta and the second tissue is from the female subject, and wherein the chromosomal abnormality is a fetal chromosomal abnormality.

102. The method of claim 93, wherein the cutoff value is based on a background methylation level associated with DNA of the second tissue from the female subject.

103. The method of claim 101, wherein the cutoff value is determined from other female pregnant subjects carrying fetuses without a chromosomal abnormality for the first chromosomal region.

104. The method of claim 101, wherein the chromosomal abnormality is trisomy 21 or trisomy 18 or trisomy 13 or Turner syndrome or Klinefelter syndrome.

105. The method of claim 93, wherein the first tissue is from a tumor within the organism and the second tissue is from a non-malignant tissue of the organism.

106. The method of claim 93, wherein the cutoff value is determined from other organisms not having cancer.

107. The method of claim 93, wherein cutoff value is based on a concentration of cell-free DNA originating from the first tissue in the biological sample.

108. The method of claim 107, wherein the cutoff value is based on a scale factor corresponding to a type of abnormality, where the type of abnormality is deletion or duplication.

109. The method of claim 93, wherein determining the location of the DNA molecule in a reference genome involves determining whether the location is within the first chromosomal region.

110. The method of claim 109, wherein determining the location of the DNA molecule in the reference genome is accomplished by determining whether the DNA molecule maps to the first chromosomal region.

111. The method of claim 93, wherein the chromosomal abnormality is a subchromosomal deletion or subchromosomal duplication or DiGeorge syndrome.

112. A computer product comprising a non-transitory computer readable medium storing a plurality of instructions that when executed control a computer system to perform the method of any one of claims 1-111.

## CLAIMS:

1. A method of analyzing a biological sample of an organism, the biological sample including nucleic acid molecules originating from normal cells and potentially from cells associated with cancer, wherein at least some of the nucleic acid molecules are cell-free in the biological sample, the method comprising:
  - analyzing a plurality of DNA molecules from the biological sample, wherein analyzing a DNA molecule includes:
    - determining a location of the DNA molecule in a genome of the organism;
    - determining whether the DNA molecule is methylated at one or more sites;
    - for each of a plurality of sites:
      - determining a respective number of DNA molecules that are methylated at the site;
      - calculating a first methylation level based on the respective numbers of DNA molecules methylated at the plurality of sites;
      - comparing the first methylation level to a first cutoff value;
      - determining a first classification of a level of cancer based on the comparison.
2. The method of claim 1, wherein determining whether the DNA molecule is methylated at one or more sites includes:
  - performing methylation-aware sequencing.
3. The method of claim 2, wherein performing methylation-aware sequencing includes:
  - treating the DNA molecules with sodium bisulfite; and
  - performing sequencing of the treated DNA molecules.
4. The method of claim 3, wherein the treatment of the cell-free DNA molecules with sodium bisulfite is part of Tet-assisted bisulfite conversion or oxidative bisulfite sequencing for the detection of 5-hydroxymethylcytosine.
5. The method of claim 2, wherein the respective numbers of DNA molecules for each region are determined by aligning sequence reads obtained from the methylation-aware sequencing.

6. The method of claim 1, wherein determining whether the DNA molecule is methylated at one or more sites includes using methylation-sensitive restriction enzymes digestion, methylation-specific PCR, methylation-dependent DNA precipitation, methylated DNA binding protein/peptide or single molecule sequencing without sodium bisulfite treatment.

7. The method of claim 1, further comprising:  
for each of a first plurality of regions of the genome:  
determining a respective number of DNA molecules as being from the region;  
calculating a respective normalized value from the respective number;  
comparing the respective normalized value to a reference value to determine whether the respective region exhibits a deletion or an amplification;  
determining a first amount of regions that are determined to exhibit a deletion or an amplification;  
comparing the first amount to a first threshold value to determine a second classification of a level of cancer; and  
using the first classification and the second classification to determine a third classification of a level of cancer.

8. The method of claim 7, wherein the first threshold value is a percentage of the first plurality of regions that are determined to exhibit a deletion or an amplification.

9. The method of claim 7, wherein the third classification is positive for cancer only when both the first classification and the second classification indicate cancer.

10. The method of claim 7, wherein the third classification is positive for cancer when either the first classification or the second classification indicate cancer.

11. The method of claim 1 or 7, wherein the first classification indicates that cancer exists for the organism, the method further comprising:  
identifying a type of cancer associated with the organism by comparing the first methylation level to a corresponding value determined from other organisms, wherein at least two of the other organisms are identified as having different types of cancer.

12. The method of claim 11, wherein the third classification indicates that cancer exists for the organism, the method further comprising:

identifying the type of cancer associated with the organism by comparing the first amount of regions to corresponding values determined from the other organisms.

5 13. The method of claim 1 or 7, wherein calculating the first methylation level includes:

identifying a second plurality of regions of the genome;

identifying one or more sites within each of the regions;

10 calculating a region methylation level for each region, wherein the first methylation level is for a first region,

the method further comprising:

comparing each of the region methylation levels to a respective region cutoff value, including comparing the first methylation level to the first cutoff value;

15 determining a second amount of regions that are determined to have a region methylation level exceeding the respective region cutoff value; and

comparing the second amount of regions to a second threshold value to determine the first classification.

14. The method of claim 13, wherein the regions that are determined to have a region methylation level exceeding the respective region cutoff value correspond to a first set of regions, the method further comprising:

20 comparing region methylation levels of the first set of regions to corresponding region methylation levels of other organisms for the first set of regions, the other organisms having at least two of: a first type of cancer, absence of cancer, and a second type of cancer; and

25 determining whether the organism has the first type of cancer, absence of cancer, or the second type of cancer based on the comparison.

15. The method of claim 14, further comprising:

30 clustering the other organisms based on the region methylation levels of the first set of regions, wherein two of the clusters correspond to any two of: the first type of cancer, absence of cancer, and the second type of cancer,

wherein the comparison of the region methylations levels determines which cluster the organism belongs.

16. The method of claim 15, wherein the clustering of the other organisms uses the region methylation levels of the organism.

5 17. The method of claim 15, wherein the clusters include a first cluster corresponding to the first type of cancer, a second cluster corresponding to the second type of cancer, and a third cluster corresponding to absence of cancer.

10 18. The method of claim 15, wherein the clustering of the other organisms is further based on respective normalized values of a second set of regions for the other organisms, the second set of regions correspond to regions that are determined to exhibit a deletion or an amplification, wherein the respective normalized value for a region is determined from a respective number of DNA molecules from the region, the method further comprising:

for each of the second set of regions:

15 determining a respective number of DNA molecules as being from the region;

calculating a respective normalized value from the respective number; and

20 comparing the respective normalized values of the second set of regions for the organism to the respective normalized values of the other organisms as part of determining which cluster the organism belongs.

19. The method of claim 18, wherein the clustering of the other organisms is further based on respective methylation densities of hypermethylated CpG islands, the method further comprising:

for each of the hypermethylated CpG islands:

25 determining a respective methylation density,  
comparing the respective methylation densities of the hypermethylated CpG islands for the organism to the methylation densities of the other organisms as part of determining which cluster the organism belongs.

20. The method of claim 13, further comprising:

30 for each of the second plurality of regions:

computing a respective difference between the region methylation level and the respective region cutoff value; and

calculating a respective probability corresponding to the respective difference;

5 wherein determining the second amount of regions includes:

calculating a cumulative score including the respective probabilities.

21. The method of claim 20, wherein calculating the cumulative score includes:

10 taking a logarithm of the respective probability to obtain a respective logarithm result; and

calculating a sum including the respective logarithm results.

22. The method of claim 21, wherein the cumulative score is a negative of the sum of the respective logarithm results.

15 23. The method of claim 20, wherein each difference is normalized with a standard deviation associated with the respective region cutoff value.

24. The method of claim 20, wherein the respective probability corresponds to a probability for the respective difference according to a statistical distribution.

20 25. The method of claim 20, wherein the second threshold value corresponds to a highest cumulative score from a reference group.

26. The method of claim 13, further comprising:  
for each of the first plurality of regions:

computing a respective difference between the respective normalized value and the reference value; and

25 calculating a respective probability corresponding to the respective difference;

wherein determining the first amount of regions includes:

calculating a first sum including the respective probabilities.

27. The method of claim 13, wherein the respective region cutoff values are a specified amount from a reference methylation level.

28. The method of claim 13, wherein the second threshold value is a percentage, and wherein comparing the second amount of regions to a second threshold value includes:

dividing the second amount of regions by a second number of the second plurality of regions before comparing to the second threshold value.

29. The method of claim 28, wherein the second number corresponds to all of the second plurality of regions.

30. The method of claim 13, wherein the first plurality of regions are the same as the second plurality of regions, and wherein the respective region cutoff values are dependent on whether the respective region exhibits a deletion or an amplification.

31. The method of claim 30, wherein a respective region cutoff value has a larger magnitude when the respective region exhibits an amplification than when no amplification is exhibited, and wherein a respective region cutoff value has a smaller magnitude when the respective region exhibits a deletion than when no deletion is exhibited.

32. The method of claim 31, wherein a respective region cutoff values test for hypomethylation of the second plurality of regions, wherein a respective region cutoff value has a larger negative value when the respective region exhibits an amplification than when no amplification is exhibited, and wherein a respective region cutoff value has a smaller negative value when the respective region exhibits a deletion than when no deletion is exhibited.

33. The method of claim 13, wherein the biological sample is taken prior to treatment, the method further comprising:

repeating the method of claim 13 for another biological sample taken after treatment to obtain:

a subsequent first amount of regions that are determined to exhibit a deletion or an amplification; and

a subsequent second amount of regions that are determined to have a region methylation level exceeding the respective region cutoff value;

comparing the first amount to the subsequent first amount and the second amount to the subsequent second amount to determine a prognosis of the organism.

5           34. The method of claim 33, wherein comparing the first amount to the subsequent first amount and the second amount to the subsequent second amount to determine the prognosis of the organism includes:

determining a first difference between the first amount and the subsequent first amount;

10           comparing the first difference to one or more first difference thresholds;

determining a second difference between the second amount and the subsequent second amount; and

comparing the second difference to one or more second difference thresholds.

15           35. The method of claim 34, wherein the prognosis is predicted to be worse when the first difference is below one first difference threshold than when the first difference is above the one first difference threshold, and wherein the prognosis is predicted to be worse when the second difference is below one second difference threshold than when the second difference is above the one second difference threshold.

20           36. The method of claim 35, wherein the one first difference threshold and the one second difference threshold are zero.

          37. The method of claim 33, wherein the treatment is immunotherapy, surgery, radiotherapy, chemotherapy, antibody-based therapy, epigenetic therapy or targeted therapy.

25           38. The method of claim 1, wherein the first cutoff value is a specified distance from a reference methylation level established from a biological sample obtained from a healthy organism.

          39. The method of claim 38, wherein the specified distance is a specified number of standard deviations from the reference methylation level.

40. The method of claim 1, wherein the first cutoff value is established from a reference methylation level determined from a previous biological sample of the organism obtained previous to the biological sample being tested.

41. The method of claim 1, wherein comparing the first methylation level to the first cutoff value includes:

determining a difference between the first methylation level and a reference methylation level; and

comparing the difference to a threshold corresponding to the first cutoff value.

42. The method of claim 1, further comprising:

determining a fractional concentration of tumor DNA in the biological sample; calculating the first cutoff value based on the fractional concentration.

43. The method of claim 1, further comprising:

determining whether a fractional concentration of tumor DNA in the biological sample is greater than a minimum value; and

if the fractional concentration is not greater than the minimum value, flagging the biological sample.

44. The method of claim 43, wherein the minimum value is determined based on an expected difference in methylation levels for a tumor relative to a reference methylation level.

45. The method of claim 1, further comprising:

measuring a size of DNA molecules at the plurality of sites; and

before comparing the first methylation level to the first cutoff value,

normalizing the first methylation level based on the measured sizes of the DNA molecules.

46. The method of claim 45, wherein normalizing the first methylation

level based on the measured sizes includes:

selecting DNA molecules having a first size;

using the selected DNA molecules to calculate the first methylation level, the first cutoff value corresponding to the first size.

47. The method of claim 46, wherein the first size is a range of lengths.

48. The method of claim 46, wherein the DNA molecules are selected based on a physical separation that is dependent on size.

49. The method of claim 46, wherein selecting DNA molecules having a first size includes:

performing paired-end massively parallel sequencing of the plurality of DNA molecules to obtain pairs of sequences for each of the DNA molecules;

determining a size of a DNA molecule by comparing the pair of sequences to a reference genome; and

selecting DNA molecules having the first size.

50. The method of claim 45, wherein normalizing the first methylation level based on the measured sizes includes:

obtaining a functional relationship between size and methylation levels; and

using the functional relationship to normalize the first methylation level.

51. The method of claim 50, wherein the functional relationship provides scaling values corresponding to respective sizes.

52. The method of claim 51, further comprising:

computing an average size corresponding to DNA molecules used to calculate the first methylation level; and

multiplying the first methylation level by the corresponding scaling value.

53. The method of claim 51, further comprising:

for each of the plurality of sites:

for each of the DNA molecules located at the site:

obtaining a respective size of the DNA molecule at the site; and

using the scaling value corresponding to the respective size to normalize a contribution of the DNA molecule to the respective number of DNA molecules that are methylated at the site.

54. The method of claim 1, wherein the plurality of sites includes CpG sites, wherein the CpG sites are organized into a plurality of CpG islands, each CpG island including one or more CpG sites, wherein the first methylation level corresponds to a first CpG island.

55. The method of claim 54, wherein the CpG islands have a mean methylation density of less than a first percentage in a reference group, and wherein the coefficient of variation for the methylation density in the reference group is less than a second percentage.

56. The method of claim 54, further comprising:  
for each of the CpG islands:  
determining whether the CpG island is hypermethylated relative to a reference group of samples of other organisms by comparing a methylation level of the CpG island to a respective cutoff value;

for each of the hypermethylated CpG islands:  
determining a respective methylation density;  
calculating a cumulative score from the respective methylation densities; and  
comparing the cumulative score to a cumulative cutoff value to determine the first classification.

57. The method of claim 56, wherein calculating the cumulative score from the respective methylation densities includes:

for each of the hypermethylated CpG islands:  
calculating a respective difference between the respective methylation density and a reference density; and  
calculating a respective probability corresponding to the respective difference; and  
using the respective probabilities to determine the cumulative score.

58. The method of claim 56, wherein the cumulative score is determined by:

taking a logarithm of the respective probability to obtain a respective logarithm result; and

calculating a sum including the respective logarithm results, the cumulative score is a negative of the sum.

59. The method of claim 56, wherein each respective difference is normalized with a standard deviation associated with the reference density.

5 60. The method of claim 56, wherein the cumulative cutoff value corresponds to a highest cumulative score from the reference group.

61. The method of claim 56, wherein determining whether the first CpG island is hypermethylated includes:

10 comparing the first methylation level to the first cutoff value and to a third cutoff value,

wherein the first cutoff value corresponds to a mean of methylation densities for the reference group plus a specified percentage, and wherein the third cutoff value corresponds to a specified number of standard deviations plus the mean of methylation densities for the reference group.

15 62. The method of claim 61, wherein the specified percentage is 2%.

63. The method of claim 61, wherein the specified number of standard deviations is three.

64. The method of claim 1, further comprising:  
for each of a first plurality of regions of the genome:

20 determining a respective number of DNA molecules as being from the region;

calculating a respective normalized value from the respective number;  
comparing the respective normalized value to a reference value to determine whether the respective region exhibits a deletion or an amplification;

25 determining a first set of regions that are determined to all exhibit one of: a deletion, an amplification, or normal representation, wherein the first methylation level corresponds to the first set of regions;

determining a second set of regions that are determined to all exhibit a second of: a deletion, an amplification, or normal representation;

calculating a second methylation level based on the respective numbers of DNA molecules methylated at sites in the second set of regions,

wherein comparing the first methylation level to the first cutoff value

includes:

- 5                    computing a parameter between the first methylation level and the second methylation; and  
                     comparing the parameter to the first cutoff value.

65.     The method of claim 64, wherein the first methylation level is a statistical value of region methylation levels calculated for each region of the first set of  
10     regions, and wherein the second methylation level is a statistical value of region methylation levels calculated for each region of the second set of regions.

66.     The method of claim 65, wherein the statistical values are determined using Student's t-test, analysis of variance (ANOVA) test, or Kruskal-Wallis test.

67.     The method of claim 64, wherein the parameter includes a ratio or a  
15     difference.

68.     The method of claim 67, wherein computing the parameter includes applying a probability distribution to the ratio or the difference.

69.     A method of determining a first methylation profile from a biological sample of an organism, the biological sample including cell-free DNA comprising a mixture  
20     of cell-free DNA molecules originating from a first tissue and from a second tissue, the method comprising:

                     obtaining a second methylation profile corresponding to DNA molecules of the second tissue, the second methylation profile providing a methylation density at each of a plurality of loci in a genome of the organism, the methylation density at a particular locus  
25     corresponding to a proportion of DNA molecules of the second tissue that are methylated;

                     determining a cell-free methylation profile from the cell-free DNA molecules of the mixture, the cell-free methylation profile providing a methylation density at each of the plurality of loci;

                     determining a percentage of the cell-free DNA molecules in the mixture that  
30     are from the first tissue; and

determining the first methylation profile of the first tissue by:

for each of the plurality of loci:

calculating a differential parameter that includes a difference between the methylation density of the second methylation profile and the methylation density of the cell-free methylation profile, the difference being scaled by the percentage.

70. The method of claim 69, further comprising:

determining a first methylation level from the first methylation profile; and comparing the first methylation level to a first cutoff value to determine a classification of a level of cancer based on the comparison.

71. The method of claim 69, further comprising:

transforming the first methylation profile to obtain a corrected first methylation profile.

72. The method of claim 71, wherein the transformation is a linear transformation.

73. The method of claim 69, wherein the differential parameter  $D$  for a locus is defined as  $D = mbc - \frac{(mbc - mp)}{f * CN}$ , where  $mbc$  denotes the methylation density of the second methylation profile at the locus,  $mp$  denotes the methylation density of the cell-free methylation profile at the locus,  $f$  is the percentage of cell-free DNA molecules from the first tissue in the biological sample, and  $CN$  represents a copy number at the locus.

74. The method of claim 73, wherein  $CN$  is one for the copy number of the first tissue being disomic at the locus.

75. The method of claim 73, further comprising:  
identifying regions where  $D$  exceeds a threshold.

76. The method of claim 69, further comprising:  
identifying the plurality of loci by selecting loci that have any one or more of the following criteria:  
a GC content of greater than 50%;

a methylation density of the second methylation profile of less than a first threshold or greater than a second threshold; and

a minimum of five CpG sites in a region defined by the locus.

5 77. The method of claim 69, wherein the biological sample is from a female subject pregnant with a fetus, and wherein the first tissue is from the fetus or a placenta and the second tissue is from the female subject.

78. The method of claim 77, further comprising:  
determining a first methylation level from the first methylation profile; and  
comparing the first methylation level to a first cutoff value to determine a  
10 classification of a medical condition.

79. The method of claim 78, wherein the medical condition is preeclampsia or a chromosomal abnormality in the first tissue.

80. The method of claim 69, wherein the first tissue is from a tumor within the organism and the second tissue is from a non-malignant tissue of the organism.

15 81. The method of claim 69, wherein the biological sample also includes DNA molecules from the second tissue, and wherein obtaining the second methylation profile includes:

analyzing the DNA molecules from the second tissue to determine the second methylation profile.

20 82. The method of claim 69, wherein the obtained second methylation profile corresponds to an average methylation profiles obtained from a group of control samples.

83. The method of claim 69, wherein determining a methylation density of the cell-free methylation profile for a first locus of the plurality of loci includes:

25 determining a first number of the cell-free DNA molecules that are from the first locus;

determining whether each DNA molecule of the first number is methylated at one or more sites to obtain a second number of methylated DNA molecules; and

calculating the methylation density from the first number and the second number.

84. The method of claim 83, wherein the one or more sites are CpG sites.

5     85. The method of claim 69, wherein determining the cell-free methylation profile includes:  
treating the cell-free DNA molecules with sodium bisulfite; and  
performing sequencing of the treated DNA molecules.

10     86. The method of claim 85, wherein the treatment of the cell-free DNA molecules is part of Tet-assisted bisulfite conversion or includes treating the cell-free DNA molecules with potassium perruthenate (KRuO<sub>4</sub>).

15     87. The method of claim 69, wherein determining the cell-free methylation profile includes:  
performing single molecule sequencing of the DNA, wherein the sequencing of a single molecule includes determining whether at least one site of a DNA molecule is methylated.

88. A computer product comprising a non-transitory computer readable medium storing a plurality of instructions that when executed control a computer system to perform the method of claim 1.

20     89. A method of determining a first methylation profile from a biological sample of an organism, the biological sample including cell-free DNA comprising a mixture of cell-free DNA originating from a first tissue and from a second tissue, the method comprising:

analyzing a plurality of DNA molecules from the biological sample, wherein analyzing a DNA molecule includes:  
25     determining a location of the DNA molecule in a genome of the organism;  
determining a genotype of the DNA molecule;  
determining whether the DNA molecule is methylated at one or more sites;

identifying a plurality of first loci at which a first genome of the first tissue is heterozygous for a respective first allele and a respective second allele and a second genome of the second tissue is homozygous for the respective first allele;

for each of the first loci:

5 for each of one or more sites associated with the locus:

determining a number of DNA molecules that are methylated at the site and correspond to the respective second allele of the locus;

calculating a methylation density based on the numbers of DNA molecules methylated at the one or more sites of the locus and corresponding to the respective  
10 second allele of the locus;

creating the first methylation profile of the first tissue from the methylation densities for the first loci.

90. The method of claim 89, wherein the biological sample is from a female subject pregnant with a fetus, and wherein the first tissue is from the fetus or a  
15 placenta and the second tissue corresponds to the female subject.

91. The method of claim 89, wherein the sites correspond to CpG sites.

92. The method of claim 89, wherein each locus includes at least one CpG site.

93. The method of claim 89, wherein each locus is adjacent to at least one  
20 CpG site.

94. A kit for analyzing fetal DNA in a biological sample containing a mixture of fetal DNA and DNA from a female subject pregnant with a fetus, the kit comprising:

one or more oligonucleotides for specifically hybridizing to at least a section  
25 of a genomic region listed in Tables S2A, S2B, S2C, S2D, S3A, S3B, S3C, and S3D.

95. The kit of claim 94, wherein at least one of the oligonucleotides is a primer corresponding to at least a section of one of the genomic regions.

96. The kit of claim 94, wherein at least one of the oligonucleotides is a probe corresponding to at least a section of one of the genomic regions.

97. The kit of claim 94, further comprising:  
one or more methylation-sensitive restriction enzymes.

98. The kit of claim 94, wherein at least one of the one or more  
methylation-sensitive restriction enzymes cuts DNA molecules that are hypomethylated.

5 99. The kit of claim 94, wherein at least one of the one or more  
methylation-sensitive restriction enzymes cuts DNA molecules that are hypermethylated.

100. The kit of claim 94, wherein at least one of the one or more  
oligonucleotides is selected from Table S2A.

10 101. The kit of claim 94, wherein at least one of the one or more  
oligonucleotides is selected from Table S2B.

102. The kit of claim 94, wherein at least one of the one or more  
oligonucleotides is selected from Table S2C.

103. The kit of claim 94, wherein at least one of the one or more  
oligonucleotides is selected from Table S2D.

15 104. The kit of claim 94, wherein at least one of the one or more  
oligonucleotides is selected from Table S3A.

105. The kit of claim 94, wherein at least one of the one or more  
oligonucleotides is selected from Table S3B.

20 106. The kit of claim 94, wherein at least one of the one or more  
oligonucleotides is selected from Table S3C.

107. The kit of claim 94, wherein at least one of the one or more  
oligonucleotides is selected from Table S3D.

108. A method of estimating a methylation level of DNA in a biological  
sample of an organism, the method comprising:

25 for each size of a plurality of sizes:

measuring an amount of a plurality of DNA fragments from the biological sample corresponding to the size;

calculating, with a computer system, a first value of a first parameter based on the amounts of DNA fragments at multiple sizes, the first parameter providing a statistical

5 measure of a size profile of DNA fragments in the biological sample;

comparing the first size value to a reference size value, the reference size value calculated from DNA fragments of a reference sample; and

estimating the methylation level based on the comparison.

109. The method of claim 108, wherein measuring amounts of DNA fragments corresponding to the sizes includes:

for each of the plurality of DNA fragments from the biological sample:

measuring a size of the DNA fragment,

wherein measuring a size of a DNA fragment includes:

15 performing paired-end sequencing of the DNA fragment to obtain paired sequence reads;

aligning the paired sequence reads to a reference genome; and

using the aligned locations to determine the size of the DNA fragment.

110. The method of claim 109, wherein the methylation level corresponds to a particular region by determining the first size value only from DNA fragments that align  
20 to the particular region.

111. The method of claim 108, wherein measuring amounts of DNA fragments corresponding to the sizes includes using electrophoresis.

112. The method of claim 108, wherein the reference sample is obtained from the organism at an earlier time than the biological sample, wherein the estimated  
25 methylation level is a relative level corresponding to an amount of increase or decrease of the first size value relative to the reference size value.

113. The method of claim 108, wherein the reference size value is part of a calibration curve.

114. The method of claim 108, wherein the methylation level is a  
30 methylation density.

115. The method of claim 114, wherein the methylation density is for CpG sites.

116. A method of detecting a chromosomal abnormality from a biological sample of an organism, the biological sample including cell-free DNA comprising a mixture  
5 of cell-free DNA originating from a first tissue and from a second tissue, the method comprising:

analyzing a plurality of DNA molecules from the biological sample, wherein analyzing a DNA molecule includes:

determining a location of the DNA molecule in a reference genome;

10 determining whether the DNA molecule is methylated at one or more

sites;

for each of a plurality of sites:

determining a respective number of DNA molecules that are methylated at  
the site;

15 calculating a first methylation level of a first chromosomal region based on the respective numbers of DNA molecules methylated at sites within the first chromosomal region;

comparing the first methylation level to a cutoff value;

20 determining a classification of an abnormality in the first tissue for the first chromosomal region based on the comparison.

117. The method of claim 116, wherein comparing the first methylation level to a cutoff value includes:

normalizing the first methylation level and comparing the normalized first methylation level to the cutoff value.

25 118. The method of claim 117, wherein the normalization uses a second methylation level of a second chromosomal region.

119. The method of claim 117, wherein the normalization uses a fractional concentration of cell-free DNA from the first tissue.

30 120. The method of claim 116, wherein calculating a first methylation level includes:

identifying a plurality of regions of a genome of the organism;  
identifying one or more sites within each of the regions;  
calculating a region methylation level for each region, wherein the first  
methylation level is for a first region, and  
5 wherein comparing the first methylation level to a first cutoff value includes  
comparing each of the region methylation levels to a respective region  
cutoff value;  
determining a first number of regions whose region methylation level  
exceeds the respective region cutoff value; and  
10 comparing the first number to a threshold value to determine the  
classification.

121. The method of claim 120, wherein the threshold value is a percentage,  
and wherein comparing the first number to a threshold value includes:

dividing the first number of regions by a second number of regions before  
15 comparing to the threshold value.

122. The method of claim 121, wherein the second number of regions is all  
of the identified plurality of regions.

123. The method of claim 120, wherein the respective region cutoff values  
are a specified amount from a reference methylation level.

20 124. The method of claim 116, wherein the biological sample is from a  
female subject pregnant with a fetus, wherein the first tissue is from the fetus or a placenta  
and the second tissue is from the female subject, and wherein the chromosomal abnormality  
is a fetal chromosomal abnormality.

25 125. The method of claim 116, wherein the cutoff value is based on a  
background methylation level associated with DNA of the second tissue from the female  
subject.

126. The method of claim 124, wherein the cutoff value is determined from  
other female pregnant subjects carrying fetuses without a chromosomal abnormality for the  
first chromosomal region.

127. The method of claim 124, wherein the chromosomal abnormality is trisomy 21 or trisomy 18 or trisomy 13 or Turner syndrome or Klinefelter syndrome.

128. The method of claim 116, wherein the first tissue is from a tumor within the organism and the second tissue is from a non-malignant tissue of the organism.

5 129. The method of claim 116, wherein the cutoff value is determined from other organisms not having cancer.

130. The method of claim 116, wherein cutoff value is based on a concentration of cell-free DNA originating from the first tissue in the biological sample.

10 131. The method of claim 130, wherein the cutoff value is based on a scale factor corresponding to a type of abnormality, where the type of abnormality is deletion or duplication.

132. The method of claim 116, wherein determining the location of the DNA molecule in a reference genome involves determining whether the location is within the first chromosomal region.

15 133. The method of claim 132, wherein determining the location of the DNA molecule in the reference genome is accomplished by determining whether the DNA molecule maps to the first chromosomal region.

20 134. The method of claim 116, wherein the chromosomal abnormality is a subchromosomal deletion or subchromosomal duplication or DiGeorge syndrome.