Europäisches Patentamt
European Patent Office
Office européen des brevets

EUROPÄISCHES PATENT | EUROPEAN PATENT BREVET EUROPÉEN

Hiermit wird bescheinigt, dass für die in der Patentschrift beschriebene Erfindung ein europäisches Patent für die in der Patentschrift bezeichneten Vertragsstaaten erteilt worden ist.

It is hereby certified that a European patent has been granted in respect of the invention described in the patent specification for the Contracting States designated in the specification.

Il est certifié par la présente qu'un brevet européen a été délivré pour l'invention décrite dans le fascicule de brevet, pour les États contractants désignés dans le fascicule.

Europäisches Patent Nr.
European patent No.
Brevet européen n°

EP3452620

Tag der Bekanntmachung des Hinweises auf die Erteilung des europäischen Patents
Date of publication of the mention of the grant of the European patent
Date de la publication de la mention de la délivrance du brevet européen

05.11.2025

CELL-FREE DETECTION OF METHYLATED TUMOUR DNA

Patentinhaber | Proprietor(s) of the patent | Titulaire(s) du brevet

Queen's University at Kingston
99 University Avenue
Kingston, Ontario K7L 3N6
CA

Institut Curie
26, Rue d'Ulm
75248 Paris Cedex
FR

Institut National de la Santé et de la Recherche Médicale
101, rue de Tolbiac
75013 Paris
FR

António Campinos

Präsident des Europäischen Patentamts | President of the European Patent Office | Président de l'Office européen des brevets

München, den | Munich, | Munich, le **05.11.2025**

URKUNDE | CERTIFICATE | CERTIFICAT

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) **EP 3 452 620 B1**

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention of the grant of the patent:
05.11.2025 Bulletin 2025/45

(21) Application number: **17801856.0**

(22) Date of filing: **04.05.2017**

(51) International Patent Classification (IPC):
C12Q 1/6886 *(2018.01)* ***C07H 21/00*** *(2006.01)*
C40B 30/04 *(2006.01)* ***G01N 33/48*** *(2006.01)*

(52) Cooperative Patent Classification (CPC):
(C-Sets available)
C12Q 1/6886; C12Q 1/6827; G16B 25/00; G16B 25/10; G16B 30/00; G16B 99/00;
C12Q 2600/154 (Cont.)

(86) International application number:
PCT/CA2017/000111

(87) International publication number:
WO 2017/201606 (30.11.2017 Gazette 2017/48)

(54) CELL-FREE DETECTION OF METHYLATED TUMOUR DNA

ZELLFREIER NACHWEIS VON METHYLIERTER TUMOR-DNA

DÉTECTION SANS CELLULES D'ADN TUMORAL MÉTHYLÉ

(84) Designated Contracting States:
AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR

(30) Priority: **04.05.2016 US 201662331585 P**

(43) Date of publication of application:
13.03.2019 Bulletin 2019/11

(73) Proprietors:
- **Queen's University at Kingston**
  Kingston, Ontario K7L 3N6 (CA)
- **Institut Curie**
  75248 Paris Cedex (FR)
- **Institut National de la Santé et de la Recherche Médicale**
  75013 Paris (FR)

(72) Inventors:
- **MUELLER, Christophe, R.**
  Kingston, Ontario K7L 2T7 (CA)
- **STERN, Marc-Henri**
  F-75014 Paris (FR)

(74) Representative: **Wichmann, Hendrik**
Wuesthoff & Wuesthoff
Patentanwälte und Rechtsanwalt PartG mbB
Schweigerstraße 2
81541 München (DE)

(56) References cited:
WO-A1-2013/177265 **WO-A1-2014/043763**
WO-A2-2014/164874 **WO-A2-2015/159292**
US-A1- 2009 305 256

- **RONI LEHMANN-WERMAN ET AL: "Identification of tissue-specific cell death using methylation patterns of circulating DNA", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 113, no. 13, 14 March 2016 (2016-03-14), pages E1826 - E1834, XP055436315, ISSN: 0027-8424, DOI: 10.1073/pnas.1519286113**
- **ANONYMOUS: "Infinium? HumanMethylation450 BeadChip", 9 March 2012 (2012-03-09), XP055401052, Retrieved from the Internet <URL:https://support.illumina.com/content/dam/illumina-marketing/documents/products/datasheets/datasheet_humanmethylation450.pdf> [retrieved on 20170824]**

Processed by Luminess, 75001 PARIS (FR)

(Cont. next page)

- **VERBEKE LIEVEN P. ET AL: "Pathway Relevance Ranking for Tumor Samples through Network-Based Data Integration", PLOS ONE, vol. 10, no. 7, 28 July 2015 (2015-07-28), US, pages e0133503, XP093199244, ISSN: 1932-6203, DOI: 10.1371/journal.pone.0133503**
- **LEGENDRE, C. ET AL.: "Whole-genome bisulfite sequencing of cell -free DNA identifies signature associated with metastatic breast cancer", CLINICAL EPIGENETICS, vol. 7, 2015, pages 100, XP055441164, ISSN: 1868-7083**
- **FARKAS, S. A. ET AL.: "Genome-wide DNA methylation assay reveals novel candidate biomarker genes in cervical cancer", EPIGENETICS, vol. 8, November 2013 (2013-11-01), pages 1213 - 1225, XP055203588**
- **HALDRUP, C. ET AL.: "DNA Methylation Signatures for Prediction of Biochemical Recurrence After Radical Prostatectomy of Clinically Localized Prostate Cancer", J CLIN ONCOL, vol. 31, 10 September 2013 (2013-09-10), pages 3250 - 3258, XP055441168, ISSN: 0936-6555**
- **TOMMASI, S. ET AL.: "Methylation of homeobox genes is a frequent and early epigenetic event in breast cancer", BREAST CANCER RESEARCH, vol. 1 1, 27 February 2009 (2009-02-27), pages R14, XP021053450, ISSN: 1465-542X**

(52) Cooperative Patent Classification (CPC): (Cont.)

C-Sets
C12Q 1/6827, C12Q 2523/125, C12Q 2531/113, C12Q 2535/122

Description

FIELD

[0001] This disclosure relates generally to tumour detection. More particularly, this disclosure relates to tumour-specific DNA methylation detection.

BACKGROUND

[0002] Cancer screening and monitoring has helped to improve outcomes over the past few decades simply because early detection leads to a better outcome as the cancer can be eliminated before it has spread. In the case of breast cancer, for instance, physical breast exams, mammography, ultrasound and MRI (in high risk patients) have all played a role in improving early diagnosis. The cost/benefit of these modalities for general screening, particularly in relatively younger women, has been controversial.

[0003] A primary issue for any screening tool is the compromise between false positive and false negative results (or specificity and sensitivity) which lead to unnecessary investigations in the former case, and ineffectiveness in the latter case. An ideal test is one that has a high Positive Predictive Value (PPV), minimizing unnecessary investigations but detecting the vast majority of cancers. Another key factor is what is called "detection sensitivity", to distinguish it from test sensitivity, and that is the lower limits of detection in terms of the size of the tumour. Screening mammography in breast cancer, for instance, is considered to have a sensitivity from 80 to 90% with a specificity of 90%. However the mean size of tumours detected by mammography remains in the range of 15 to 19 mm. It has been suggested that only 3-13% of women derive an improved treatment outcome from this screening suggesting that the detection of smaller tumours would provide increased benefit. For women at high risk of developing breast cancer the use of MRI has offered some benefit with sensitivities in the range of 75 to 97% and specificities in the area of 90 to 96% and in combination with mammography offering 93-94% sensitivity and 77 to 96% specificities. However, MRI is acknowledged to have a poor PPV, in the area of 10-20%, leading to a large number of false positives and as a consequence unnecessary invasive investigations. All of these screens have likely reached their limit of detection sensitivity (or size of the tumour) and in the case of mammography still involve exposure to radiation, which may be of particular concern in women with familial mutations which render them more sensitive to radiation damage. There are no effective blood based screens for breast cancer based on circulating analytes.

[0004] While the above discussion focusses on breast cancer as an example, many of the same challenges exist for other types of cancers as well.

[0005] The detection of circulating tumour DNA is increasingly acknowledged as a viable "liquid biopsy" allowing for the detection and informative investigation of tumours in a non-invasive manner. Typically using the identification of tumour specific mutations these techniques have been applied to colon, breast and prostate cancers. Due to the high background of normal DNA present in the circulation these techniques can be limited in sensitivity. As well, the variable nature of tumour mutations in terms of occurrence and location (such as p53 and KRAS mutations) has generally limited these approaches to detecting tumour DNA at 1% of the total DNA in serum. Advanced techniques such as BEAMing have increased sensitivity, but are still limited overall. Even with these limitations the detection of circulating tumour DNA has recently been shown to be useful for detecting metastasis in breast cancer patients.

[0006] The detection of tumour specific methylation in the blood has been proposed to offer distinct advantages over the detection of mutations[1-5]. A number of single or multiple methylation biomarkers have been assessed in cancers including lung[6-10], colon[11,12] and breast[13-16]. These have suffered from low sensitivities as they have tended to be insufficiently prevalent in the tumours. Several multi-gene assays have been developed with improved performance. A more advanced multi-gene system using a combination of 10 different genes has been reported and uses a multiplexed PCR based assay[17]. It offers combined sensitivity and specificity of 91% and 96% respectively, due to the better coverage offered and it has been validated in a small cohort of stage IV patients. However, it has a very high background in normal blood which will limit its detection sensitivity. Methylated markers have been used to monitor the response to neoadjuvant therapy[18,19], and recently a methylation gene signature associated with metastatic tumours has been identified[20]. Patent application US 2009/305256 A1 describes measuring methylation level of CpG islands comprised in chromosomal regions preferably unmethylated in normal samples and heavily methylated in a large fraction of the tumors. Patent application WO 2014/043763 A1 describes systems, methods, and apparatuses that can determine and use methylation profiles of various tissues and samples. In WO 2015/159292 A1, a method of detecting death of a cell type or tissue in a subject is outlined, wherein the method comprises determining whether cell-free DNA comprised in a fluid sample of the subject is derived from the cell type or tissue by ascertaining the methylation status. In a scientific publication by Lehmann-Werman, Roni, et al. titled "Identification of tissue-specific cell death using methylation patterns of circulating DNA." (Proceedings of the National Academy of Sciences 113.13 (2016): E1826-E1834) authors describe a method of detecting tissue-specific cell death in humans based on tissue-specific methylation patterns in cell-free circulating DNA. Additionally, chips are also

available for the detection of genome-wide DNA methylation sites, such as Illumina's Infinium® HumanMethylation450 BeadChip (https://support.illumina.com/content/dam/illumina-marketing/documents/products/datasheets/datasheet_humanmethylation450.pdf)

[0007] There remains a need for more sensitive and specific screening tools, as well as for straightforward tests that allow for the assessment of tumour burden, chemotherapy response, detection of residual disease, relapse and primary screening in high risk populations.

SUMMARY

[0008] It is an object of this disclosure to obviate or mitigate at least one disadvantage of previous approaches.

[0009] As defined in the claims, in a first aspect, this disclosure provides a method for detecting a tumour, comprising: extracting DNA from a cell-free sample obtained from a human subject, bisulphite converting at least a portion of the DNA, amplifying regions methylated in cancer from the bisulphite converted DNA present in the sample using a plurality of PCR primers, wherein (i) amplifying with at least one primer set designed to amplify at least one methylation site having a methylation value at or below -0.3 in normal tissue; and detecting signals wherein at least one signal comprises methylation of at least two adjacent methylation sites within at least one region of the multiple regions; wherein the multiple regions are methylated in at least 50% of the tumours in a population of samples for a selected tumour type or tumour subtype and are not methylated in tissues and blood of subjects without tumours;wherein individual primers of the plurality of PCR primers are designed based on methylated DNA within individual regions of the multiple regions; wherein the presence of a tumour in the subject is indicated when signals comprising methylation of at least two adjacent methylation sites within the at least one region of the multiple regions are detected.

[0010] In another aspect, there is provided a use of the method for determining response to treatment.

[0011] In another aspect, there is provided a use of the method for monitoring tumour load.

[0012] In another aspect, there is provided a use of the method for detecting residual tumour post-surgery.

[0013] In another aspect, there is provided a use of the method for detecting relapse.

[0014] In another aspect, there is provided a use of the method as a secondary screen.

[0015] In another aspect, there is provided a use of the method as a primary screen.

[0016] In another aspect, there is provided a use of the method for monitoring cancer development.

[0017] In another aspect, there is provided a use of the method for monitoring cancer risk.

[0018] In another aspect, there is provided a kit for detecting a tumour comprising reagents for carrying out the method, and instructions for detecting the tumour signals, as defined by the claims.

[0019] Other aspects and features of this disclosure will become apparent to those ordinarily skilled in the art upon review of the following description of specific embodiments in conjunction with the accompanying figures.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020] Embodiments of this disclosure will now be described, by way of example only, with reference to the attached Figures.

Fig. 1 depicts a schematic of the method.

Fig. 2 depicts a schematic of the amplification of multiple target regions.

Fig. 3 lists 47 CpG targets selected to identify differentially methylated regions, and shows the results of Receiver Operator Curve (ROC) analysis.

Fig. 4 depicts histograms showing the frequency of patients binned according to positive (methylated) probe frequency. Panel A depicts results for luminal tumours. Panel B depicts results for basal tumours.

Fig. 5 depicts sequencing results to assess methylation status of a region near the CHST11 gene (CHST11 Probe C) in breast cancer cell lines.

Fig. 6 depicts sequencing results to assess methylation status of CHST11 Probe A in breast cancer tumors and normal breast tissue.

Fig. 7 depicts sequencing results to assess methylation status of FOXA Probe A in breast cancer cell lines.

Fig. 8 depicts sequencing results to assess methylation status of CHST Probe A and Probe B in prostate cancer cell lines.

Fig. 9 depicts sequencing results to assess methylation status of FOXA Probe A in prostate cancer cell lines.

Fig. 10 depicts sequencing results to assess methylation status of NT5 Probe E in breast cancer cell lines.

Fig. 11 depicts a summary of BioAnalyzer electrophoresis summary for amplification product generated from various cell lines.

Figs. 12A and 12B depict a numerical summary of validation data generated for 98 different probes by bisulphite sequencing six different cell lines. # Reads is indicative of the number of reads exported, and Mean Me is indicative of

the mean methylation.

Figs. 13A and 13B depict a numerical summary of generated methylation data for tumour samples. # Reads is indicative of the number of reads exported, and Mean Me is indicative of the mean methylation.

Fig. 14 depicts a numerical summary generated methylation data for prostate cell lines. # Reads is indicative of the number of reads exported, and Mean Me is indicative of the mean methylation.

Fig. 15 is a diagram showing validation of various uveal melanoma (UM) probes in two cell lines MP38 (with loss of 3p) and MP41 (3p WT). Negative controls were cell free DNA (cfDNA) consisting of a pool of 18 individuals without cancer and peripheral mononuclear cells (PBMC). Probes for the indicated regions were PCR amplified individually and sequenced. Darker shading indicates higher level of methylation. OST3F was methylated in PBMCs while LDL3F was not methylated in tumours, with the majority showing strong methylation in the UM lines but not in the PBMCs or cfDNA.

Fig. 16 is a diagram showing methylation of cfDNA from patients with metastatic uveal melanoma. Methylated reads for each probe were extracted and all reads were normalized for the total number of reads in the sample. Stacked columns represent the total reads from all of the individual probes with different probes identified by shading. The patients are sorted by PAP measurements with high values on the left and lower values on the right. cfDNA is a pool of cell free DNA from 18 normal donors.

Fig. 17 is a diagram showing methylation of cfDNA from patients with metastatic uveal melanoma. Methylated reads for each probe were extracted and all reads were normalized for the total number of reads in the sample. Stacked columns represent the total reads from all of the individual probes with different probes identified by shading. The patients are sorted by tumour volume with larger volume on the left and lower volume on the right, and the volume indicated at the bottom. PAP values obtained from these patients is indicated. <5 refers to no detection of ctDNA in these samples. cfDNA is a pool of cell free DNA from 18 normal donors.

Figs. 18A and 18B are diagrams showing methylation of cfDNA from sequential blood samples of two patients who were part of the patient groups shown in Figs. 17 and 18. In Fig. 19A the patient was retested after seven months and the tumour at that time was assessed as being 0.5 cm^3 in volume. In Fig. 19B the patient was retested after four months where the initial tumour volume was 483 cm^3.

Fig. 19 is a log-log plot showing assay values (methylated reads) are correlated with tumour volume. The character of the metastatic tumour such as whether it is a solid mass or dispersed (miliary) was not taken into account.

Fig. 20 is a log-log plot showing relationship between test results and PAP signal, where PAP and methylation signals were correlated at higher PAP levels (trend line), although below the detection threshold of PAP at 5 copies/ml (vertical dashed line) the PAP signals were not correlated (ellipse).

Fig. 21 is a heat map of gene methylation in indicated prostate cancer cell lines.

Fig. 22 is a heat map of multiplexed probes for each prostate cancer patient sample. Patient samples were taken before the initiation of ADT (START) and 12 months after (M12). A black square indicates that methylated reads having greater than 80% methylation per read were detected for that probe but does not take into consideration the number of reads for each.

Fig. 23 is a diagram showing number of methylated reads per probe for each prostate cancer patient sample. Different probes are shown in different shading. The number of reads that were at least 80% methylated were determined for each sample and all probes are stacked per sample. Patient samples were taken before the initiation of ADT (START) and 12 months after (M12).

Fig. 24 is a plot showing normalized methylation reads per sample verses PSA levels for each patient. The totals of normalized methylated reads for all probes are plotted with solid lines. Patients initiated androgen deprivation therapy (START) and PSA levels measured at that time and after 12 months of treatment (M12) and are indicated with dashed lines. The methylation detection of circulating tumour DNA (mDETECT) test was performed on 0.5 ml of plasma from these same time points. The Gleason score for each patient at initial diagnosis is shown along with grading, as is the treatment applied as primary therapy (RRP, radical retropubic prostatectomy; BT, brachytherapy; EBR, external beam radiation; RT, radiotherapy).

Fig. 25 is a plot of TCGA prostate cancer tumour data, showing the average methylation for each of various Gleason groups, as well as for normal tissue from breast, prostate, lung, and colon, verses position on the genome (in this case on chromosome 8 for the region upstream of the TCF24gene, a transcription factor of unknown function and PRSS3, a serine protease gene on chromosome 9).

Figs. 26A, 26B, and 26C are charts showing regions used to develop a breast cancer test according to one embodiment. The chromosomal location and nucleotide position of the first CpG residue in the region is indicated. The TCGA breast cancer cohort was divided into sub-groups based on PAM-50 criteria. The fraction of each group that is positive for that probe is indicated. "Tissue" indicates results from normal tissue samples.

Fig. 27 shows theoretical area under the curve analyses of blood tests using the top 20 probes for each breast cancer subtype (LumA, LumB, Basal, HER2). These values were compared against normal tissue samples for the same probes.

Fig. 28 is a heatmap of multiplexed probes for each TNBC tumour sample and selected normal samples. A black square indicates that methylated reads having greater than 80% methylation per read were detected for that probe but does not take into consideration the number of reads for each.

Fig. 29 is a diagram showing results of a sensitivity test for TNBC to detect low levels of tumour DNA, using HCC1937 DNA diluted into a fixed amount of PBMC DNA (10 ng). Shaded squares indicate a distinct methylation signature.

Fig. 30 is a flowchart illustrating a method for determining biological methylation signatures, and for developing probes for their detection.

DETAILED DESCRIPTION

[0021] Generally, this disclosure provides a method for detecting a tumour that can be applied to cell-free samples, e.g., to detect cell-free circulating tumour DNA. The method utilizes detection of adjacent methylation signals within a single sequencing read as the basic "positive" tumour signal.

[0022] In one aspect, there is provided a method for detecting a tumour, comprising: extracting DNA from a cell-free sample obtained from a subject, bisulphite converting at least a portion of the DNA, amplifying regions methylated in cancer from the bisulphite converted DNA, generating sequencing reads from the amplified regions, and detecting tumour signals comprising at least two adjacent methylated sites within a single sequencing read, wherein the detection of at least one of the tumour signals is indicative of a tumour, as further defined in the claims.

[0023] By **"cell-free DNA (cfDNA)"** is meant DNA in a biological sample that is not contained in a cell. cfDNA may circulate freely in in a bodily fluid, such as in the bloodstream.

[0024] **"Cell-free sample",** as used herein, is meant a biological sample that is substantially devoid of intact cells. This may be a derived from a biological sample that is itself substantially devoid of cells, or may be derived from a sample from which cells have been removed. Example cell-free samples include those derived from blood, such as serum or plasma; urine; or samples derived from other sources, such as semen, sputum, feces, ductal exudate, lymph, or recovered lavage.

[0025] **"Circulating tumour DNA",** as used herein, accordingly refers to cfDNA originating from a tumour.

[0026] By **"region methylated in cancer"** is meant a segment of the genome containing methylation sites (CpG dinucleotides), methylation of which is associated with a malignant cellular state. Methylation of a region may be associated with more than one different type of cancer, or with one type of cancer specifically. Within this, methylation of a region may be associated with more than one subtype, or with one subtype specifically.

[0027] The terms cancer **"type"** and **"subtype"** are used relatively herein, such that one "type" of cancer, such as breast cancer, may be "subtypes" based on e.g., stage, morphology, histology, gene expression, receptor profile, mutation profile, aggressiveness, prognosis, malignant characteristics, etc. Likewise, "type" and "subtype" may be applied at a finer level, e.g., to differentiate one histological "type" into "subtypes", e.g., defined according to mutation profile or gene expression.

[0028] By **"adjacent methylated sites"** is meant two methylated sites that are, sequentially, next to each other. It will be understood that this term does not necessarily require the sites to actually be directly beside each other in the physical DNA structure. Rather, in a sequence of DNA including spaced apart methylation sites A, B, and C in the context A-$(n)_n$-B-$(n)_n$-C, wherein $(n)_n$ refers to the number of base pairs (bp) (e.g., up to 300bp), sites A and B would be recognized as "adjacent" as would sites B and C. Sites A and C, however, would not be considered to be adjacent methylated sites.

[0029] In one aspect, the regions methylated in cancer comprise CpG islands.

[0030] **"CpG islands"** are regions of the genome having a high frequency of CpG sites. CpG islands are usually 300-3000bp in length and are found at or near promotors of approximately 40% of mammalian genes. They show a tendency to occur upstream of so-called "housekeeping genes". A concrete definition is elusive, but CpG islands may be said to have an absolute GC content of at least 50%, and a CpG dinucleotide content of at least 60% of what would be statistically expected. Their occurrence at or upstream of the 5' end of genes may reflect a role in the regulation of transcription, and methylation of CpG sites within the promoters of genes may lead to silencing. Silencing of tumour suppressors by methylation is, in turn, a hallmark of a number of human cancers.

[0031] In one aspect, the regions methylated in cancer comprise CpG shores.

[0032] **"CpG shores"** are regions extending short distances from CpG islands in which methylation may also occur. CpG shores may be found in the region 0 to 2kb upstream and downstream of a CpG island.

[0033] In one aspect, the regions methylated in cancer comprise CpG shelves.

[0034] **"CpG shelves"** are regions extending short distances from CpG shores in which methylation may also occur. CpG shelves may generally be found in the region between 2kb and 4kb upstream and downstream of a CpG island (i.e., extending a further 2kb out from a CpG shore).

[0035] In one aspect, the regions methylated in cancer comprise CpG islands and CpG shores.

[0036] In one aspect, the regions methylated in cancer comprise CpG islands, CpG shores, and CpG shelves.

[0037] In one aspect, the regions methylated in cancer comprise CpG islands and sequences 0 to 4kb upstream and downstream. The regions methylated in cancer may also comprise CpG islands and sequences 0 to 3kb upstream and

downstream, 0 to 2kb upstream and downstream, 0 to 1kb upstream and downstream, 0 to 500bp upstream and downstream, 0 to 400bp upstream and downstream, 0 to 300bp upstream and downstream, 0 to 200bp upstream and downstream, or 0 to 100bp upstream and downstream.

[0038] The step of amplifying may be carried out with primers designed to anneal to bisulphite converted target sequences having at least one methylated site therein. Bisulphite conversion results in unmethylated cytosines being converted to uracil, while 5-methylcytosine is unaffected. **"Bisulphite converted target sequences"** are thus understood to be sequences in which cytosines known to be methylation sites are fixed as "C" (cytosine), while cytosines known to be unmethylated are fixed as "U" (uracil; which can be treated as "T" (thymine) for primer design purposes). Primers designed to target such sequences may exhibit a degree of bias towards converted methylated sequences. However, in one embodiment, the primers are designed without preference as to location of the at least one methylated site within target sequences. Often, to achieve optimal discrimination, it may be desirable to place a discriminatory base at the ultimate or penultimate 3' position of an oligonucleotide PCR primer. In this embodiment, however, no preference is given to the location of the discriminatory sites of methylation, such that overall primer design is optimized based on sequence (not discrimination). This results in a degree of bias for some primer sets, but usually not complete specificity towards methylated sequences (some individual primer pairs, however, may be specific if a discriminatory site is fortuitously placed). As will be described herein, this permits some embodiments of the method to be quantitative or semiquantitative.

[0039] In one embodiment, the PCR primers are designed to be methylation specific. This may allow for greater sensitivity in some applications. For instance, primers may be designed to include a discriminatory nucleotide (specific to a methylated sequence following bisulphite conversion) positioned to achieve optimal discrimination, e.g. in PCR applications. The discriminatory may be positioned at the 3' ultimate or penultimate position.

[0040] In one embodiment, the primers are designed to amplify DNA fragments 75 to 150bp in length. This is the general size range known for circulating DNA, and optimizing primer design to take into account target size may increase the sensitivity of the method according to this embodiment. The primers may be designed to amplify regions that are 50 to 200, 75 to 150, or 100 or 125bp in length.

[0041] In some embodiments, concordant results provide additional confidence in a positive tumour signal. By **"concordant"** or **"concordance",** as used herein, is meant methylation status that is consistent by location and/or by repeated observation. As has already been stated, the basic "tumour signal" defined herein comprises at least two adjacent methylated sites within a single sequencing read. However, additional layers of concordance can be used to increase confidence for tumour detection, in some embodiments, and not all of these need be derived from the same sequencing read. Layers of concordance that may provide confidence in tumor detection may include, for example:

(a) detection of methylation of at least two adjacent methylation sites;
(b) detection of methylation of more than two adjacent methylation sites;
(c) detection of methylation at adjacent sites within the same section of a target region amplified by one primer pair;
(d) detection of methylation at non-adjacent sites within the same section of a region amplified by one primer pair;
(e) detection of methylation at adjacent sites within the same target region;
(f) detection of methylation at non-adjacent sites within the same target region;
(g) any one of (a) to (f) in the same sequencing read;
(h) any one of (a) to (f) in at least two sequencing reads;
(i) any one of (a) to (f) in a plurality of sequencing reads;
(j) detection over methylation at sets of adjacent sites that overlap;
(k) repeated observation of any one of (a) to (j); or
(l) any combination or subset of the above.

[0042] In one embodiment, each of the regions is amplified in sections using multiple primer pairs. In one embodiment, these sections are non-overlapping. The sections may be immediately adjacent or spaced apart (e.g. spaced apart up to 10, 20, 30, 40, or 50bp). Since target regions (including CpG islands, CpG shores, and/or CpG shelves) are usually longer than 75 to 150bp, this embodiment permits the methylation status of sites across more (or all) of a given target region to be assessed.

[0043] A person of ordinary skill in the art would be well aware of how to design primers for target regions using available tools such as Primer3, Primer3Plus, Primer-BLAST, etc. As discussed, bisulphite conversion results in cytosine converting to uracil and 5'-methyl-cytosine converting to thymine. Thus, primer positioning or targeting may make use of bisulphite converted methylate sequences, depending on the degree of methylation specificity required.

[0044] Target regions for amplification are designed to have at least two CpG dinucleotide methylation sites. In some embodiments, however, it may be advantageous to amplify regions having more than one CpG methylation site. For instance, the amplified regions may have 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 CpG methylation sites. In one embodiment, the primers are designed to amplify DNA fragments comprising 3 to 12 CpG methylation sites. Overall this permits a larger number of adjacent methylation sites to be queried within a single sequencing read, and provides

additional certainty (exclusion of false positives) because multiple concordant methylations can be detected within a single sequencing read. In one embodiment, the tumour signals comprise more than two adjacent methylation sites within the single sequencing read. Detecting more than two adjacent methylation sites provides additional concordance, and additional confidence that the tumour signal is not a false positive in this embodiment. For example, a tumour signal may be designated as 3, 4, 5, 6, 7, 8, 9, 10 or more adjacent detected methylation sites within a single sequencing read. The detection of more than one of the tumour signals can be indicative of a tumour. Detection of multiple tumour signals, can increase confidence in tumour detection. Such signals can be at the same or at different sites. The detection of more than one of the tumour signals at the same region can be indicative of a tumour. Detection of multiple tumour signals indicative of methylation at the same site in the genome, in this embodiment, can increase confidence in tumour detection. So too can detection of methylation at adjacent sites in the genome, even if the signals are derived from different sequencing reads. This reflects another type of concordance. The detection of adjacent or overlapping tumour signals across at least two different sequencing reads can be indicative of a tumour. In one aspect, the adjacent or overlapping tumour signals are within the same CpG island. In another aspect, the detection of 5 to 25 adjacent methylated sites can be indicative of a tumour.

[0045] Methylated regions can be selected according to the purpose of the intended assay. In one aspect, the regions can comprise at least one region listed Table 1 and/or Table 2. In another aspect, the regions may comprise all regions listed in Table 1 and/or Table 2.

[0046] Likewise, primer pairs can be designed based on the intended target regions.

[0047] The step of amplification may be carried out with more than 100 primer pairs. The step of amplification may be carried out with 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, or more primer pairs. The step of amplification can be a multiplex amplification. Multiplex amplification permits large amount of methylation information to be gathered from many target regions in the genome in parallel, even from cfDNA samples in which DNA is generally not plentiful. The multiplexing may be scaled up to a platform such as ION AmpliSeq™, in which, e.g. up to 24,000 amplicons may be queried simultaneously. The step of amplification can be nested amplification. A nested amplification may improve sensitivity and specificity.

[0048] The nested reaction may be part of a next generation sequencing approach. Barcode and/or sequencing primers may be added in the second (nested) amplification. Alternatively, these may added in the first amplification.

[0049] In one embodiment, the method further comprises quantifying the tumour signals, wherein a number in excess of a threshold is indicative of a tumour. In one embodiment, the steps of quantifying and comparing are carried out independently for each of the sites methylated in cancer. Accordingly, a count of positive tumour signals may be established for each site. The method may further comprise determining a proportion of the sequencing reads containing tumour signals, wherein the proportion in excess of a threshold is indicative of a tumour. The step of determining may be carried out independently for each of the sites methylated in cancer.

[0050] By **"threshold",** as used herein, is meant a value that is selected to discriminate between a disease (e.g., malignant) state, and a non-disease (e.g., healthy) state. Thresholds can be set according to the disease in question, and may be based on earlier analysis, e.g., of a training set. Thresholds may also be set for a site according to the predictive value of methylation at a particular site. Thresholds may be different for each methylation site, and data from multiple sites can be combined in the end analysis.

[0051] Various design parameters may be used to select the regions subject to amplification in some embodiments. According to the claims, the regions are having a methylation value at or below -0.3 in healthy normal tissue. Healthy tissue would be understood to be non-malignant. Healthy tissue is often selected based on the origin of the corresponding tumour.

[0052] Regions may be selected based on desired aims or required specificity, in some embodiments. For instance, it may be desirable to screen for more than one cancer type. Thus, the regions may be collectively methylated in more than one tumour type. It may be desirable to include regions methylated generally in a group of cancers, and regions methylated in specific cancers in order to provide different tiers of information. Thus, the regions can comprise regions that are specifically methylated in specific tumours, and regions that are methylated in more than one tumour type. Likewise, it may be desirably to include a second tier of regions that can differentiate between tumour types. In one embodiment, the regions specifically methylated in specific tumours comprise a plurality of groups, each specific to one tumour type. However, it may be desirable in some contexts to have a test that is focused on one type of cancer. Thus, in one embodiment, the regions are methylated specifically in one tumour type. The regions may be selected from those listed in Table 3 and the tumour is one carrying a BRCA1 mutation.

[0053] More specifically, in some embodiments regions may be selected that are methylated in particular subtypes of a cancer exhibiting particular histology, karyotype, gene expression (or profile thereof), gene mutation (or profile thereof), staging, etc. Accordingly, the regions to be amplified may comprise one or more groups of regions, each being established to be methylated in one particular cancer subtype. In one embodiment the regions to be amplified may be methylated in a cancer subtype bearing particular mutations. With breast cancer in mind, one example subtype defined by mutation is cancer bearing BRCA1 mutations. Another subtype is cancer bearing BRCA2 mutations. Other breast cancer subtypes for

which methylated regions may be determined include Basal, Luminal A, Luminal B, HER2 and Normal-like tumours. For uveal melanoma, for example, subtypes may include tumours that have retained or lost chromosome 3 (monosomy 3).

[0054] Within the context of such a test of some embodiments, information about not only the presence, but also the pattern and distribution of tumour signals both within specific regions and between different regions may help to detect or validate the presence of a form of cancer. In one embodiment, the method further comprises determining a distribution of tumour signals across the regions, and comparing the distribution to at least one pattern associated with a cancer, wherein similarity between the distribution and the pattern is indicative of the cancer.

[0055] **"Distribution",** as used herein in this context, is meant to indicate the number and location of tumour signals across the regions. Statistical analysis may be used to compare the observed distribution with, e.g., pre-established patterns (data) associated with a form of cancer. In other embodiments, the distribution may be compared to multiple patterns. In one embodiment, the method further comprises determining a distribution of tumour signals across the regions, and comparing the distribution to a plurality of patterns, each one associated with a cancer type, wherein similarity between the distribution and one of the plurality of patterns is indicative of the associated cancer type.

[0056] The step of generating sequencing reads may be carried out by next generation sequencing. This permits a very high depth of reads to be achieved for a given region. These are high-throughput methods that include, for example, Ilumina (Solexa) sequencing, Roche 454 sequencing, Ion Torrent sequencing, and SOLiD sequencing. The depth of sequencing reads may be adjusted depending on desired sensitivity.

[0057] The step of generating sequencing reads can be carried out simultaneously for samples obtained from multiple patients, wherein the amplified CpG islands from is barcoded for each patient. This permits parallel analysis of a plurality of patients in one sequencing run.

[0058] A number of design parameters may be considered in the selection of regions methylated in cancer. Data for this selection process may be from a variety of sources such as, e.g., The Cancer Genome Atlas (TCGA) (http://cancergenome.nih.gov/), derived by the use of, e.g., Illumina Infinium HumanMethylation450 BeadChip (http://www.illumina.com/products/methylation 450 beadchip kits.html) for a wide range of cancers, or from other sources based on, e.g., bisulphite whole genome sequencing, or other methodologies. For instance, **"methylation value"** (understood herein as derived from TCGA level 3 methylation data, which is in turn derived from the beta-value, which ranges from -0.5 to 0.5) may be used to select regions. The step of amplification is carried out with primer sets designed to amplify at least one methylation site having a methylation value of below -0.3 in normal issue. This can be established in a plurality of normal tissue samples, for example 4. The methylation value may be at or below -0.3, -0.4, or -0.5. In one embodiment, the primer sets are designed to amplify at least one methylation site having a difference between the average methylation value in the cancer and the normal tissue of greater than 0.3, as defined in the claims. The difference may be greater than 0.3, 0.4, or 0.5. Proximity of other methylation sites that meet this requirement may also play a role in selecting regions, in some embodiments. In one embodiment, the primer sets include primer pairs amplifying at least one methylation site having at least one methylation site within 200 bp that also has a methylation value of below -0.3 in normal issue, and a difference between the average methylation value in the cancer and the normal tissue of greater than 0.3, as defined in the claims. The adjacent site having these features may be 300 bp. The adjacent site may be within 100, 200, 300, 400, or 500bp.

[0059] The target regions are selected for amplification based on the number of tumours in the validation set having methylation at that site. For example, a region may be selected if it is methylated in at least 50%, 55%, 60%, 65%, 70%, 75%, 80, 85%, 90, or 95% of tumours tested. For example, regions may be selected if they are methylated in at least 75% of tumours tested, including within specific subtypes. For some validations, it will be appreciated that tumour-derived cell lines may be used for the testing.

[0060] The method may further comprise oxidative bisulphite conversion. In addition to the analysis of methylation of CpG residues, additional information that may be of clinical significance may be derived from the analysis of hydroxymethylation. Bisulphite sequencing results in the conversion of unmethylated cytosine residues into uracil/thymidine residues, while both methylated and hydroxymethylated cytosines remain unconverted. However, oxidative bisulphite treatment allows for the conversion of hydroxymethylated cytosines to uracil/thymidine allowing for the differential analysis of both types of modifications. By comparison of bisulphite to oxidative bisulphite treatments the presence of hydroxymethylation can be deduced. This information may be of significance as its presence or absence may be correlated with clinical features of the tumor which may be clinically useful either as a predictive or prognostic factor. Accordingly, in some embodiments, information about hydroxymethylation could additionally be used in the above-described embodiments.

[0061] In one aspect, the presence of specific patterns of methylation is linked to underlying characteristics of particular tumours. In these cases, the methylation patterns detected by the method are indicative of clinically relevant aspects of the tumours such as aggressiveness, likelihood of recurrence, and response to various therapies. Detection of these patterns in the blood may thus provide both prognostic and predictive information related to a patient's tumor.

[0062] In another aspect, the forgoing method may be applied to clinical applications involving the detection or monitoring of cancer.

[0063] In one embodiment, the forgoing method may be applied to determine and/or predict response to treatment.

[0064] In one embodiment, the forgoing method may be applied to monitor tumour load.

[0065] In one embodiment, the forgoing method may be applied to detect residual tumour post-surgery.

[0066] In one embodiment, the forgoing method may be applied to detect relapse.

[0067] In one aspect, the forgoing method may be applied as a secondary screen.

[0068] In one aspect, the forgoing method may be applied as a primary screen.

[0069] In one aspect, the forgoing method may be applied to monitor cancer development.

[0070] In one aspect, the forgoing method may be applied to monitor cancer risk.

[0071] In another aspect, there is provided a kit for detecting a tumour comprising reagents for carrying out the aforementioned method, and instructions for detecting the tumour signals, as defined in the claims. Reagents may include, for example, primer sets, PCR reaction components, and/or sequencing reagents, as defined in the claims.

[0072] In one embodiment of the forgoing methods, the regions comprise C2CD4A, COL19A1, DCDC2, DHRS3, GALNT3, HES5, KILLIN, MUC21, NR2E1/OSTM1, PAMR1, SCRN1, and SEZ6, and the tumour is uveal melanoma. In one embodiment, the probes comprise C2C5F, COL2F, DCD5F, DGR2F, GAL1F, GAL3F, HES1F, HES3F, HES4F, KIL5F, KIL6F, MUC2F, OST3F, OST4F, PAM4F, SCR2F, SEZ3F, and SEZ5F.

[0073] In one embodiment, the regions comprise ADCY4, ALDH1L1, ALOX5, AMOTL2, ANXA2, CHST11, EFS, EPSTI1, EYA4, HAAO, HAPLN3, HCG4P6, HES5, HIF3A, HLA-F, HLA-J, HOXA7, HSF4, KLK4, LOC376693, LRRC4, NBR1, PAH, PON3, PPM1H, PTRF, RARA, RARB, RHCG, RND2,TMP4, TXNRD1, and ZSCAN12, and the tumour is prostate cancer. In one aspect, the probes comprise ADCY4-F, ALDH1L1-F, ALOX5-F, AMOTL2-F, ANXA2-F, CHST11-F, EFS-F, EPSTI1-F, EYA4-F, HAAO-F, HAPLN3-F, HCG4P6-F, HES5-F, HIF3A-F, HLA-F-F, HLA-J-1-F, HLA-J-2-F, HOXA7-F, HSF4-F, KLK4-F, LOC376693-F, LRRC4-F, NBR1-F, PAH-F, PON3-F, PPM1H-F, PTRF-F, RARA-F, RARB-F, RHCG-F, RND2-F,TMP4-F, TXNRD1-F, and ZSCAN12-F. In another aspect, the probes additionally include C1Dtrim, C1Etrim, CHSAtrim, DMBCtrim, FOXAtrim, FOXEtrim, SFRAtrim, SFRCtrim, SFREtrim, TTBAtrim, VWCJtrim, and VWCKtrim.

[0074] In one embodiment, the regions comprise ASAP1, BC030768, C18orf62, C6orf141, CADPS2, CORO1C, CYP27A1, CYTH4, DMRTA2, EMX1, HFE, HIST1H3G/1H2BI, HMGCLL1, KCNK4, KJ904227, KRT78, LINC240, Me3, MIR1292, NBPF1, NHLH2, NRN1, PPM1H, PPP2R5C, PRSS3, SFRP2, SLCO4C1, SOX2OT, TUBB2B, USP44, Intergenic (Chr1), Intergenic (Chr2), Intergenic (Chr3), Intergenic (Chr4), Intergenic (Chr8), and Intergenic (Chr10), and the tumour is aggressive prostate cancer. In one embodiment, the aggressive prostate cancer has a Gleason Score greater than 6. In one embodiment, the aggressive prostate cancer has a Gleason Score of 9 or greater. In one embodiment, the probes comprise ASAP1/p, BC030768/p, C18orf62/p, C6orf141/p-1, C6orf141/p-2, CADPS2/p, CORO1C/p-1, CORO1C/p-2, CYP27A1/p, CYTH4/p, DMRTA2/p, EMX1/p, HFE/p-1, HFE/p-2, HIST1H3G/1H2BI/p, HMGCLL1/p, KCNK4/p, KJ904227/p, KRT78/p, LINC240/p-1, LINC240/p-2, Me3/p-1, Me3/p-2, MIR129, NBPF1/p, NHLH2/p, NRN1/p, PPM1H/p-1, PPM1H/p-2, PPP2R5C/p, PRSS3/p, SFRP2/p-1, SFRP2/p-2, SLCO4C1/p, SOX2OT/p, TUBB2B/p, USP44/p, Chr1/p-1, Chr2/p-1, Chr3/p-1, Chr4/p-1, Chr8/p-1, and Chr10/p-1.

[0075] In one embodiment, the regions comprise the regions depicted in Figures 26A, 26B, and 26C, and the tumour is breast cancer.

[0076] In another aspect , the regions comprise ALX1, ACVRL1, BRCA1,C1orf114, CA9, CARD11, CCL28, CD38, CDKL2, CHST11, CRYM, DMBX1, DPP10, DRD4, ERNA4, EPSTI1, EVX1, FABP5, FOXA3, GALR3, GIPC2, HINF1B, HOXA9, HOXB13, Intergenic5, Intergenic 8, IRF8, ITPRIPL1, LEF1,LOC641518, MAST1, BARHL2, BOLL, C5orf39, DDAH2, DMRTA2, GABRA4, ID4, IRF4, NT5E, SIM1, TBX15, NFIC, NPHS2, NR5A2, OTX2, PAX6, GNG4, SCAND3, TAL1, PDX1, PHOX2B, POU4F1,PFIA3, PRDM13, PRKCB, PRSS27, PTGDR, PTPRN2, SALL3, SLC7A4, SOX2OT, SPAG6, TCTEX1D1, TMEM132C, TMEM90B, TNFRSF10D, TOP2P1, TSPAN33, TTBK1, UDB, and VWC2., and the tumour is triple negative breast cancer (TNBC). In one embodiment, the probes comprise ALX1, AVCRL1, BRCA1-A, C1Dtrim, C1Etrim, CA9 - A, CARD11 - B, CCL28-A, CD38, CDKL2 - A, CHSAtrim, CRYM-A, DMBCtrim, DMRTA2exp - A, DPP10-A, DPP10-B, DPP10-C,DRD4 - A, EFNA4 - B, EPSTI1, EVX1, FABP5, FOXAtrim, FOXEtrim, GALR3 - A, GIPC2 - A, HINF C trim, HOXAAtrim, HOXACtrim, HOXB13-A, Int5, Int8, IRF8-A, ITRIPL1, LEF1 - A, MAST1 A trim, mbBARHL2 Trim, mbBOLL Trim, mbC5orf Trim, mbDDAH Trim, mbDMRTA Trim, mbGABRA A Trim, mbGABRA B Trim, mbGNG Trim, mbID4 Trim, mbIRF Trim, mbNT5E Trim, mbSIM A Trim, mbTBX15 Trim, NFIC - B, NFIC -A, NPSH2-B, NR5A2 - B, OTX2-A, PAX6-A, pbDMRTA Trim, pbGNG Trim, pbSCAND Trim, pbTAL Trim, PDX1exp - B, PHOX2B - A, POU4F1 A trim, PPFIA3-A, PRDM13, PRKCB-A, PRKCB-C, PRSS27 - A, PTGDR, PTPRN2 - A, PTPRN2 - B, SALL3-A, SALL3-B, SLC7A4 - A, SOX2OT - B, SPAG6 A trim, TCTEX1D1 - A, TMEM - A, TMEM - B, TMEM90B - A, TNFRSF10D, TOP2P1 - B, TSPAN33 - A, TTBAtrim, UBD - A, VWCJtrim, and VWCKtrim.

[0077] In one embodiment, each region is amplified with primer pairs listed for the respective region in **Table 15.**

[0078] The method may further comprise administering a treatment for the tumour detected.

[0079] In one aspect, there is provided a method for identifying a methylation signature indicative of a biological characteristic, the method comprising: obtaining data for a population comprising a plurality of genomic methylation data sets, each of said genomic methylation data sets associated with biological information for a corresponding sample, segregating the methylation data sets into a first group corresponding to one tissue or cell type possessing the biological characteristic and a second group corresponding to a plurality of tissue or cell types not possessing the biological characteristic, matching methylation data from the first group to methylation data from the second group on a site-by-site

basis across the genome, identifying a set of CpG sites that meet a predetermined threshold for establishing differential methylation between the first and second groups, identifying, using the set of CpG sites, target genomic regions comprising at least two differentially methylated CpGs with 300bp that meet said predetermined criteria, extending the target genomic regions to encompass at least one adjacent differentially methylated CpG site that does not meet the predetermined criteria, wherein the extended target genomic regions provide the methylation signature indicative of the biological trait.

[0080] The method may further comprise validating the extended target genomic regions by testing for differential methylation within the extended target genomic regions using DNA from at least one independent sample possessing the biological trait and DNA from at least one independent sample not possessing the biological sample.

[0081] In one embodiment, the step of identifying further comprises limiting the set of CpG sites to CpG sites that further exhibit differential methylation with peripheral blood mononuclear cells from a control sample.

[0082] In one embodiment, the plurality of tissue or cell types of the second group comprises at least some tissue or cells of the same type as the first group.

[0083] In one embodiment, the plurality of tissue or cell types of the second group comprises a plurality of non-diseased tissue or cell types.

[0084] In one embodiment, the predetermined threshold is indicative of methylation in the first group and non-methylation in the second group.

[0085] In one embodiment, the predetermined threshold is at least 50% methylation in the first group.

[0086] In one embodiment, the predetermined threshold is a difference in average methylation between the first and second groups of 0.3 or greater.

[0087] In one embodiment, the biological trait comprises malignancy.

[0088] In one embodiment, the biological trait comprises a cancer type.

[0089] In one embodiment, the biological trait comprises a cancer classification.

[0090] In one embodiment, the cancer classification comprises a cancer grade.

[0091] In one embodiment, the cancer classification comprises a histological classification.

[0092] In one embodiment, the biological trait comprises a metabolic profile.

[0093] In one embodiment, the biological trait comprises a mutation.

[0094] In one embodiment, the mutation is a disease-associated mutation.

[0095] In one embodiment, the biological trait comprises a clinical outcome.

[0096] In one embodiment, the biological trait comprises a drug response.

[0097] The method may further comprise designing a plurality of PCR primers pairs to amplify portions of the extended target genomic regions, each of the portions comprising at least one differentially methylated CpG site.

[0098] The step of designing the plurality of primer pairs may comprise converting non-methylated cytosines uracil, to simulate bisulphite conversion, and designing the primer pairs using the converted sequence.

[0099] The primer pairs may be designed to have a methylation bias.

[0100] The primer pairs may be methylation-specific.

[0101] The primer pairs may have no CpG residues within them having no preference for methylation status.

[0102] In one aspect, there is provided a method for synthesizing primer pairs specific to a methylation signature, the method comprising: carrying out the forgoing method, and synthesizing the designed primer pairs.

[0103] In one aspect, there is provided a non-transitory computer-readable medium comprising instructions that direct a processor to carry out the forgoing method.

[0104] In one aspect, there is provided a computing device comprising the computer-readable medium.

EXAMPLE 1

Concept Summary

[0105] The embodiments detect circulating tumour DNA using a highly sensitive and specific methylation based assay with detection limits 100 times better than other techniques.

[0106] **Fig. 1** depicts a schematic of the overall strategy. CpG dinucleotides are often clustered into concentrated regions in the genome referred to as CpG islands (grey box) and are often, but not always, associated with the promoter or enhancer regions of genes. These regions are known to become abnormally methylated in tumours (CmpG) as compared to normal tissue (CpG) which may be linked to the inactivation of tumour suppressor genes by this methylation event. Methylation of CpG islands and the boundary regions (CpG island shores) is extensive and coordinated such that most or all of the CpG residues in that region become methylated. The detection of this methylation involves bisulphite conversion, PCR amplification of the relevant region (arrows), and sequencing where un-methylated CpG residues are converted to TpG dinucleotides while methylated CpG residues are preserved as CpGs. Sequencing of these PCR-amplified "probes" (BISULFITE SEQUENCING) from tumour DNA (arrows) results in the detection of multiple CpG residues being

methylated within the same DNA fragment (Dashed Box) which can easily be distinguished from DNA from normal tissue (Boxes). The co-ordinated/concordant nature of this methylation produces a strong signal which can be detected over random or background changes from DNA sequencing. This is accomplished by first identifying regions of tumour specific DNA methylation with multiple correlated CpG methylation sites within the same region.

[0107] **Fig. 30** depicts a flowchart showing how a methylation signature for a biological trait may be determined. One or more steps of this method may be implemented on a computer. Accordingly, another aspect of this disclosure relates to a non-transitory computer-readable medium comprising instructions that direct a processor to carry out steps of this method.

[0108] Generally "probe" is used herein to refer to a target region for amplification and/or the ensuing amplified PCR product. It will be understood that each probe is amplified by a "primer set" or "primer pair".

[0109] **Fig. 2** depicts a schematic for amplification of target regions. Multiple regions from across the human genome have been identified as being differentially methylated in the DNA from various types of tumours compared to the normal DNA from a variety of different tissues. These regions can be fairly extensive spanning 100s to 1000s of base pairs of DNA. These target regions (black boxes, bottom) exhibit coordinated methylation where most or all of the CpG dinucleotides in these regions are methylated in tumour tissue with little or no methylation in normal tissues. As shown in Fig. 2, when sequencing across these regions (arrows) multiple CpG residues are seen to be methylated together in the tumour creating a concordant signal identifiable as being tumour specific. By targeting multiple PCR-amplified probes across individual regions (middle) and across the entire genome (top) large numbers of probes can be designed with the advantage that with more probes comes greater sensitivity due to the greater likelihood of detecting a tumour specific fragment in a given sample. Primers for these probes are designed to amplify regions from 75 to 150 bp in length, corresponding to the typical size of circulating tumour DNA. The primers may include CpG dinucleotides or not, which in the former case can make these primers biased towards the amplification of methylated DNA or exclusively amplify only methylated DNA.

[0110] Multiple methylation-biased PCR primer pairs can be created, which are able to preferentially amplify these regions. These multiple regions are sequenced using next generation sequencing (NGS) at a high read depth to detect multiple tumour specific methylation patterns in a single sample. As described herein, features have been incorporated into a blood based cancer detection system that provides advantages over other tests which have been developed, and provides an unprecedented level of sensitivity and specificity as well as enables the detection of minute quantities of DNA (detection sensitivity).

EXAMPLE 2

Probe and Primer Set Development

[0111] The detection of circulating tumour DNA is hampered by both the presence of large amounts of normal DNA as well as by the very low concentrations of tumour DNA in the blood. Compounding this issue, both PCR and sequencing based approaches suffer from the introduction of single nucleotide changes due to the error prone nature of these processes. To deal with these issues, regions of the genome have been identified that exhibit concerted tumour specific methylation over a significant expanse of DNA so that each CpG residue is concordant[21]. Methylation-biased PCR primer pairs were designed for multiple segments of DNA across these regions each containing multiple CpG residues. Sample protocols for selection of differentially methylated regions and design of region specific PCR primers are provided.

Protocol for the Selection of Differentially Methylated Regions

Use of TCGA DATA for identifying Breast Specific Probes

[0112] Level 3 (processed) Illumina Infinium HumanMethylation450 BeadChip array data (http://www.illumina.com/techniques/microarrays/methylation-arrays.html) was downloaded from The Tumour Genome Atlas (TCGA) site (https://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp) for the appropriate tumour types (e.g., breast, prostate, colon, lung, etc.). Tumour and normal samples were separated and the methylation values (from -0.5 to + 0.5) for each group were averaged. The individual methylation probes were mapped to their respective genomic location. Probes that fulfilled the following example criteria were then identified:

1. The average methylation values for the normal breast, prostate, colon and lung tissues all below -0.3;
2. The difference between the average breast tumour and average breast normal values greater than 0.3, or at least 50% methylation in the tumour group; and
3. Two probes within 300 bp of each other fulfill criteria 1 and 2.

[0113] These criteria establish that the particular probe is not methylated in normal tissue, that the difference between

the tumour and normal is significant, and that multiple probes in a relatively small area are co-ordinately methylated. Regions which had multiple positive consecutive probes (i.e., 3 or more) were prioritized for further analysis. Average values for approximately 10 other probes to either side of the positive region were plotted for all tumour and normal tissue samples to define the region exhibiting differential methylation. Regions exhibiting concerted differential methylation between tumour and normal for single or multiple tumour types were identified.

[0114] A secondary screen for a lack of methylation of these regions in blood was carried out by examining the methylation status of the defined regions in multiple tissues using nucleotide level genome wide bisulphite sequencing data. Specifically the UCSC Genome Browser (https://genome.ucsc.edu/) was used to examine methylation data from multiple sources.

[0115] Data was processed by the method described in Song Q, et al., A reference methylome database and analysis pipeline to facilitate integrative and comparative epigenomics. PLOS ONE 2013 8(12): e81148 (http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0081148) for use in the UCSC Browser and to identify hypo-methylated regions (above blue lines).

[0116] The following data sources were used:

Gertz J, et al., Analysis of DNA methylation in a three-generation family reveals widespread genetic influence on epigenetic regulation. PLoS Genet. 2011 7(8):e1002228 (http://journals.plos.org/plosgenetics/article?id=10.1371/journal.pgen.1002228).

Heyn H, et al., Distinct DNA methylomes of newborns and centenarians. Proc. Natl. Acad. Sci. U.S.A. 2012 109(26):10522-7 (http://www.pnas.org/content/109/26/10522).

Hon GC, et al., Global DNA hypomethylation coupled to repressive chromatin domain formation and gene silencing in breast cancer. Genome Res. 2012 22(2):246-58 (http://genome.cshlp.org/content/22/2/246).

Heyn H, et al., Whole-genome bisulfite DNA sequencing of a DNMT3B mutant patient. Epigenetics. 2012 7(6):542-50 (http://www.tandfonline.com/doi/abs/10.4161/epi.20523#.VsS_gdIUVIw).

Hon GC, et al., Global DNA hypomethylation coupled to repressive chromatin domain formation and gene silencing in breast cancer. Genome Res. 2012 22(2):246-58 (http://genome.cshlp.org/content/22/2/246).

[0117] All of the regions identified exhibited hypo-methylation in normal blood cells including Peripheral Blood Mononuclear Cells (PBMC), the prime source of non-tissue DNA in plasma.

Protocol for the Design of Region Specific Primers for PCR Amplification and Next Generation Sequencing

[0118] For regions identified as being differentially methylated in tumours, PCR primers were designed that are able to recognize bisulphite converted DNA which is methylated. Using Methyprimer Express™ or PyroMark™, or other web based programs, the DNA sequence of the region was converted to the sequence obtained when fully methylated DNA is bisulphite converted (i.e., C residues in a CpG dinucleotide remain Cs, while all other C residues are converted to T residues). The converted DNA was then analysed using PrimerBlast™ (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to generate optimal primers. Primers were not expressly selected to contain CpG residues but due to the nature of the regions, generally CpG islands, most had 1 to 3 CpGs within them. This renders them biased towards the amplification of methylated DNA but in many cases they do recognize and amplify non-methylated DNA as well. The region between the primers includes 2 or more CpG residues. Primers were chosen to amplify regions from 75 to 150 base pairs in size with melting temperatures in the range of 52 - 68°C. Multiple primers were designed for each region to provide increased sensitivity by providing multiple opportunities to detect that region. Adapter sequences (CS1 and CS2) were included at the 5' end of the primers to allow for barcoding and for sequencing on multiple sequencing platforms by the use of adaptor primers for secondary PCR.

[0119] Primers were characterized by PCR amplification of breast cancer cell line DNA and DNA from various primary tumours. PCR amplification was done with individual sets of primers and Next Generation Sequencing carried out to characterize the methylation status of specific regions. Primer sets exhibiting appropriate tumour specific methylation were then combined into a multiplex PCR reaction containing many primers.

Results

[0120] **Fig.** 3 lists the 47 CpG probes used to identify differentially methylated regions. These were analyzed by Receiver Operator Curve analysis (ROC). Normal and tumour samples from the entire TCGA breast cancer database were compared. The Area Under the Curve (AUC) analysis for each probe is shown with the standard error, 95% confidence interval and P-value. All of them where shown to have excellent discriminatory capabilities.

[0121] **Fig. 4** depicts the results of analysis methylation level for each patient in the TCGA database for the 47 CpG. Those exceeding the threshold of -0.1 were considered to be positive for methylation in that patient. The number of probes

exceeding this methylation threshold were calculated for each patient. Patients were divided into those with Luminal A and B subtypes (Luminal Tumours; **Fig. 4, Panel A)** and those with Basal cancers (Basal Tumours; **Fig. 4, Panel B)** or and the number of patients with a specific range of positive probes was calculated. The histogram shows the frequency of patents within each range of positive probes. While these probes give excellent coverage in both populations, there are more positive probes amongst the Luminal tumours than the Basal tumours. Additional probes specific to the different breast cancer subtypes have been identified and appropriate probe development and validation is underway.

EXAMPLE 3

Selection of Regions for Cancer and Cancer Types

[0122] For breast cancer, 52 regions in the genome were identified that are highly methylated in tumours but where multiple normal tissues do not exhibit methylation of these regions. These serve as highly specific markers for the presence of a tumour with little or no background signal.

[0123] **Table 1** depicts regions selected for breast cancer screening.

Table 1

Chromosome	Start (hg18)	End (hg18)	General Location	Tumour	Size
2nd Generation					
chr1	167663259	167663533	C1orf114	P/B	274
chr7	49783577	49784309	VWC2	P/B/C	732
chr14	23873519	23873993	ADCY4	P/B/C	474
chr11	43559012	43559541	MIR129-2	B/C	529
3rd Generation					
chr6	43319186	43319213	TTBK1	P/B	27
chr1	46723905	46724176	DMBX1	P/B/C	271
chr7	27171684	27172029	HOXA9	B	345
chr8	120720175	120720579	ENPP2	P/B	404
chr10	99521635	99521924	SFRP5	P/B	289
chr12	103376281	103376485	CHST11	P/B/C	204
chr19	51071603	51072234	FOXA3	P/B	631
4th Generation					
chr1	47470535	47470713	TAL1	B	178
chr1	50658998	50659557	DMRTA2	B	559
chr1	66030610	66030634	PDE4B	B	24
chr1	90967262	90967924	BARHL2	B	662
chr1	119331667	119332616	TBX15	B/C	949
chr1	153557070	153557585	RUSC1,C1orf104	B	515
chr1	233880632	233880962	GNG4	B	330
chr2	104836482	104837226	POU3F3	B	744
chr2	198359230	198359743	BOLL	B/C	513
chr3	32834103	32834562	TRIM71	B/C	459
chr3	172228723	172228985	SLC2A2	B	262
chr4	5071985	5072137	CYTL1	B	152
chr4	42094549	42094615	SHISA3	B	66
chr4	46690266	46690578	GABRA4	B	312

(continued)

4th Generation					
chr5	38293273	38293312	EGFLAM	B	39
chr5	43076195	43076642	C5orf39	B	447
chr5	115179918	115180393	CDO1	B	475
chr6	336189	337131	IRF4	B/C	942
chr6	19944994	19945298	ID4	B	304
chr6	28618285	28618318	SCAN D3	B	33
chr6	31806197	31806205	DDAH2	B	8
chr6	33269254	33269355	COL11A2	B	101
chr6	86215822	86215929	NT5E	B	107
chr6	101018889	101019751	SIM1	B	862
5th Generation					
chr6	153493505	153494425	RGS17	B	920
chr7	121743738	121744126	CAPDS2	B	388
chr8	72918338	72918895	MSC	B/C	557
chr10	22674438	22674584	SPAG6	B/C	146
chr10	105026601	105026737	INA	B	136
chr11	128068895	128069316	FLI1	B/C	421
chr12	52357158	52357378	ATP5G2	B	220
chr12	94466892	94467095	USP44	B/C	203
chr13	78075521	78075764	POU4F1	B	243
chr14	55656275	55656325	PELI2	B	50
chr17	33176853	33178091	HNF1B	B	1238
chr17	32368343	32368604	LHX1	B/C/L	261
chr17	44154844	44155027	PRAC,C17orf93	B/C	183
chr18	73090725	73091121	GALR1	B/C	396
chr19	12839383	12839805	MAST1	B	422
chr20	2729122	2729438	CPXM1	B/C	316
chr20	43952209	43952500	CTSA, NEU RL2	B	291

[0124] In Table 1, 'Start' and 'End' designate the coordinates of the target regions in the hg18 build of the human genome reference sequence. The 'General Location' field gives the name of one or more gene or ORF in the vicinity of the target region. Examination of these sequences relative to nearby genes indicates that they were found, e.g., in upstream, in 5' promoters, in 5' enhancers, in introns, in exons, in distal promoters, in coding regions, or in intergenic regions. The 'Tumour' field indicates whether a region is methylated in prostate (P), breast (B), colon (C), and/or lung (L) cancers. The 'Size' field indicates the size of the target region.

[0125] In the discussion here, it should be recognized that reference to genes such as CHST11, FOXA, and NT5 are not intended to be indicative of the genes in question per se, but rather to the associated methylated regions described in Table 1.

[0126] In total, 52 regions were found to be methylated in association with breast cancer, 17 were found to be methylated in association with prostate cancer, 9 were found to be methylated in association with prostate cancer, and 1 region was found to be methylated in association with lung cancer. Thus, some regions appear to be generally indicative of the various types of cancers assessed. Other regions methylated in subgroups of these, while others are specific for cancers. In the context of this assay and the types of cancers examined, 25 regions may be described as being "specifically methylated in

breast cancer". However, it is noted that the same approach may be used to identify regions methylated specifically in other cancers.

[0127] Assays may be developed for cancer generally, or to detect groups of cancers or specific cancers. A multi-tiered assay may be developed using "general" regions (methylated in multiple cancers) and "specific" regions (methylated in only specific cancers). A multi-tiered test of this sort may be run together in one multiplex reaction, or may have its tiers executed separately.

Probes for Breast Cancer

[0128] Over 150 different PCR primer pairs were developed to the 52 different regions in the genome shown to exhibit extensive methylation in multiple breast cancer samples from the TCGA database but with no or minimal methylation in multiple normal tissues and in blood cells (Peripheral Blood Mononuclear Cells and others).

[0129] As proof of concept, these were then used to amplify bisulphite converted DNA from breast cancer cell lines MCF-7 (ER+, PR+), T47-D (ER+, PR+), SK-BR-3 (HER2+), MDA-MD-231 (Triple Negative) and normal breast lines MCF-10A and 184-hTERT. Sequencing adapters were added and Next Generation Sequencing carried out on an Ion Torrent sequencer. The sequencing reads were then separated by region and the sequence reads were analyzed using the BiqAnalyzer HT program.

Results

[0130] Example results of methylation analysis will be discussed herein. CHST11 is an example of a region methylated in prostate, breast, and colon cancer. FOXA is a region methylated in breast and prostate cancer. NT5 is a region methylated specifically in breast cancer.

[0131] **Fig. 5** depicts sequencing results from a region from near the CHST11 gene (Probe C) is shown. For each cell line the results of a single sequencing read is depicted as a horizontal bar with each box representing a single CpG residue from between the PCR primers (in this case there being 6 CpG residues, Illustration at bottom right). Methylated bases are shown in dark grey while un-methylated bases are shown in light grey. Where a CpG could not be identified by the alignment program it is shown as a white box. Multiple sequence reads are shown for each cell line, stacked on top of each other. The numbers at the bottom of each stack indicates the number of sequence reads (Reads) and the overall methylation level determined from these reads (Meth).

[0132] When sequenced, these probes produced strong concordant signals that consisted of multiple methylated CpGs (5 to 25) where there is a strong correlation between individual sites being methylated in tumours. This eliminates false positive results due to PCR and sequencing errors. These tumour specific multiple methylated sites can be detected against a high background of normal DNA, being limited only by the read depth of the sequencing. Based on bioinformatic analysis of TCGA tumours, this essentially eliminates false positive signals.

[0133] **Fig. 6** depicts results for CHST11 Probe A. Methylation in the region was characterized for a variety of breast cancer tumour samples (T) and in normal breast tissue samples (N) from the same patient. As in Fig. 5 the methylated bases are shown in dark grey while unmethylated bases are shown in light grey (illustration bottom left). Tumours of various subtypes were analysed including A02324 which is positive for HER2 amplification (HER2+), A02354 and B02275 which are Triple Negative Breast Cancer (TNBC), and D01333, D02291, D02610 which are all Estrogen and Progesterone Receptor positive tumours (ER+ PR+). The values below each column refer to the number of sequence reads obtained by Next Generation Sequencing (Reads) and the overall level of methylation of all of the CpG residues (Meth) based on these reads. Where no sequence reads were obtained for a given sample and box is shown as for sample D01333 N (Normal).

[0134] **Fig. 7** depicts results of similar analysis of FOXA Probe A in breast cancer cell lines.

[0135] **Fig. 15** depicts a numerical summary generated methylation data for prostate cell lines. # Reads is indicative of the number of reads exported, and Mean Me is indicative of the mean methylation.

[0136] **Fig. 8** depicts results of similar analysis of the CHST11 Probe A and CHST11 Probe B in prostate cancer cell lines. DU145 is an Androgen Receptor (AR-) negative cell line which is able to generate metastases in the mouse. PC3 is also AR- and also metastatic. LNCaP is an Androgen Receptor positive line (AR+) which does generate metastases in the mouse while RWPE cells are AR+ and non-metastatic.

[0137] **Fig. 9** depicts results of similar analysis of FOXA Probe A in prostate cell lines.

[0138] **Fig. 10** depicts sequencing results to assess methylation status NET5 Probe E in breast cancer cell lines.

[0139] These results exemplify probes of differing specificities that can be selected using the approach outlined herein.

EXAMPLE 4

Probes for Uveal Cancer

[0140] Using the above-described methodologies, regions were selected for uveal cancer screening. **Table 2** depicts these regions.

Table 2

Chromosome	Start	Stop	General Location	Descriptor	Size
chr10	89611399	89611920	PTEN,KILLIN	Shore CGI	521
chr11	35503400	35504124	PAMR1	small CGI	724
chr11	1.18E+08	1.18E+08	MPZL2	Prox Prom	599
chr15	60146043	60147120	C2CD4A	Shore CGI	1077
chr17	24370858	24371386	SEZ6	small CGI	528
chr19	11060476	11060965	LDLR	Prox Prom	489
chr2	1.66E+08	1.66E+08	GALNT3	CGI	1465
chr2	2.23E+08	2.23E+08	ccdc140/pax3	Shore CGI	4724
chr6	21774638	21775386	FU22536/casc15	small CGI	748
chr6	24465699	24466545	KAAG1,DCDC2	CGI	846
chr6	31031220	31031651	MUC21	CGI	431
chr6	70632889	70633262	COL19A1	Proc Prom	373
chr6	1.09E+08	1.09E+08	NR2E1/OSTM1	small CGI	1001
chr7	29996242	29996333	SCRN1	Shore CGI	91
chr1	2450725	2452224	HES5	CGI	1499
chr1	12601228	12601893	DHRS3	Shore CGI	665

EXAMPLE 5

Tests for Breast Cancer Subtypes

[0141] The screen that has been described above, which originally incorporated all breast tumours in the TCGA database, can also be done on subsets of the tumour database.

[0142] BRCA1 carriers were taken out of the dataset and analyzed individually to identify target methylated regions specific to this subgroup. Breast cancer can also be divided in other ways: e.g., into five subtypes, Basal, Luminal A., Luminal B, HER2 and Normal-like. Patients in each of these groups were identified and analyzed to identify target methylated regions for each subset.

[0143] The screen can also be changed to look at individual patients using the previously described criteria to see who are positive or negative. Target methylated regions can then be ranked based on how many individuals are positive. This can help to remove biasing due to amalgamation (averaging). Targets can then be selected, e.g., if they are present in greater than 75% of patients for each subtype, and then rationalize amongst these.

Test for BRCA Carriers

[0144] Current monitoring practices for women at high risk of developing breast cancer due to familial BRCA1 or 2 mutations involve yearly MRI, however the high false positive rates result in a large number of unnecessary biopsies. Using the methodology described herein, a test may be developed to serve as a secondary screen, e.g., to be employed after a positive MRI finding; or to be used for primary screening of high risk patients. The blood test is designed to detect all types of breast cancer but because ER+ breast cancer is the most frequent it is biased towards these cancers, though some of the constituent probes do recognize HER2+ and TNBC tumours. In order to provide optimal sensitivity for the monitoring of BRCA1 and 2 an assay optimized for these patients may be developed.

[0145] Both TNBC and BRCA1 and 2 patients were selected from the TCGA 450k methylation database. Generally, most BRCA1 and 2 tumours will present as TNBC but many non-familial cancers are also TNBC. These patients were

analyzed using the above-described tumour specific methylation region protocol on both the overall TNBC population and on the BRCA1 and 2 patients. 85 tumour specific regions were identified for TNBC, 67 for BRCA1 and 13 for BRCA2 populations. Of these 39 were present in any two populations and they constitute the starting point for the development of this assay. Appropriate regions for a BRCA1 specific test were identified and assessed in individual patients with known mutations. This population is surprisingly uniform and most patients are recognized by a large number of probes. AUCs for individual probes are for the most part very high. Based on these results, an assay can be developed to detect all three, i.e., TNBC, BRCA1 and 2. If additional detection sensitivity is required, then individual tests can be constructed. For high risk women who are BRCA1 or 2 mutation carriers, their mutation status should be known so that the appropriate test can be applied.

Test for BRCA1 Carriers

[0146] Probes have been developed for the detection of cancer in carriers of the BRCA1 mutation. Methylation data from the TCGA Breast cancer cohort were selected from patients known to be carriers of pathogenic BRCA1 mutations. This data was then analyzed as described to identify regions of the genome specifically methylated in this sub-set of breast cancers. **Table** 3 lists appropriate regions identified and their genomic locations.

Table 3

Target Region (hg18 reference)				
chr	**Nearest Gene**	**Start (nt)**	**End (nt)**	**Size**
chr1	LOC105378683	43,023,840	43,023,487	353
chr1	NPHS2	177,811,942	177,811,671	271
chr1	NR5A2	198,278,599	198,278,409	190
chr11	PAX6	31,783,955	31,782,545	1,410
chr11	KCNE3	73,856,332	73,855,762	570
chr12	KCNA6	4,789,491	4,789,342	149
chr12	TMEM132C	127,318,539	127,317,001	1,538
chr13	PDX1	27,390,265	27,389,540	725
chr13	EPSTI1	42,464,618	42,463,901	717
chr16	A2BP1	6,009,930	6,009,020	910
chr16	CRYM	21,202,914	21,202,448	466
chr16	PRKCB	23,755,504	23,754,826	678
chr16	IRF8	84,490,354	84,490,167	187
chr18	SALL3	74,842,145	74,839,705	2,440
chr19	LYPD5	49,016,848	49,016,696	152
chr2:	DPP10	115,636,420	115,635,215	1,205
chr20	C20orf56	22,507,867	22,507,676	191
chr3	SOX2OT	182,919,993	182,919,839	154
chr4	CDKL2	76,774,880	76,774,658	222
chr5	March 11	16,233,072	16,232,633	439
chr5	CCL28	43,433,329	43,432,559	770
chr5	AP3B1	77,304,644	77,304,208	436
chr7	CARD11	3,050,299	3,049,859	440
chr7	BLACE	154,859,799	154,859,051	748
chr7	PTPRN2	157,176,806	157,176,096	710
chr8	RUNX1T1	93,183,481	93,183,326	155

[0147] 52 different probes were then developed to various parts of these regions and the methylation pattern in tumor cell lines was characterized, including MDA-MB-436 and HCC1937 which are known to carry BRCA1 mutations. These probes will be combined with previously characterized probes to other regions which are also methylated in tumours from BRCA1 patients. This would provide for a highly sensitive assay able to detect cancer in these high risk women at the earliest possible stage.

Tests for Other Subtypes

[0148] A number of breast cell lines from women with known BRCA1 mutations have been isolated such as MDA-MB-436, HCC1937 and HCC1395 (all available from ATCC). These may be used to validate the assay as was done for the general blood test. For BRCA2 mutant lines there is only one ATCC cell line at present, HCC1937. There are several BRCA2 mutant ovarian cancer lines that have been identified and they may be used if the bioinformatic analysis confirms that these methylation markers are also found in ovarian cancer. The development of a single assay that detects both breast and ovarian cancer in BRCA2 carriers represents a distinct advantage as it would simultaneously monitor the two primary cancer risks in these patients.

[0149] The development of these assays follows the same course the above-described general assay proceeding from TCGA data to cells lines to patient samples. Tumour banks (some of which have mutation data) can be used for this, and analysis of these tumours provides an indication of their likely BRCA mutation. These samples can also be sequenced to confirm the prediction.

EXAMPLE 6

Testing of Cell-Free Samples

[0150] Proof of concept testing was carried out using cell lines for ease of analysis. However, the assay can be applied to test for cell-free DNA, e.g., circulating cell-free tumour DNA in blood, and finds wide application in this context. A sample protocol for circulating tumour DNA is provided.

Sample Protocol: Test for Circulating Tumour DNA

DNA Preparation

[0151] The following example protocol may be used to detect circulating tumour DNA (tDNA).

- Obtain DNA to be used for bisulfite conversion and downstream PCR amplification (i.e., cell line, tumour or normal DNA). Determine DNA purity on 0.8% agarose gel.
- Determine genomic DNA (gDNA) for concentration in ug/uL by UV spectrophotometry.
- Prepare a 1:100 dilution with TE buffer.
- Remove RNA contaminates, if necessary, using the purification protocol for the GenElute Mammalian Genomic DNA Miniprep Kit, Sigma Aldrich, CAT# G1N350 (http://www.sigmaaldrich.com/technical-documents/protocols/biology/genelute-mammalian-genomic-dna-miniprep-kit.html). Follow purification protocol from steps A: 2a-3a, step 4-9.
- OPTIONAL: For gDNA from a cell line, sonicate gDNA to approximately 90-120bp (this represents general size of circulating tDNA). To do this, sonicate 5-10ug of sample (50-100ng/100uL) using a sonicator. Use setting 4, and 15 pulses for 30 seconds with 30 seconds rest on ice in between. Determine sonicated DNA purity and bp size on 0.8% agarose gel.
- Bisulfite convert DNA - EpiTect Fast Bisulfite Conversion Kit, QIAgen, CAT# 59824 (https://www.qiagen.com/us/resources/resourcedetail?id=15863f2d-9d1c-4f12-b2e8-a0c6a82b2b1e&lang=en). Follow bisulfite conversion protocol on pages 1-18, 19-23. Refer to trouble shooting guide pages 30-32. Modifications to the protocol include: 1. Prepare reactions in 1.5mL tubes, 2. High concentration samples at 2ug, and low concentration samples at 500ng-1ug, 3. Perform the bisulfite conversion using 2 heat blocks set at 95°C and 60°C, 4. Incubation at 60°C extended to 20 minutes, to achieve complete bisulfite conversion, 5a Elute DNA in 10-20uL of elution buffer for ~50-100ng/uL final concentration, and 5b Dilute DNA to 10ng/uL for use in PCR.
- Perform nested PCR with Hot Star *Taq* Plus DNA Polymerase, Qiagen, CAT# 203605 (https://www.qiagen.com/ca/resources/resourcedetail?id=c505b538-7399-43b7-ad10-d27643013d10&lang=en).

Singleplex PCR Amplification

[0152]

- For singleplex PCR amplification of individual probes, carry out a primary PCR reaction with methylation-biased primers (MBP), (primer forward and reverse).

[0153] **Table 4** recites reaction components.

Table 4

Component	1X (uL)
10X PCR Buffer	2.5
5mM dNTP's	1
5U Hot Star Taq	0.1
25mM MgCl2	3
PCR Grade H2O	17
[10ng/uL] DNA	1
10pmol FWD Primer	0.2
10pmol REV Primer	0.2
Total	25

[0154] **Table 5** lists thermocycler conditions.

Table 5

Thermocycler Cond itions		
Temp.	Time	
95°C	15 min	
95°C	30 sec	⌐
58°C	30 sec	| X 40
72°C	30 sec	⌙
72°C	7 min	
4°C	∞	

- Carry out a secondary PCR reaction with universal primers CS1 (Barcode) and CS2 (P1 Adapter). To do this, remove an aliquot from the primary reaction, use as template DNA, this method serves as a two-step dilution PCR reaction

[0155] **Table 6** recites reaction components.

Table 6

Component	1X (uL)
10X PCR Buffer	5
5mM dNTP's	2
5U Hot Star Taq	0.2
25mM MgCl2	6
PCR Grade H2O	34.4
MBP PCR Template	2
10pmol CS1 Primer	0.2
10pmol CS2 Primer	0.2
Total	50

[0156] **Table 7** recites thermocycler conditions.

Table 7

Thermocycler Conditions		
Temp.	Time	
95°C	15 min	
95°C	30 sec	
58°C	30 sec	X 3
72°C	30 sec	
72°C	7 min	
4°C	∞	

- Determine PCR specificity on 2% agarose gel. Run the methylation-biased PCR product and the CS1 CS2 sequencing PCR product beside one another on the agarose to visualize the banding pattern and increase in bp size. PCR product should be between 200-300bp

[0157] For Singleplex PCR products, pool 5-10uL of each PCR reaction (CS1 CS2 Secondary RXN) into a single tube for each sample type. Purify the pooled PCR with Agencourt AMPure XP beads at a 1.2:1 ratio (90uL beads + 75uL sample), e.g., as below.

Agencourt Ampure XP Bead Purification

[0158] Use freshly prepared 70% ethanol. Allow the beads and pooled DNA to equilibrate to room temperature.

1. Add indicated volume of Agencourt AMPure XP beads to each sample: 90uL beads + 75uL Pool (1.2:1)
2. Pipet up and down 5 times to thoroughly mix the bead suspension with the DNA. Incubate the suspension at RT for 5 minutes.
3. Place the tube on a magnet for 5 minutes or until the solution clears. Carefully remove the supernatant and store until purified library has been confirmed.
4. Remove the tube from the magnet; add 200uL of freshly prepared 70% EtOH. Place the tube back on the magnet and incubate for 30 seconds; turn the tube around twice in the magnet to move the beads through the EtOH solution. After the solution clears, remove and discard the supernatant without disturbing the pellet.
5. Repeat step #4 for a second EtOH wash.
6. To remove residual EtOH, pulse-spin the tube. Place the tube back on the magnet, and carefully remove any remaining EtOH with a 20uL Pipette, without disturbing the pellet.
7. Keeping the tube on the magnet, air-dry the beads at RT for ~5 minutes.
8. Remove the tube from the magnet; add 50uL of TE directly to the pellet. Flick the tube to mix thoroughly. Incubate at RT for 5 minutes.
9. Pulse-spin and place the tube back on the magnet for ~2 minutes or until the solution clears. Transfer the supernatant containing the eluted DNA to a new 1.5mL Eppendorf LoBind tube.
10. Remove the tube from the magnet; add 50uL of TE directly to the pellet. Flick the tube to mix thoroughly. Store the beads, along with the supernatant, at 4^{O}c until purified library has been confirmed.
11. Visualize the sample pre- and post-purification on an 8% acrylamide gel (higher resolution). Pooled PCR product should be visualized as multiple bands (as each PCR product is a slightly different bp size). Purified sample should eliminate product beneath 150bp.

Fig. 11 depicts a summary of BioAnalyzer electrophoresis summary for amplification product generated from various cell lines.

12. Perform nested PCR with Multiplex PCR Plus Kit, Qiagen, CAT# 206152 (https://www.qiagen.com/ca/resources/resourcedetail?id=beb1f99e-0580-42c5-85d4-ea5f37573c07&lang=en), e.g., as below.

[0159] Multiplex PCR Amplification of up to 50 Probes in a Single Reaction

- Create multiplex primer mix by aliquot 1uL of each forward and reverse primer at 10pmol/uL into a single 1.5mL tube. Calculate the final concentration of each primer by dividing the initial primer concentration by the final volume of primer

mix in the tube, i.e., 15 probes to be multiplexed into a single reaction, would total 30 primers and at 1uL each, 30uL final volume. Thus ((10pmol)(1uL))/30uL=0.333pmol. Primer concentration requires optimization during PCR amplification, as the number of primers in a single reaction can influence the efficiency of the product, e.g.

15 primer sets ~2pmol final [ ] in PCR
50 primer sets ~0.5pmol final [ ] in PCR

- Carry out primary PCR reaction with methylation-biased primers.

[0160] **Table 8** lists reaction components for multiple amplifications of 15 probes, and **Table** 9 lists reaction components for multiple amplifications of 50 probes. **Table 10** list reaction conditions.

Table 8

15 primer pairs at 2pmol	
Component	1X (uL)
2X Multiplex MM	25
PCR H2O	18
Primer Mix	6
[10ng/uL] DNA	1
Total	50

Table 9

50 primer pairs at 0.5pmol	
Component	1X (uL)
2X Multiplex MM	25
PCR H2O	19
Primer Mix	5
[10ng/uL] DNA	1
Total	50

Table 10

Thermocycling Conditions	
Temp.	Time
95°C	5 min
95°C	30 sec ㄱ
58°C	90 sec | X 35
72°C	90 sec ㄴ
68°C	10 in

- Determine PCR specificity on 2% agarose gel. Multiplex products should be visualized with multiple banding pattern between 100-300bp.
  Pooling is not required for multiplex products, as the probes have already been combined and amplified into a single tube/ reaction.
- Purify the pooled PCR with Agencourt AMPure XP beads at a 1.2:1 ratio (60uL beads + 50uL sample) (refer within document for purification protocol).
- After PCR amplification, along with pooling and purifying, the samples can be quantified by qPCR, e.g., Ion Library Quantification Kit, TaqMan assay quantification of Ion Torrent libraries, Thermo Fisher Scientific, CAT# 4468802

(https://tools.thermofisher.com/content/sfs/manuals/4468986_IonLibraryQuantitationKit_UG.pdf).

1. Create a standard curve of 6.8pM, 0.68pM, 0.068pM, 0.0068pM
2. Dilute samples 1:1000, and run in duplicate
3. Perform qPCR assay on the Step One Plus Real Time machine by Life Technologies
4. Sample libraries quantified ≥100pM can proceed to be sequenced on the Life Technologies Ion Torrent Sequencing platform

Life Technologies Ion Torrent PGM Sequencing

[0161] Ion PGM Template OT2 200.

- Perform template reaction with Ion PGM Template OT2 200 Kit, Thermo Fisher Scientific, CAT# 4480974. Kit contents to be used on the One Touch 2 and Enrichment system (https://tools.thermofisher.com/content/sfs/manuals/MAN0007220_Ion_PGM_Template OT2 200_ Kit_UG.pdf_
- Utilizing library quant. obtained from qPCR, dilute libraries appropriately to 100pM. Follow Life Technologies guide on how to further dilute libraries for input into final template reaction.
- Follow reference guide to complete template reaction
- Run the Ion One Touch 2 instrument
- Recover the template positive ISPs
- Enrich the template positive ISPs with the Ion One Touch ES

Ion PGM Sequencing 200

[0162]

- Perform sequencing reaction with Ion PGM Sequencing 200 kit, Thermo Fisher Scientific, CAT# 4482006. Kit contents to be used on the Ion PGM system (https://tools.thermofisher.com/content/sfs/manuals/MAN0007273_IonPGMSequenc_200Kit_v2_UG .pdf).
- Plan sequencing run
    - Select chip capacity (314, 316 or 318)
    - Determine sequencing flows and bp read length (i.e., 500 flows and 200bp read length)
- Follow reference guide to complete PGM sequencing
    - Prepare enriched template positive ISPs
    - Anneal the sequencing primer
    - Chip check
    - Bind sequencing polymerase to the ISPs
    - Load the chip
    - Select the planned run and perform sequencing analysis

[0163] Sequencing data analysis and work flow

- Obtain run report generated by the PGM and Torrent Browser
- Run report includes the following information
    - ISP Density and loading quality
    - Total reads generated and ISP summary
    - Read length distribution graph
    - Barcoded samples: reads generated per sample and mean read length
- Obtain uBAM files generated by the PGM, available for download to an external hard drive
- Bioinformatics data analysis
    - Upload uBAM files to a web based bioinformatics platform, Galaxy GenAp

- Perform quality control analysis (i.e., basic statistics and sequence quality check)
- Convert data files: BAM SAM FastQ
- Filter FastQ file: select bp size to trim (i.e., trim sequence <100bp)
- Convert data files: FastQ FastA
- Download FastA file

- Upload FastA files to BiqAnalyzer software platform

- Create project
- Add sample
- Load reference sequence
- Set gap extension penalty and minimal sequence identity
- Link in FastA files to samples and reference sequences
- Analyze and collect data files (pattern maps and pearl necklace diagrams)

EXAMPLE 7

Uveal Melanoma Test

[0164] The molecular biology of uveal melanoma (UM) is simpler than that of breast cancer, with minimal mutations and rearrangements, and only two major sub-types which correspond to the retention or loss of chromosome 3p. A test was developed for UM which is superior to current state of the art blood assays.

[0165] Analysis of 450k methylation TCGA data for 80 UMs allowed for the identification of regions of tumour specific methylation in both 3p- and 3pWT tumours using our algorithm. **Table 11** shows 16 hypermethylated regions in both 3p- and 3pWT tumours used for probe development and testing, according to one embodiment.

Table 11

Gene	Chr	start	stop		Size		CGI CpGs
PTEN,KILLIN	chr10	89611399	89611920		521	Shore CGI	171
PAMR1	chr11	35503400	35504124		724	small CGI	19
MPZL2	chr11	117640011	117640610		599	Prox Prom	
C2CD4A	chr15	60146043	60147120		1077	Shore CGI	127
SEZ6	chr17	24370858	24371386		528	small CGI	34
LDLR	chr19	11060476	11060965		489	Prox Prom	
GALNT3	chr2	166358156	166359521		1465	CGI	98
ccdc140/pax3	chr2	222881305	222886029		4724	Shore CGI	72
FLJ22536/casc15	chr6	21774638	21775386		748	small CGI	18
KAAG1,DCDC2	chr6	24465699	24466545		846	CGI	56
MUC21	chr6	31031220	31031651		431	CGI	46
COL19A1	chr6	70632889	70633262		373	Proc Prom	
NR2E1/OSTM1	chr6	108542808	108543809		1001	small CGI	34
SCRN1	chr7	29996242	29996333		91	Shore CGI	133
HES5	chr1	2450725	2452224		1499	CGI	111
DHRS3	chr1	12601228	12601393		665	Shore CGI	133

[0166] The top 14 of these common regions were carried forward for probe development and a total of 26 different probes were characterized, with several regions having up to three probes targeting them. Each of these probes was then

validated using six different UM cell lines to assess their methylation status. As negative controls, DNA from peripheral blood mononuclear cells (PBMCs), which are the main source of contaminating DNA in blood samples, as well as a pool of cell free DNA (cfDNA) from 16 individuals, were also tested **(Fig. 15).** These results indicated that the majority of the probes tested showed tumour specific methylation with little or no methylation in the negative controls. A total of 18 probes from 12 different regions were combined into a multiplex PCR reaction and used to analyze cell free DNA from plasma for a previously characterized cohort of metastatic UM patients.

[0167] The validated regions were C2CD4A, COL19A1, DCDC2, DHRS3, GALNT3, HES5, KILLIN, MUC21, NR2E1/OSTM1, PAMR1, SCRN1, and SEZ6. The validated probes were C2C5F, COL2F, DCD5F, DGR2F, GAL1F, GAL3F, HES1F, HES3F, HES4F, KIL5F, KIL6F, MUC2F, OST3F, OST4F, PAM4F, SCR2F, SEZ3F, and SEZ5F.

[0168] These patients were previously tested using the pyrophosphorolysis-activated polymerization (PAP) assay[26], which detects the frequent GNAQ or GNA11 mutations in UM[27]. In all cases the test detected cancer in these patients even when the PAP assay failed to register a signal **(Figs. 16 and 17).** Most of the probes functioned like methylation specific PCR reactions, only giving product when there was tumour DNA present though with the additional validation that the specificity of each probe was guaranteed by the presence of multiple methylated CpG residues within each read. In two patients from which serial blood samples were obtained **(Figs. 18A and 18B)** the test showed increased tumour levels over time even when the final tumour volume was 0.5 cm^3 (Fig. **18A).** The test was also generally correlated with the volume of tumour, though the nature of the metastatic tumour as either a solid mass or dispersed has not yet been accounted for **(Fig.** 19). The levels detected by the test were generally in line with those of the PAP assay and notably gave a signal where PAP failed due to the lack of a mutation **(Fig. 16,** UM32). Where no or limited amounts of tumour DNA were detected by PAP, the test still gave significant signals **(Fig. 20).** Even greater sensitivity is expected when the total number of reads analyzed per patient is increased, as this run had less than optimal overall reads due to the presence of large amounts of primer dimer, an issue that has now been resolved. The specificity of the test was demonstrated by the extremely low levels of methylation seen in the pool of 16 cfDNA controls. Overall, the test has been validated in a patient population, and it has been shown to be superior to a state of the art mutation based assay.

EXAMPLE 8

Prostate Cancer Test

[0169] An important aspect of any test is that it should be applicable to all patients. Based on our experience it is essential to consider specific subtypes of a given cancer to ensure that all patients are detected by the assay. The TCGA analysis of a large prostate cohort revealed sub-groups based on specific mutations and transcriptional profiles[28]. Four subtypes were identified based on the overall pattern of methylation found in these tumours. In this example the TCGA prostate cohort was divided into groups based on the methylation pattern and subjected to methylation analysis.

[0170] **Table 12** lists 40 regions associated with all sub-types of prostate cancer.

Table 12

HES5	ANXA2	HLA-F	HAAO
LOC376693	RHCG	PON3	RARB
CSRP1	RARA	LRRC4	ALDH1L1
ALOX5	PTRF	HLA-J	HIST1H3G
PPM1H	RND2	PAH	ZSCAN12
MON2	TMP4	EPSTI1	HCG4P6
KIAA0984	HIF3A	ADCY4	EYA4
TXNRD1	KLK5	HAPLN3	HOXA7
CHST11	AMOTL2	AX747633	HSF4
EFS	SCGB3A1	NBR1	TMEM106A

[0171] These regions common to all four methylation subtypes were identified and a total of 38 probes from 33 regions were selected and appropriate "biased" PCR probes were generated. These were characterized using four different prostate cancer lines. DU145 is an androgen receptor (AR-) negative cell line that is able to generate metastases in the

mouse. PC3 is also AR- and also metastatic. LNCaP is an androgen receptor positive line (AR+) that is non-metastatic in the mouse while RWPE cells are AR+ and non-metastatic. DNA from PBMC was also tested as this represents the primary source of cell free DNA in the circulation.

[0172] A total of 34 probes from 33 regions were validated in that they showed little or no methylation in PBMCs while showing large scale methylation in one or more of the tumour cell lines **(Fig. 21).**

[0173] The validated regions were ADCY4, ALDH1L1, ALOX5, AMOTL2, ANXA2, CHST11, EFS, EPSTI1, EYA4, HAAO, HAPLN3, HCG4P6, HES5, HIF3A, HLA-F, HLA-J, HOXA7, HSF4, KLK4, LOC376693, LRRC4, NBR1, PAH, PON3, PPM1H, PTRF, RARA, RARB, RHCG, RND2,TMP4, TXNRD1, and ZSCAN12.

[0174] The validated probes were ADCY4-F, ALDH1L1-F, ALOX5-F, AMOTL2-F, ANXA2-F, CHST11-F, EFS-F, EPSTI1-F, EYA4-F, HAAO-F, HAPLN3-F, HCG4P6-F, HES5-F, HIF3A-F, HLA-F-F, HLA-J-1-F, HLA-J-2-F, HOXA7-F, HSF4-F, KLK4-F, LOC376693-F, LRRC4-F, NBR1-F, PAH-F, PON3-F, PPM1H-F, PTRF-F, RARA-F, RARB-F, RHCG-F, RND2-F, TMP4-F, TXNRD1-F, and ZSCAN12-F.

[0175] To these 34 probes an additional 12 probes (from 7 regions) were added that had previously been characterized in breast cancer, which were also able to detect prostate cancer, for a total of 46 probes.

[0176] The added probes were C1 Dtrim, C1 Etrim, CHSAtrim, DMBCtrim, FOXAtrim, FOXEtrim, SFRAtrim, SFRCtrim, SFREtrim, TTBAtrim, VWCJtrim, and VWCKtrim.

[0177] These probes were multiplexed together and were then used to analyze plasma samples from five patients before they had initiated androgen deprivation therapy (ADT) and 12 months after starting treatment. These patients were part of a small cohort (~40 patients) being followed for depression and the plasma samples at 0.5 ml were much smaller than normally used for the assay (2 mls). All of the patients were M0 with no sign of metastatic disease when placed on ADT.

[0178] A variety of probes were positive depending on the particular patient **(Fig. 22).** The total number of positive probes was in keeping with the total number of methylated reads, which were normalized for total reads for each sample **(Fig. 23).** In all cases significant ctDNA signals were observed with results that were notably different than PSA results **(Fig. 24).** Two of the patients, TM19 and RM26 were started on ADT due to their aggressive diseases (T3A and T3B) despite having low PSA levels. PSA levels for both remained low but methylation detection of circulating tumour DNA (mDETECT) either decreased slightly (TM19) or rose dramatically (RM26) suggesting their diseases did not express PSA but had stable or increasing disease. HS29 showed decreased PSA levels which mDETECT paralleled. Both GL20 and GP27 trended in opposite directions to PSA levels with mDETECT increasing even with dramatic drops in PSA levels. GL20 did develop a radiation induced secondary cancer which may be what is detected. Ongoing analysis of additional clinical data is expected to help explain these results.

[0179] Based on the literature, three of these regions appear to have prognostic significance as well. C1orf114 or CCDC1 has been shown to be correlated with biochemical relapse. HES5 is a transcription factor that is regulated by the Notch pathway and methylation of its promoter occurs early in prostate cancer development. KLK5 is part of the Kallikrein gene complex that includes KLK3 (the PSA gene). We can demonstrate that KLK5 expression is correlated with methylation and KLK5 expression has previously been shown to be increased in higher grade tumours. These results strongly suggest that the examination of a large number of methylation markers may yield significant insight into the specific processes involved in prostate cancer development and produce diagnostic and prognostic information that would be vital for management of the disease.

EXAMPLE 9

Predictive Prostate Cancer Methylation Biomarkers

[0180] The 50 region assay according to embodiments described herein is sufficiently sensitive to easily detect metastatic disease and to follow changes in tumour size over time and, as indicated, has predictive value in itself. As described above, at least three regions, KLK5, HER5, and C1orf114 have potential to predict progression. In order to develop additional probes that are able to predict outcome in this patient population, the prostate cancer TCGA data was reanalysed to divide the patients by Gleason score. An inter-cohort comparison was conducted to identify regions frequently methylated in higher score cancers. Initially, Gleason grades 6 and 9 were compared as these typically represent less and more aggressive tumours and both groups had sufficient numbers of patients to ensure significance of the results. Probe development was carried out under the same criteria as with the original probe sets so that they could be used with ctDNA. No single probe will be absolutely specific for a given grade but a number of the probes showed excellent division between Gleason scores with the proportion of the cohort positive for a given grade increasing with increasing grade **(Fig. 25).** One of these, PSS3, is a gene whose expression has previously been associated with prostate cancer and particularly metastasis. It should be noted that not all methylation is associated with gene repression. Forty-three new probes were developed based on selection criteria to target the 36 regions shown in **Table 13,** which are associated with aggressive prostate cancer.

Table 13

ASAP1	EMX1	MIR1292	SOX2OT
BC030768	HFE	NBPF1	TUBB2B
C18orf62	HIST1H3G/1H2BI	NHLH2	USP44
C6orf141	HMGCLL1	NRN1	Intergenic (Chr1)
CADPS2	KCNK4	PPM1H	Intergenic (Chr8)
CORO1C	KJ904227	PPP2R5C	Intergenic (Chr2)
CYP27A1	KRT78	PRSS3	Intergenic (Chr3)
CYTH4	LINC240	SFRP2	Intergenic (Chr4)
DMRTA2	Me3	SLCO4C1	Intergenic (Chr10)

[0181] The probes were ASAP1/p, BC030768/p, C18orf62/p, C6orf141/p-1, C6orf141/p-2, CADPS2/p, CORO1C/p-1, CORO1C/p-2, CYP27A1/p, CYTH4/p, DMRTA2/p, EMX1/p, HFE/p-1, HFE/p-2, HIST1H3G/1H2BI/p, HMGCLL1/p, KCNK4/p, KJ904227/p, KRT78/p, LINC240/p-1, LINC240/p-2, Me3/p-1, Me3/p-2, MIR129, NBPF1/p, NHLH2/p, NRN1/p, PPM1H/p-1, PPM1H/p-2, PPP2R5C/p, PRSS3/p, SFRP2/p-1, SFRP2/p-2, SLCO4C1/p, SOX2OT/p, TUBB2B/p, USP44/p, Chr1/p-1, Chr2/p-1, Chr3/p-1, Chr4/p-1, Chr8/p-1, and Chr10/p-1.

[0182] It is expected that it will be an overall pattern of hypermethylation, rather than a single probe, that will have the greatest predictive power.

EXAMPLE 10

Breast Cancer Test

[0183] One approach described herein for identifying hypermethylated regions in breast cancer focused on the most frequently methylated regions within the TCGA database. Due to the large number of LumA and LumB patients in this dataset there was a significant under-detection particularly of the Basal class of tumours.

[0184] Accordingly, the data were reanalyzed based on the four molecular subtypes LumA, LumB, Her2 and Basal. The Normal-like subtype is not very frequent in the dataset and as expected is very close to normal tissue, however a small number of regions recognizing this subtype were also included. Overall, methods and probes were developed and tested for over 230 different regions (some with multiple probes), and these have been validated using a variety of breast cancer cell lines and tumour samples. Some regions are subtype-specific but most recognize multiple subtypes. These have been assembled into a single test incorporating 167 different probes which recognize all subtypes **(Fig. 26A, 26B, and 26C),** with all patients being recognized by a significant number of probes. By looking at just the top 20 probes for each subtype this test has an area under the curve (AUC) per subgroup from 0.9078 to 0.9781, indicating that high detection rates have been achieved for all types of tumours **(Fig. 27).** This also means that the test is able to identify the subtype of tumour based on the distribution of probe methylation.

[0185] Another test specific for the triple negative breast cancer (TNBC) subtype was developed from the larger set of general regions identified as described above. This test incorporates 86 probes from 71 regions, listed in **Table 14.**

Table 14

CCL28	PTPRN2	UDB	IRF4	HOXA9	HINF1B	POU4F1
PAX6	BARHL2	TMEM90B	SOX2OT	NT5E	TNFRSF10D	VWC2
PPFIA3	PRSS27	C1orf114	TSPAN33	DPP10	CD38	BRCA1
SPAG6	DMRTA2	ITPRIPL1	CA9	FOXA3	CHST11	HOXB13
TMEM132C	NR5A2	GIPC2	IRF8	C5orf39	FABP5	OTX2
DMBX1	BOLL	ERNA4	CRYM	PTGDR	Intergenic5	
TAL1	SLC7A4	MAST1	GNG4	SALL3	EVX1	
TOP2P1	LEF1	DRD4	DDAH2	ID4	ACVRL1	
PRDM13	CARD11	Intergenic 8	EPSTI1	GABRA4	TBX15	
GALR3	NFIC	TCTEX1D1	TTBK1	PRKCB	ALX1	
CDKL2	PDX1	PHOX2B	SCAND3	NPHS2	SIM1	

[0186] The probes were ALX1, AVCRL1, BRCA1-A, C1Dtrim, C1Etrim, CA9 - A, CARD11 - B, CCL28-A, CD38, CDKL2 - A, CHSAtrim, CRYM-A, DMBCtrim, DMRTA2exp - A, DPP10-A, DPP10-B, DPP10-C,DRD4 - A, EFNA4 - B, EPSTI1, EVX1, FABP5, FOXAtrim, FOXEtrim, GALR3 - A, GIPC2 - A, HINF C trim, HOXAAtrim, HOXACtrim, HOXB13-A, Int5, Int8, IRF8-A, ITRIPL1, LEF1 - A, MAST1 A trim, mbBARHL2 Trim, mbBOLL Trim, mbC5orf Trim, mbDDAH Trim, mbDMRTA Trim, mbGABRA A Trim, mbGABRA B Trim, mbGNG Trim, mbID4 Trim, mbIRF Trim, mbNT5E Trim, mbSIM A Trim, mbTBX15 Trim, NFIC - B, NFIC -A, NPSH2-B, NR5A2 - B, OTX2-A, PAX6-A, pbDMRTA Trim, pbGNG Trim, pbSCAND Trim, pbTAL Trim, PDX1exp - B, PHOX2B - A, POU4F1 A trim, PPFIA3-A, PRDM13, PRKCB-A, PRKCB-C, PRSS27 - A, PTGDR, PTPRN2 - A, PTPRN2 - B, SALL3-A, SALL3-B, SLC7A4 - A, SOX2OT - B, SPAG6 A trim, TCTEX1D1 - A, TMEM - A, TMEM - B, TMEM90B - A, TNFRSF10D, TOP2P1 - B, TSPAN33 - A, TTBAtrim, UBD - A, VWCJtrim, and VWCKtrim.

[0187] The ability of this test to detect TNBC was validated by the analysis of 14 TNBC primary tumours as well as matched normal tissue from four of these patients. Large scale methylation was observed for the majority of probes and was distinctly different from the normal samples **(Fig. 28).**

EXAMPLE 11

Sensitivity of the Tests

[0188] The tests described herein are designed to detect less than one genome's worth of DNA in a sample through the use of multiple regions where a single probe out of many can signal the presence of a tumour. The more regions and probes incorporated into a test the greater is the sensitivity. This is in contrast to mutation detection where the presence of a single mutation per genome equivalent means that random sampling effects rapidly limit sensitivity when the concentration of the tumour DNA falls below one genome equivalent per sample. The presence of large amounts of normal DNA in fluid samples also creates problems for the detection of mutations through the relatively high error rates for PCR and sequencing. To assess the limits of methods and tests described herein, a dilution experiment was performed wherein DNA from a TNBC cell line (HCC1937 DNA) was diluted into a constant amount of PBMC DNA (10 ng) from a normal patient **(Fig. 29).** These samples were then tested using the TNBC test. A conclusive signal was obtained from the test even when as little as 0.0001 ng of TNBC DNA was present in 10 ng of PBMC DNA. This represents a detection of 0.03 genome equivalents of tumour DNA against a background of 100,000 times more normal DNA.

EXAMPLE 12

Discussion

[0189] The sensitivity of mutation based detection tests is limited by their detection of single unknown mutations in genes, such as p53 or *ras.* As only a single mutation is present per genome equivalent, this dramatically limits the sensitivity of these assays. Once the concentration of tumour DNA in the blood decreases to less than one genome equivalent per volume of blood analysed, the probability of detecting a mutation decreases dramatically as that particular segment of DNA may not be present in the blood sample. The assay described herein incorporates multiple probes for multiple regions from across the genome to dramatically increase sensitivity. For example, up to 100 or more probes may be incorporated into the assay, making it up to 100 or more times more sensitive than mutation based tests.

[0190] Circulating tumour DNA may be produced by the apoptotic or necrotic lysis of tumour cells. This produces very small DNA fragments in the blood. With this in mind, PCR primer pairs were designed to detect DNA in the range of 75 to 150 bp in length, which is optimal for the detection of circulating tumour DNA.

[0191] The use of DNA methylation offers one more advantage over mutation based approaches. Mutated genes are typically expressed in the cells (such as p53). They are thus in loosely compacted euchromatin, in comparison to methylated DNA which is in tightly compacted heterochromatin. This methylated and compacted DNA may be protected from apoptotic nucleases, increasing its concentration in the blood in comparison to these less compacted genes.

[0192] Extensive analysis of the genome wide methylation patterns in breast, colon, prostate and lung cancers and normal tissue in each of these organs based on TCGA data was carried out. 52 regions were identified for breast cancer which fulfill design criteria, which looks for an optimal difference in methylation between tumour and normal breast tissue, and where there is no methylation in any of the other normal tissues. As well, there should optimally be at least 2 CpG residues within 200 basepairs of each other. This ensured that regions of coordinated tumour specific methylation have been identified.

[0193] Within these 52 regions, 17 were found in common with colon cancer, and 9 in common with prostate cancer. Interestingly there were few appropriate regions identified in lung cancer, with only 1 overlapping with breast cancer. Most of these regions are associated with specific genes, though several are distantly intergenic, and almost all were found in CpG islands of various sizes. Probes were first developed for those regions with some commonality between cancers and designed PCR primers which recognize the methylated DNA sequence. This provides a bias in the amplification process for tumour DNA, enriching the tumour signal. These primer pairs amplify regions of 75 to 150 bp in accordance with our design criteria. Typically these regions contain from 3 to 12 CpG residues each, ensuring a robust positive signal when these regions are sequenced. Multiple non-overlapping probes were used as the CpG islands are generally larger than 150 bp, allowing for multiple probes for each appropriate region, providing more power to detect these regions and increasing the detection sensitivity of the assay.

[0194] Six different breast cancer lines were used in this validation analysis that have been shown to generally retain tumour specific methylation patterns[22]. MCF-7 and T47D lines are classic ER+ positive cell lines representing the most frequent class of breast cancer. SK-BR-3 cells are a HER2+ line and MDA-MB-231 cells represent a Triple Negative Breast cancer (TNBC), thus the 3 main categories of breast cancer are represented covering 95% of all tumours. Two "normal" lines were also used, the MCF10A line, though this line has been shown to contain some genomic anomalies, and the karyotypically normal 184-hTERT line. DNA was bisulphite converted, and the probes were amplified individually, barcoded then pooled according to cell line and subject to Next Generation Sequencing on an Ion Torrent sequencer. Not all PCR primer pairs produced a product due to the methylation-based nature of the primers, but in general, where a signal was detected, around 1000 reads were obtained per probe for each cell line. These reads were processed through our NGS pipeline using Galaxy and then loaded into the NGS methylation program BiqAnalyzer[23,24]. This program extracts probe specific reads, aligns them against the probe reference sequence, and calls methylated and unmethylated CpGs. It also carries out quality control measures related to bisulphite conversion and alignment criteria. In all of these probes there are several CpG residues within the primer sequence producing a bias towards amplifying methylated DNA. The analysis shown only includes CpGs outside of the primers which are solely representative of the methylation status of the sample being analysed.

[0195] **Figs. 5 and 6** depict results for the CHST11 gene, which is a good example where robust PCR primers are able to recognize tumour specific methylation. Four different primer pairs were assessed, three of which amplify probes that partially overlap. In all four cases these regions are completely methylated at all CpGs (not including CpGs in the primers) and are essentially completely unmethylated in the normal lines. CHST11 primers do not recognize the Her2 or TNBC lines, but other primers such as ADCY and MIRD do. The corresponding probes cover a small region of the CpG island and information about the status of the rest of the CpG island is limited due to the relatively coarse resolution of the 450K methylation data. Clearly the remaining part of the CpG island can be developed for additional probes that would increase the sensitivity of detection.

[0196] **Fig. 7** shows that FOXA probe A had similar characteristics and recognized all but one TNBC tumour. This proves that the target and probe development pipeline moving from TCGA data to cell lines and then to patient normal and tumour tissue successfully identified primer pairs that are able to specifically recognize tumour DNA based on their methylation patterns.

[0197] Validation work continues to validate potential probe regions. A further 24 regions were characterized using 52 different probes in the cell lines as an initial screen for their suitability.

[0198] **Fig. 4** shows the results of analysis of all of the potential CpGs identified in the TCGA cohort for individual patients indicates most patients are recognized by a large proportion of these probes.

[0199] **Fig. 3** shows the results of ROC analysis[25] and indicates each of these probes has a very high AUC, suggesting excellent performance individually and presumably even better when combined.

[0200] It has been noted that there does appear to be a population of patients with relatively few positive probes. This is not subtype specific and other probes specific for this population have been identified. As appropriate, additional probes

will be developed for all suitable regions and expanded to include other parts of the associated CpG islands. Overall it is expected that 100-150 separate probes in the assay will provide optimal sensitivity.

[0201] **Figs. 12A and 12B** depict a numerical summary of validation data, wherein "# Reads" indicates the number of reads, and "Mean" Me indicates the mean methylation observed in results. Approximately half of the probes met the design criteria of having complete methylation of all CpG residues in the tumour samples and little or no methyation in the normal lines.

[0202] The next step in validating each of these probes was to examine their methylation patterns in actual patient tumour samples. A small cohort of patient samples was used to investigate GR methylation. From this group three ER+ tumours (one of which is positive for GR methylation), one HER2+ tumour and two TNBC tumours were chosen, as well as their corresponding normal controls. Taking the CHST11A probe as an example, Fig. 6 shows that all six of the normal breast tissue samples had either no reads due to the methylation biased amplification yielding no product or minimal methylation. In no case was there any concerted methylation signal where all CpGs were methylated. In contrast, in one TNBC and one ER+PR+ tumour a strong concordant methylation signal was seen at all six CpG sites. The other 2 ER+PR+ tumours also showed consistent methylation at four or five CpGs with their normal breast tissue controls having minimal reads with only one CpG showing any methylation.

[0203] **Figs. 13A and 13B** depict a numerical summary of generated methylation data for tumour samples for all probes tested to date. # Reads is indicative of the number of reads exported, and Mean Me is indicative of the mean methylation.

[0204] Initial proof of concept work involved mixing experiments where non-methylated and methylated DNA was mixed in increasing ratios. This demonstrated that based in the presence of multiple CpG signatures methylated DNA could easily be detected in the presence of at least a 500 fold excess of unmethylated DNA. These probes were amplified with PCR primers that were not methylation specific or biased, and the probes developed to date do incorporate a bias towards methylated DNA, which further increases the detection sensitivity. However, they do amplify non-methylated DNA (in part because primers were designed with no preference as to the location of methylation sites within the primers). This was done intentionally as it provides for a potential quantitative aspect to this assay. Some of the circulating normal DNA in blood samples is likely from the lysis of nucleated blood cells, which is why serum is preferred over plasma as a source of DNA. However the ratio of tumour to normal DNA in blood may provide some quantitation of the actual concentration of tumour DNA present in the blood, which is thought to be correlated with tumour load. Since tumour can be distinguished from normal DNA reads, the ratio between them can be used as a proxy for the tumour DNA concentration. The number of tumour specific reads per volume of blood, regardless of the number of normal reads, may also prove to be closely linked to circulating tumour DNA levels.

[0205] Optimizing this test may include multiplexing to allow all of the probes the opportunity to amplify their targets in a given sample of DNA. Through the use of limited concentrations of primers and cycles, excellent amplification of all probes was obtained within a set of 17 primer pairs. Expanding this to include all of the optimized primers is not expected to be an issue.

[0206] The test may be implemented as a blood based breast cancer detection system in patient blood samples.

[0207] Based on development and validation work to date, the assay offers significant advantages other current and developing tests based on sensitivity, specificity, and detection sensitivity.

[0208] Some potential applications of the embodiments described herein are listed below by level of detection sensitivity:

- Determining response to neo-adjuvant chemotherapy;
- Monitoring tumour load in diagnosed patients;
- Detecting residual disease post-surgery;
- Detecting relapse;
- Secondary screen after positive MRI in high risk patients;
- Direct monitoring of high risk patients; and
- Primary population screening.

[0209] The analysis of patients with active breast cancer offers the ability to assess a number of different aspects of this blood based test. Patients with locally advanced disease can be recruited preferentially, as these patients generally have larger tumours, receive neo-adjuvant therapy, are more likely to have residual disease and are at higher risk of relapse. By analysing blood samples from these patients upon diagnosis, after any neo-adjuvant treatments, pre-surgery, and at followup visits post-surgery it is possible to follow the relative tumour burden in these patients over the course of treatment. This will allow the tumour size and type to be correlated with the results of the test described herein.

[0210] Patients can be recruited in the clinic after a biopsy confirmed positive diagnosis. Blood can be drawn in conjunction with other routine blood work at diagnosis, after neo-adjuvant treatment, before surgery, within a month after surgery and every 3-6 months following that. Blood from 50 aged matched women without disease can also be collected from the community to provide control samples for the patient cohort. Relevant clinical data can be collected including

radiological assessments and/or pathology reports. In particular, the receptor status of the tumours, the size of the tumour based on both radiological assessment and examination of the excised tumour, as well as treatments and response to therapy can be correlated with the circulating DNA analysis.

[0211] The assay described herein is expected to be quantitative at different levels. At very low levels of tumour DNA, the random presence of the tumour DNA in a sample will result in a subset of individual probes being positive, with the number of positive probes increasing with greater tumour DNA levels. At higher levels of tumour DNA the number of tumour specific reads will increase, either as an absolute number or in relation to the number of normal DNA reads. As a result methylation data can be treated in three ways:

(1) As a binary outcome where each probe will be considered to be positive if it has any tumour specific methylation pattern present;
(2) An individual threshold of methylation will be established for each probe based on the minimum number of reads required to call a tumour; or
(3) Tumour specific reads per number of normal reads for each probe (or, e.g., per 100,000 total reads).

[0212] Each of these approaches may be used to carry out logistic regression on the patient and control sets. Receiver Operating Characteristic (ROC) analysis may be used to define thresholds for each probe that maximizes the sensitivity and sensitivity of the assay. The performance of the entire assay may be characterized using Area Under the Curve (AUC) analysis for overall sensitivity, specificity, classification accuracy and likelihood ratio. Pearson or Spearman correlations may be used to compare patient parameters with the test outcomes.

[0213] Changes in methylation may be important drivers of breast cancer development and that these occur very early during the process of transformation. This may explain why many of the observed methylations are common amongst different breast cancer sub-types, while some are even common to other cancers. This may mean that these changes predate the development of full malignancy and suggests that they could also have value in assessing the risk of a women developing breast cancer. It is envisaged that the assay described herein can be used to track the accumulation of risk in the form of increasing gene specific methylation levels and could be used to develop a risk assessment tool. This would be useful for the development and assessment of risk mitigation and prevention strategies.

[0214] **Table 15** lists the primers used herein for each probe.

Table 15 (1/23)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
C1orf114/ CCDC181	C1Df	1	TTGAGGTAAAGGAGATTTCGGT	chr1: 167663228 -	134
	C1Dr	2	ACATACGCCTACGCAAATTTTTA	167663361	
	C1Ef	3	TTCGGTGTTTGCGAAGGGTTA	chr1: 167663398 -	111
	+ C1Er	4	TCACAACCAACACAACGACACTT	167663508	
	C1Er	5	ACAACCAACACAACGACACTT		
	C1Ff	6	TCGGTATTTGTTTTCGCGGT	chr1: 167663245 -	112
	C1Fr	7	CGCCTACGCAAATTTTTATCGC	167663356	
	C1Gf	8	CGAGAGCGATAAAAATTTGCGT	chr1: 167663330 -	88
	C1Gr	9	ACCCTTCGCAAACACCGAAA	167663417	
	C1 eAf	10	GGTAATAGCGTGTTTTTGC	chr1:167663285-	82
	C1 eAr	11	ATATTACATTCGCCTACGCAAA	167663366	
	C1 eBf	12	TTTGTGTAALATGCGGCGGT	chr1:167663149-	118
	C1 eBr	13	CTACCGCGAAAACAAATACCGA	167663266	
	C1 eCf	14	ATTTCGGTGTTTGCGAAGGG	chr1:167663395-	112
	C1 eCr	15	ACAACCAACACAACGACACT	167663506	

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
VWC2	VWCJf	16	TTTCGGTTGTCGGGTTTGGA		
	+ VWCJf	17	TATTTCGGTTGTCGGGTTTGGA	chr7: 49783871 -	133
	VWCJr	18	CCCTCAATCGCTCATCCTCC	49784003	
	VWCKf	19	TCGTCGGTCGGTTTAGGATG	chr7: 49784151 -	129
	+ VWCKr	20	AAAACCGACGCCAAACCTACAT	49784279	
	VWCKr	21	AACCGACGCCAAACCTACAT		
	VWCLf	22	CGGAGGATGAGCGATTGAGG	chr7: 49783983 -	118
	VWCLr	23	TAACGCGCACACCGAACTAA	49784100	
	VWCMf	24	CGAGTTGGGGTCGCGATTAT	chr7: 49784021 -	150
	VWCMr	25	CATCCTAAACCGACCGACGA	49784170	
	VWCNf	26	CGACGCGTTACGGTTGTTTA	chr7: 49783849 -	125
	VWCNr	27	CCGCTTCTCCGAAACCAAAC	49783973	
	VWC2 eAf	28	TAAGGCGGGGTTTTTAGAGC	chr7:49783687-	106
	VWC2 eAr	29	TAAAAACTAACGCGCCCG	49783792	
	VWC2 eBf	30	GGTTTCGGTGTTATTCGC	chr7:49783797-	126
	VWC2 eBr	31	CTCCTCTCCGCGAAAAAAT	49783922	
	VWC2 eCf	32	CGGAGGATGAGCGATTGAGG	chr7:49783983-	118
	VWC2 eCr	33	TAACGCGCACACCGAACTAA	49784100	
	VWC2 eDf	34	TCGTCGGTCGGTTTAGGATG	chr7:49784151-	127
	VWC2 eDr	35	AACCGACGCCAAACCTACAT	49784277	
	VWC2 eEf	36	GTCGGACGCGTTTTAGTTGG	chr7:49784315-	110
	VWC2 eEr	37	TCCCTACCGACCTCAACACT	49784424	

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
	MIRBf	38	TGGTTGGGGGATTTTGAGGG	chr11: 43559089 -	141
	MIRBr	39	AAACCTCCCCGCCTACCTAT	43559229	
	MIRCF	40	GCGGACGGTTTGGAGAAATG	chr11: 43559343 -	82
	MIRCr	41	CGCGACTCAATCTCACCACT	43559424	
	MIRDf	42	GGAGGTTGGGTTTCGGGATT	chr11: 43559257 -	127
	MIRDr	43	GCGCCCCTAAACTCGTATCT	43559383	
	MIREf	44	GCGGAGTGGTGAGATTGAGT	chr11: 43559401 -	113
	MIREr	45	ACCGACTTCTTCGATTCGCC	43559513	
MIR129-2	MIRFf	46	ATAGGTAGGCGGGGAGGTTT	chr11: 43559205 - 43559343	139
	MIRFr	47	CGATCCCCCAACTCAACCC		
	MIR eAf	48	TGAGTTGGCGGTTTCGTTTG	chr11:43559004-43559125	122
	MIR eAr	49	CCCGAATCCCCTCTTATCCC		
	MIR eBf	50	CGCGATTTTGTAGTCGGGGT	chr11:43559156-43559251	96
	MIR eBr	51	TTTCCTATCGCCCCAACACC		
	MIR eCf	52	GGAGGTTGGGTTTCGGGATT	chr11:43559257-43559383	127
	MIR eCr	53	GCGCCCCTAAACTCGTATCT		
	MIR eDf	54	GATTGAGTCGCGATGGAACG	chr11:43559413-43559494	81
	MIR eDr	55	GCCGCCTTCAACCCAAAATA		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
ADCY4	ADCYFf	56	CGCGAGCGTATAGAGTACGA	chr14: 23873573 23873735	163
	ADCYFr	57	ACCCTAACCAACCCCGAAAC		
	ADCYGf	58	TAGCGTCGCGAGCGTATAGA	chr14: 23873567 - 23873754	188
	ADCYGr	59	AAAAATAACCCGACGCCCGA		
	ADCYHf	60	GGTTTCGTAGAAGAGGTTTTC	chr14: 23873642 - 23873815	174
	ADCYHr	61	CGCGAAATAATAACGACTTT		
	ADCY4 eAf	62	AGAAGAGGTTTTCGTTGGGGG	chr14:23873650-23873729	80
	ADCY4 eAr	63	ACCAACCCCGAAACTCGAAA		
	ADCY4 eBf	64	TAGGATTTGGGGTTGGTGCG	chr14:23873975-23874115	141
	ADCY4 eBr	65	AACGCAACGACGAACGTAAC		
	ADCY4 eCf	66	TGGTAGTGGGGAGATCGAGG	chr14:23874376-23874474	99
	ADCY4 eCr	67	AAACGCCCCCAACTCTAACC		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
DMBX1	DMBAf	68	GTTGCGGACGGCGTAGAT	chr1: 46723984 - 46724132	149
	DMBAr	69	ACGCTCCCCGAAACAATAACT		
	DMBBf	70	TTGTTAGTTTTGTTAGCGCGG	chr1: 46723919 - 46723993	75
	DMBBr	71	CGTCCGCAACGATTCATCATC		
	DMBCf	72	TGTTTAGGAGATGGTTCGTGGT	chr1: 46723889 - 46724003	115
	+ DMBCr	73	GCATCTACGCCGTCCGCAAC		
	DMBCr	74	ATCTACGCCGTCCGCAAC		
	DMBX1 eAf	75	TGTTTAGACGTGGGTTGGGG	chr1:46723237-46723323	87
	DMBX1 eAr	76	TCAACTCCACTCACCCCGTA		
	DMBX1 eBf	77	GAGGAGGGTGGAGAGGGTAG	chr1:46723478-46723610	133
	DMBX1 eBr	78	ATACCGCACGTACTCCCAAC		
	DMBX1 eCf	79	GGAGTGGAGAGGTAGCGGT	chr1:46723635-46723751	117
	DMBX1 eCr	80	TTCCTAACCCTCTCCGACCA		
	DMBX1 eDf	81	TTTTTGAGCGGTGAAGGGGA	chr1:46723764-46723888	125
	DMBX1 eDr	82	AATTATTAACGCGACCGCCG		
HOXA9	HOXAAf	83	GTAATAATTGGTGGTATCGGGGG	chr7: 27171666 - 27171765	100
	HOXAAr	84	TCTACTAAACGAACACGTAACGC		
	HOXABf	85	ATAATTTGGTGGTATCGGGGG	chr7: 27171669 - 27171777	109
	HOXABr	86	ACGCGTTATTATTCTACTAAACGAA		
	HOXACf	87	TGGGGTTTGTTTTAATTGTGGTT	chr7: 27171878 - 27172029	152
	+ HOXACr	88	GCGAAACCCGCGCCTTCTTAAT		
	HOXACr	89	GAAACCCGCGCCTTCTTAAT		
	HOXADf	90	GGGGAAGTATAGTTATTTAATAAGTTG	chr7: 27171688 - 27171815	128
	HOXADr	91	ACAAAACATCRAACCATTAATAA		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
	HOXA9 eAf	92	TTCGCGAAGGAGAGCGTATC	chr7:27171234-27171334	101
	HOXA9 eAr	93	CCCTACGTACACCCCCAAAC		
	HOXA9 eBf	94	CGTTTGGGGTGTACGTAGG	chr7:27171314-27171401	88
	HOXA9 eBr	95	AAACCCAATACACGCGACGA		
	HOXA9 eCf	96	TTTGTCGGGGAGGTTGGTTT	chr7:27171478-27171559	82
	HOXA9 eCr	97	TTCCTACTAAACGCCGACGC		
	HOXA9 eDf	98	TAGCGTTTGGTTCGTTCGGT	chr7: 27171611-27171733	123
	HOXA9 eDr	99	ATAAAAACGCGAACGCCGAC		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
SFRP5	SFRAf	100	GCGGGCGTTTCGATTGATTT		
	+ SFRAf	101	TTGCGGGCGTTTCGATTGATTT	chr10: 99521730 - 99521860	131
	SFRAr	102	TAAAAACCGCCCCCACTACC		
	SFRBf	103	TGTTCGGCGGTTTAGGTGTT	chr10: 99521628 - 99521751	124
	SFRBr	104	AAATCAATCGAAACGCCCGC		
	SFRCf	105	TAGTTCGGGTTTCGTCGTGC	chr10: 99521776 - 99521865	90
	+ SFRCr	106	AAAACTAAAAACCGCCCCCACT		
	SFRCr	107	AACTAAAAACCGCCCCCACT		
	SFRDF	108	GTGGGTGGTAGTTTGCGTTG	chr10: 99521713 - 99521847	135
	SFRDr	109	CACTACCTCCCCGCCTTAAA		
	SFREf	110	GCGTGCGTTTTCGGTTTTGA		
	+ SFREF	111	CGGCGTGCGTTTTCGGTTTTGA	chr10: 99521649 - 99521731	83
	SFREr	112	AACGCAAACTACCACCCACC		
	SFRP5 eAf	113	GGACGTTGGGTTGAGTTAGGA	chr10:99520910-99521018	109
	SFRP5 eAr	114	ACGACCCTACAACTCCCCTA		
	SFRP5 eBf	115	GGTGTTCGAATTGTACGGCG	chr10:99521073-99521179	107
	SFRP5 eBr	116	CTACGCGCCGCTCATAAAAA		
	SFRP5 eCf	117	GCGCGTACGGTTTCGTATAG	chr10:99521183-99521257	75
	SFRP5 eCr	118	ATACTCGCTCTTTACGCCCG		
	SFRP5 eDf	119	TAGAGCGGTAGGTCGGTAGG	chr10:99521393-99521471	79
	SFRPS eDr	120	AACAAACCGAACCGCTACAC		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
CHST11	CHSAf	121	GCGGCGTGGGAATGAATTTT		
	+ CHSAf	122	GGGCGGCGTGGGAATGAATTTT	chr12: 103376278 - 103376397	120
	CHSAr	123	CTTTCCCTCGCACCCCTAAA		
	CHSBf	124	TGCGAGGGAAAGTTTGGGTT	chr12: 103376386 - 103376508	123
	CHSBr	125	CCGCGTTACCCGAAAAACTT		
	CHSCf	126	TTTTAGGGGTGCGAGGGAAA	chr12: 103376377 - 103376462	86
	CHSCr	127	CGCAACCGAACTACTCACCC		
	CHSDf	128	GTGCGAGGGAAAGTTTGGGT	chr12: 103376385 - 103376510	126
	CHSDr	129	ACCCGCGTTACCCGAAAAA		
	CHST11 eAf	130	TTTTTTTGGTTGTCGGGTC	chr12:103375901-103376009	109
	CHST11 eAr	131	CGAAACCCGAAACACGTA		
	CHST11 eBf	132	AGAGTGGTCGGGTGTTTAGC	chr12:103376031-103376179	149
	CHST11 eBr	133	ACGTAACCCAAAAACTCGAAA		
	CHST11 eCf	134	GTCGTTTTTTAGGGGTGC	chr12:103376371-103376469	99
	CHST11 eCr	135	TAAACTTCGCAACCGAACTA		
	CHST11 eDf	136	TATTAAGTTGCGTTTGGGTC	chr12:103376781-103376889	109
	CHST11 eDr	137	AAAACCGTATCCCTACGC		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
FOXA3	FOXAf	138	CGAGGTAGGAAGTTTTGCGG	chr19: 51071936 - 51072038	103
	FOXAr	139	CGACTCCTCCCGCGAAATAA		
	FOXBf	140	CGGGGTGTTGTTGTAGGGTT	chr19: 51072158 - 51072250	93
	FOXBr	141	AATCACACCTACCCACGCC		
	FOXCf	142	TAGGGCGGTTAGGTTTGGGG	chr19: 51072076 - 51072203	128
	FOXCr	143	GACGAATAACCCCACCCTCC		
	FOXDf	144	TTGTCGCGTTGGTTTTTCGT	chr19: 51071765 - 51071867	103
	FOXDr	145	ACCTTTCTCTCGACCCCAAT		
	FOXEf	146	CGTTTTGTCGGTTGCGTGTTA	chr19: 51071734 - 51071824	91
	FOXEr	147	ATTCCCCGACCTACCCAAAAC		
	FOXA3 eAf	148	GGTAGGTGATAACGTTAGTGGGTT	chr19:51068615-51068724	110
	FOXA3 eAr	149	ACCTCCATCCCCTACCCAAC		
	FOXA3 eBf	150	AGTAGGGGGAGGTGGTTTTG	chr19:51069110-51069244	135
	FOXA3 eBr	151	TCCTCCTCCCCAACTTAACC		
	FOXA3 eCf	152	AGTTTGGGTGTGGCGGTTTA	chr19:51070046-51070156	111
	FOXA3 eCr	153	ACCAACTTCGCCATATTAACCA		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
TTBK1	TTBAf	154	CGCGGTGTATTGTGGGTAGT	chr6: 43319189 - 43319287	99
	TTBAr	155	CCTTCCGACCCGAATCATCC		
	TTBBf	156	GGTCGTCGGAACGTGATGT	chr6: 43319101 - 43319186	86
	TTBBr	157	GCCAACATCAACACCAACCC		
	TTBCf	158	TCGTTTTGTCGTTGTCGTCG	chr6: 43319212 - 43319318	107
	TTBCr	159	TTAAATAACCCGCTCCCTCCG		
	TTBDf	160	GTCGTGATGTTAGAGCGGGC	chr6: 43319130 - 43319255	126
	TTBDr	161	ACCCCGATCCTCCTTAAACG		
	TTBK1 eAf	162	TTAAGGAGGATCGGGGTC	chr6:43319239-43319329	91
	TTBK1 eAr	163	TCAATACGACGTTAAATAACCC		
	TTBK1 eBf	164	TGGAGTTAAGCGGGTGGTAG	chr6:43319008-43319148	141
	TTBK1 eBr	165	CCCGCTCTAACATCACGACTC		
TAL1	pbTAL f	166	GTATTGTCGCGGGTTCGTTC	chr1: 47470631 - 47470738	129
	pbTALr	167	CTCAACCAATCCCCACTCCC		
	mbTAL f	168	GTTTTAGGTTTCGTTAGTATGGG	chr1: 47470570 - 47470698	129
	+ mbTAL r	169	CAAATTAAAATAAATCATTTAACCCATAA		
	mbTAL r	170	TTAAAATAAATCATTTAACCCATAA		
DMRTA2	pbDMRTA f	171	CGAAGATTTCGTAGGCGGGT	chr1: 50659325 - 50659469	145
	+ pbDMRTA r	172	ACGACGCAAATAACGCTACGCA		
	pbDMRTA r	173	GACGCAAATAACGCTACGCA		
	mbDMRTA f	174	TGTTTTAGAAGCGGGAGAAAG		
	mbDMRTA r	175	AAATAAAACCCCCGTATCCAAT		
	+ mbDMRTA f	176	AATGTTTTAGAAGCGGGAGAAAG	chr1: 50659041 - 50659153	113
	+ mbDMRTA r	177	AAAAATAAAACCCCCGTATCCAAT		
	DMRTAexp Af	178	GCGGCGGTTAGCGTTAGTTTTTCGGTAG	chr1: 50659366 - 50659489	124
	DMRTAexp Ar	179	CGAAACGCCAACGTATCATAACGACGCA		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
PDE4B	pbPDE f	180	ACGTTTTAGGGACGGCGAAT	chr1: 66030622 - 66030698	77
	pbPDE r	181	AATCCCAACGACCGTCTACC		
	mbPDE f	182	TTTCGTTTTGTATTTATGGTAGATGT	chr1: 66030580 - 66030694	115
	mbPDE r	183	CCAACGACCGTCTACCACTA		
BARHL2	pbBARHL f	184	CGTGGTATGGATTTCGGGGT	chr1: 90967266 - 90967376	111
	pbBARHL r	185	ACTCCTAACCCTAAACGCGA		
	mbBARHL f	186	GTTTTTTTCGGTTTTTGTTCGA		
	mbBARHL r	187	TTTCTCCCAATTCCAATATCCA		
	+ mbBARHL f	188	TGGTTTTTTCGGTTTTTGTTCGA	chr1: 90967815 - 90967900	86
	+ mbBARHL r	189	ACTTTCTCCCAATTCCAATATCCA		
TBX15	pbTBX f	190	GCGATCGGCGATTGGTTTTT	chr1: 119331668 - 119331767	100
	pbTBX r	191	GCGACGACACACGACCTAAA		
	mbTBX f	192	TGAGGTTTTAGGTCGTGTGT		
	+ mbTBX f	193	GGTGAGGTTTTAGGTCGTGTGT	chr1: 119331740 - 119331881	142
	mbTBX r	194	AAAACCTTAATCGACTCAAATAAAA		
RUSC1,C1orf104	pbRUSC f	195	GGGTGTAGTTGCGTAGCGTA	chr1: 153557280 - 153557421	142
	pbRUSC r	196	CCGAACCCTCCTCACCAAAA		
	mbRUSC f	197	TAGTTGCGTAGCGTAGGGTA	chr1: 153557285 - 153557410	126
	mbRUSC r	198	TCACCAAAATCCTCCTAAAAC		
GNG4 B	pbGNG f	199	ACGTAGTGTTGGTAAGATTTGTAGA	chr1: 233880823 - 233880971	149
	pbGNG r	200	ACAAAAACCGCTTATAAACGACGA		
	mbGNG f	201	GTAGGTTTTUGCGTIGGAGATT	chr1: 233880677 - 233880817	141
	mbGNG r	202	ATTTTCGTTACTTCTICTATTCCCAAA		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
POU3F3	pbPOU3F f	203	GGGGTTTCGCGTTTTGAGTT	chr2: 104836866 - 104836944	79
	pbPOU3F r	204	AACACCAAAACCCCCGCTAA		
	mbPOU3F f	205	AAAAGTAATTAATCGGAACGGT	chr2: 104836837 - 104836970	134
	mbPOU3F r	206	ACACTTTCCCAAATACAAAAAAA		
BOLL B/C	pbBOLL f	207	TTTCGAGTCGGGGCGTTTTA	chr2: 198359264 - 198359401	138
	pbBOLL r	208	TACCTAACCGCTCGCTCTCT		
	mbBOLL f	209	GTTCGGTTTTGGGATTTTT		
	mbBOLL r	210	AATCCCAAAAACCGACTCT		
	+ mbBOLL f	211	GAGGGTTCGGTTTTGGGATTTTT	chr2: 198359331 - 198359461	131
	+ mbBOLL r	212	ACCAATCCCAAAAACCGACTCT		
TRIM71	pbTRIM f	213	CGGAGGAATUTGTGTCGTCG	chr3: 32834331 - 32834440	110
	pbTRIM r	214	CACCAAAACAACGCTACCCG		
	mbTRIM Af	215	TTGGGAATTTTTTCGTTTAT	chr3: 32834188 - 32834337	150
	mbTRIM Ar	216	TCCTCCGAATAACTTAAAAACC		
	mbTRIM Bf	217	TCGTTGGATAGTGGTATTTAATGT	chr3: 32834348 - 32834497	150
	mbTRIM Br	218	AAAATCACCGACTCACTCAA		
SLC2A2	pbSLC f	219	CGGAGTACGC;CGGTAGGAA	chr3: 172228914 - 172228993	80
	+ pbSLC r	220	AATACCCCGAAAACCCGCTAATA		
	pbSLC r	221	ACCCCGAAAACCCGCTAATA		
	mbSLC f	222	ATGATATTTTGTAGGAAAGCGT	chr3: 172228748 - 172228850	103
	mbSLC r	223	CAAATTCCGTTTCTAAAAAAAC		
CYTL1	pbCYTL f	224	GGGTTCGTATGCGGGAGTAG	chr4: 5071974 - 5072099	126
	pbCYTL r	225	ACGAAACTACACCAACGCCT		
	mbCYTL f	226	GGGGGTTTTCGTTAGGAGTAG	chr4: 5072020 - 5072142	123
	mbCYTL r	227	AAACCGCCCTAAACCACC		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
SHISA3	pbSHISA f	228	GAAGGGCGGTAGCGATAGTT	chr4: 42094543 - 42094650	108
	+ pbSHISA r	229	CTACGAATTCCGCAAACCGAAA		
	pbSHISA r	230	ACGAATTCCGCAAACCGAAA		
	mbSHISA f	231	ATTGTTTTTGTCGGCGTT	chr4: 42094569 - 42094654	86
	mbSHISA r	232	TACACTACGAATTCCGCAA		
GABRA4	pbGAB f	233	GCGTGCGTATATTCGCGTTT		
	+ pbGAB f	234	CGGCGTGCGTATATTCGCGTTT	chr4: 46690291 - 46690385	95
	pbGAB r	235	AAATTCCGCCTCCCCTAACC		
	mbGAB Af	236	TTTAGCGTTTAATGTGTATGTAGA	chr4: 46690411 - 46690545	135
	+ mbGAB Ar	237	CGAAATTACTATCGAAACAAACTTAC		
	mbGAB Ar	238	AAATTACAATCGAAACAAACTTAC		
	mbGAB Bf	239	GTTTTGAGTAGGGTGCGAG		
	mbGAB Br	240	AAAAAAACAAAATTCCGCCT		
	+ mbGAB Bf	241	GATGTTTTGAGTAGGGTGCGAG	chr4: 46690248 - 46690398	151
	+ mbGAB Br	242	AAACGAAAAAAACAAATTCCGCCT		
EGFLAM	pbEGF f	243	TGGTAGCGTTGTAAGGTGGG	chr5: 38293231 - 38293359	129
	pbEGF r	244	AAAAACAAACGCGACCCTCG		
	mbEGF f	245	TCGAGTTTTGGTAGCGTTGTAA	chr5: 38293223 - 38293306	84
	+ mbEGF r	246	AATACCCCGCAAAAAAAATCTACA		
	mbEGF r	247	CCCCGCAAAAAAAATCTACA		
C5orf39	pbC5orf f	248	ACGAGAAATTGGCGCGTTGA	chr5: 43076304 - 43076404	101
	pbC5orf r	249	AACAACACCCTTTACGACGC		
	mbC5orf f	250	TGTTTGTTAGGGTTTTGTTTTAA		
	mbC5orf r	251	CGCCAAAACGAATATTTATTTA		
	+ mbC5orf f	252	AATTGTTTGTTAGGGTTTTGTTTTAA	chr5: 43076267 - 43076390	124
	+ mbC5orf r	253	CGACGCCAAAACGAATATTTATTTA		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
CDO1 B	pbCDO f	254	GGTAGCGTAGTGGATTCGGG	chr5: 115180192 - 115180333	142
	pbCDO r	255	CTCGTCCTCCCTCCGAAAAC		
	mbCDO f	256	GTTTGTTTTATTTCGTGGGGAG	chr5: 115179983 - 115180067	85
	mbCDO r	257	CCAACTCCTTAACTCGCTCAA		
IRF4 B/C	pbIRF f	258	TCGCGGGAAACGGTTTTAGT		
	pbIRF r	259	GCCCTTAACGACCCTCCG		
	+ pbIRF f	260	TTTTCGCGGGAAACGGTTTTAGT	chr6: 336451 - 336550	100
	+ pbIRF r	261	GCGCCCTTAACGACCCTCCG		
	mbIRF f	262	CGTTTTGTAAAGCGAAGTTT		
	+ mbIRF f	263	GTTATACGTTTTGTAAAGCGAAGTTT	chr6: 336298 - 336405	108
	mbIRF r	264	AAACCAATCAATCACTAAACTACA		
ID4 B	pbID Af	265	GGTTTTTGGGCGTCGTGTTA	chr6: 19945064 - 19945170	107
	pbID Ar	266	AAATTCACTCTCCACCGCCC		
	pbID Bf	267	AGGCGAATAATGAAACGGAGGA	chr6: 19944950 - 19945083	134
	pbID Br	268	TAACACGACGCCCAAAAACC		
	mbID f	269	ATTTTACGGATGGAGTGATG		
	+ mbID f	270	GGAATTTTACGGATGGAGTGATG	chr6: 19945031 - 19945148	118
	mbID r	271	CTTATCCCGACTAAACTACTAAAAAA		
SCAND3, GPX5	pbSCAND f	272	AATTCGTTTCGCGACGTGAG		
	+ pbSCAND f	273	TTAATTCGTTTCGCGACGTGAG	chr6: 28618249 - 28618359	111
	pbSCAND r	274	ACACGCCTTAAAACCTACTCAT		
	mbSCAND f	275	CGTGAGGGAGAATTTAGGAG	chr6: 28618265 - 28618368	104
	mbSCAND r	276	TAAAAAAACACACGCCTTAAAACCTA		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
DDAH2	pbDDAH f	277	TCGTTTAGCGAGCGTTGTTT	chr6: 31806112 - 31806210	99
	pbDDAH r	278	GATCCGCCGTTACGCTATTC		
	mbDDAH f	279	TGTTAGAAATCGGTATCGTTTA		
	mbDDAH r	280	TCTACGAAACGTTTACAACC		
	+ mbDDAH f	281	TTTTTITGTTAGAAATCGGTATCGTTTA	chr6: 31806097 - 31806189	97
	+ mbDDAH r	282	AAAATCTACGAAACGTTTACAACC		
COL11A2	pbCOL f	283	TTTAGGGATCGCGTTCGGAG	chr6: 33269259 - 33269402	144
	pbCOL r	284	AAACTCCTTTCCCTCTCATAC		
	mbCOL f	285	CGGAGTTTTTAATCGGATAT	chr6: 33269274 - 33269415	142
	mbCOL r	286	TCCCTTCTCTTTAAAACTCCT		
NT5E B	mbNT5E f	287	GTCGGATTTTATTTTAATCGTG		
	mbNTSE r	288	AAACAAAAAAATCTCAAAAACTAAAA		
	+ mbNTSE f	289	GTTGTCGGATTTTATTTTAATCGTG	chr6: 86215769 - 86215912	144
	+ mbNTSE r	290	CTTAAACAAAAAAATCTCAAAAACTAAAA		
SIM1 B	pbSIM Af	291	GTTAGGGGCGAGGCGTTTAT	chr6: 101019614 - 101019695	82
	pbSIM Ar	292	CGAAACCTAAACGCGCGAAA		
	pbSIM Bf	293	AGGTTAATAGGTGGCGCGTT	chr6: 101019077 - 101019171	95
	pbSIM Br	294	CCCGCAACTCCGCGATAATA		
	pbSIM Cf	295	AGTCGTTTTTCGCGCGTTTA		
	+ pbSIM Cf	296	CGAGTCGTTTTTCGCGCGTTTA	chr6: 101019667 - 101019756	90
	pbSIM Cr	297	GACCCGACACCCTAAACTCAT		
	mbSIM Af	298	AGGCGTTTATTGGTTAATAGGG	chr6: 101019624 - 101019757	134
	+ mbSIM Ar	299	CGACCCGACACCCTAAACTCAT		
	mbSIM Ar	300	ACCCGACACCCTAAACTCAT		
	mbSIM Bf	301	TTTAATTTGGGTTTTAAGTTTGAGG	chr6: 101018944 - 101019075	132
	mbSIM Br	302	ACGCTACTAAACCCCGCTTAT		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
RGS17	RGS17 Af	303	GCGTTTAGGTGCGACGC	chr6: 153493700 - 153493820	121
	RGS17 Ar	304	ATACCCCGACGAAAACGAC		
	RGS17 Bf	305	TTTGGGATTTGGTCGAGC	chr6: 153493620 - 153493730	111
	RGS17 Br	306	AAAATTAAATCCCGCGTCG		
CAPDS2	CAPDS Af	307	CGTTTAGGTTTGTGGACGC	chr7: 121743823 - 121743951	129
	CAPDS Ar	308	AAAAACGAAATCGCTAATACGC		
MSC	MSC Af	309	TTTTTCGAATTTTGCGC		
	MSC Ar	310	AACACGCTCCGACTAACTTC		
	+ MSC Af	311	GGTTGTTTTTCGAATTTTTGCGC +	chr8: 72918397 - 72918531	135
	+ MSC Ar	312	TAAACACGC'-'CCGACTAACTTC		
	MSC Bf	313	CGTTCGCGTTATTATTTGC		
	MSC Br	314	CGCCCAATAACAACTCGT		
	+ MSC Bf	315	ATTATCGTTCGCGTTATTATTTGC	chr8: 72918698 - 72918852	155
	+ MSC Br	316	CCTCGCCCAATAACAACTCGT		
SPAG6	SPAG6 Af	317	GTCGAGTCGCCTTACGATC	chr10: 22674453 - 22674529	77
	SPAG6 Ar	318	CTACCCTCCTCGAACTCTACG		
INA	INA Af	319	GTTTTCGGAGGGAAATTTTAG		
	INA Ar	320	AAACCATCTACATCGAAATCGC		
	+ INA Af	321	GTGGTTTTCGGATGGGAAATTTTAG	chr10: 105026593 - 105026715	123
	+ INA Ar	322	AACAAAACCATCTACATCGAAATCGC		
FLI	FLI Af	323	TTTTTAGGAGTAAGTATTTTGTGTG	chr11: 128068870-128068981	112
	FLI Ar	324	CCCTCTTCCCCCCTACTAAT		
ATPSG2	ATP5G2 Af	325	TAGGTATATTTCGGTCGGC	chr12: 52357363 - 52357478	116
	ATP5G2 Ar	326	AACTCGAAACCTCATCCG		
USP44	USP44 Af	327	ACGGGAGGGTAAATTTAGC	chr12: 94466977 - 94467090	114
	USP44 Ar	328	TACCAAACAATTCGACGTTA		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
POU4F1	POU4F1 Af	329	GCGTACGTCGGTTTATTC		
	POU4F1 Ar	330	ACGCTCTACGCGATCAAA		
	+ POU4F1 Af	331	AAGTGCGTACGTCGGTTTATTC	chr13: 78075512 - 78075652	141
	+ POU4F1 Ar	332	GCGACGCTCTACGCGATCAAA		
LHX1	LHX Af	333	CGAGCGATTGTGGGGTTAGA	chr17: 32368543 - 32368624	82
	LHX Ar	334	CAACTCGCGACCGCCTAAA		
HINF1B	HINF Af	335	TTCGGGCGTTTATAGAGTTC	chr17: 33176898 - 33177017	120
	HINF Ar	336	AAAATCAAAACGCGAACG		
	HINF Bf	337	TAGCGTCGCGTTAGAAAGC		
	HINF Br	338	ATCGCTCAANACCTAACGAA		
	+ HINF Bf	339	TTTTAGCGTCGCGTTAGAAAGC	chr17: 33177225-33177341	117
	+ HINF Br	340	AAAAATCGCTCAAAACCTAACGAA		
	HINF Cf	341	AGGTTTAGTTTCGAAATCGC		
	HINF Cr	342	AACCGAACGATCCCTAA		
	+ HINF Cf	343	GTTAAGGTTTAGTTTCGAAATCGC	chr17: 33177654 - 33177773	120
	+ HINF Cr	344	CTAAAAAACCGAACGATTCCCTAA		
GALR1	GALR1 Af	345	GAATTTTTGGAAAAGTCGGGA		
	GALR1 Ar	346	CTCCTACAAAAAAAACTCCC		
	+ GALR1 Af	347	TTCGGAATTTTTGGAAAAGTCGGGA	chr18: 73090886 - 73090989	104
	+ GALR1 Ar	348	CGACTCCTACAAAAAAAACTCCC		
MAST1	MAST1 Af	349	AGAAGGTGGCGGTAAGC		
	MAST1 Ar	350	ACGTAATTAAAAAAACACGCC		
	+ MAST1 Af	351	GGAGAAGGTGGTCGGTAAGC	chr19: 12839386 - 12839533	148
	+ MAST1 Ar	352	AAAACGTAATTAATTATAAAAAACACGCC		
	MAST1 Bf	353	TAGTTTTTTGGAGGCAGAGG	chr19: 12839568 - 12839670	103
	MAST1 Br	354	ATCCTCGTCCTCTTAAAAAAC		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
CPXM1	CPXM1 Af	355	GTCGAGTTTGGGATTTTGGT		
	CPXM1 Ar	356	AAACTCCTACTCGCCCTAACC		
	+ CPXM1 Af	357	GGGGTCGAGTTTGGGATTTTGGT	chr20: 2729097 - 2729214	118
	+ CPXM1 Ar	358	AAAAACTCCTACTCGCCCTAACC		
NEURL2	NEURL2 Af	359	TCGAGTTGGATAAGGCGTAC	chr20: 43952304 - 43952445	142
	NEURL2 Ar	360	CCGATAACACGACCGACATA		
	NEURL2 Bf	361	TGTATGTCGGTCGTGTTATC	chr20: 43952424 - 43952505	82
	NEURL2 Br	362	TAAACGTACTACCTCCGACC		
ACVRL1	ACVRL1f	363	GGATGTGGGHGGTTCGGTTCGGGTG	chr12:50587308-50587443	136
	ACVRL1r	364	CCGCTCGCCCCTCGCTAAAACTACA		
AFF3	AFF3f	365	GGCGCGAGGTAGTTTTAGTACGTAGTTTTT	chr2:99542180-99542257	78
	AFF3r	366	ATAACAACGTCGTCCTTTCCGCAAAACG		
	AKR1B1f	367	GGGGATTTTGTAAGTTCGCGCGTGGTTT	chr7:133794143-133794250	108
	AKR1B1r	368	ACACTCTCCGCGCGACCTATATTAACGA		
AKR1B1	AKR1B1R_f	369	GGAGACGGTTTGTTATGGTTGTTGCGTT	chr15:43266838-43266959	122
	AKR1B1R_r	370	ACGCCCTTTCTACCGACCTCACGAACTA		
ALDOC	ALDOCf	371	TTTTTCGGGGGCGTGGTTTGTATGTTT	chr17:23928071-23928193	123
	ALDOCr	372	TACCTAACGAAACGCTCACTCCACCTCG		
ALOX5	ALOX5f	373	TTTTGCGGTAGGTGAAGGCGTAGAGGT	chr10:45234654-45234759	106
	ALOX5r	374	GACCGAATACCCCGCTTTCTCTCTCGAC		
	ALOX5R_f	375	GAGGTCGAGAGAGAAAGCGGGGTATTCG	chr10:45234729-45234838	110
	ALOX5R_r	376	AACGCTCTCAACCCAACCCCTAAACTCA		
ALX1	ALX1f	377	AGGATAGTAGCGGTGAGTCGTTAGCGTT	chr12:84198385-84198501	117
	ALX1r	378	CGCTCCCACTTTTCTCCTTTCTCCCTCC		
ALX4	ALX4f	379	TTTTGATAAAGTGGGGAGGGCGTAGGGG	chr11:44289270-44289375	106
	ALX4r	380	ACACTCTCAAATACCCGTCGCGCTCTAT		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
C1orf230	C1orf230f	381	TTTTGATAAAGTGGGGAGGGCGTAGGGG	chr1:149960830-149960921	92
	C1orf230r	382	ACACTCTCAAATACCCGTCGCGCTCTAT		
	C1orf230R_f	383	AGCGTAGCGAGTTGGAGTAGTTGCGAA	chr1:149960685-149960805	121
	C1orf230R_r	384	CGACGACTCTCTTCCCAATCTAAAACCCCA		
C6orf186	C6orf186f	385	CGGAGTTTAGAAGGGCGTTCGGTTACGG	chr6:110785585-110785700	116
	C6orf186r	386	CTCCACGAATCGCATCTTTCAATACCCA		
C17orf64	C17orf64f	387	AAAGGTGGTTCGAGTGAGGAAATTGCGG	chr17:55853711-55853789	79
	C17orf64r	388	GCGTCCCTAAACGACACACGACGAAATC		
	C17orf64R_f	389	GTCGACGGCGGTTTTATCGTATTGTCGC	chr17:55853578-55853689	112
	C17orf64R_r	390	CCTTCTCCCGAACCTTCCTTCGTATCCT		
C19orf41	C19orf41f	391	TTAGAGGTATGGCGGGGTTTTTGTGACG	chr19:55358254-55358348	95
	C19orf41r	392	AATACTCCCTAAACCTCCTAACCGCGCC		
CCDC67	CCDC67f	393	GAGGTTTAATGTTTCGTTGGTCGC	chr11:92703424-92703546	123
	CCDC67r	394	ACGCAAAACCGCGTATATCACCT		
CCDC8	CCDC8f	395	GGTTTTAGGGACGCGGTTGGAATTTGGG	chr19:51608460-51608548	89
	CCDC8r	396	CCCAACGCCTCGACCATATTAAATAACTT		
CD38	CD38f	397	GCGATTAAGGCGTATCGGTGGGTATTGC	chr4:15389377-15389501	125
	CD38r	398	AACACCACCCGACGAACTCTCGACTAAC		
CD8A	CD8Af	399	TAGGACGTTGTTTGGTTCGAAGTTCGGG	chr2:86871471-86871569	99
	CD8Ar	400	CTCCGAACCGACCGAAAAACGCAACTTT		
CDH23	CDH23f	401	GGCGGGGTATTGTTTTGTTTC	chr10:72826313-72826423	111
	CDH23r	402	TCTACCGATATCATAACACCGACT		
CDK5R2	CDK5R2f	403	AAAGGTAGAGGGAAGGAGAGTTGTTTTT	chr2:219532251-219532354	104
	CDK5R2r	404	ACTCCTACCTCCTCCGAATCCTAAAACCT		
CHST2	CHST2f	405	CGGAATGAAG GTGTTTCGTAGGAAGGCG	chr3:144322486-144322636	151
	CHST2r	406	GCTACGACACCCAACGACCCATCGAAA		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
CLCN1	CLCN1f	407	AATGATTTTGTTGGGTTCGGTGGAGCGG	chr7:142752740-142752852	113
	CLCN1r	408	CCGACAACTCCGCGCCATCTCTTAAAC		
	CLCN1R_f	409	TTGTGTTTTGAGCGTAGGTTGCGCGTAG	chr7:142752798-142752874	77
	CLCN1R_r	410	GCCTTCCCGTCGTAAAACAACTCCGACA		
COL16A1	COL16A1f	411	GTTTTAGGGGGTTGGGGGTTTGTTAGGGA	chr1:31942237-31942382	146
	COL16A1r	412	AACCCGAAACGAAACTATACACCCCGCA		
CPNE8	CPNE8f	413	TCGATGTTCGTAGTGTTGTTGTAGCGGT	chr12:37585569-37585689	121
	CPNE8r	414	CCATCCCCGUCTAACGAAAACTAACCCT		
DIO3	DIO3f	415	CGTTTCGAGAAGAAGTTTCGCGGTTGGT	chr14:101095917-101096005	89
	DIO3r	416	ATCTAAACCCAAATCGAAAACCGCCGCC		
DNM3	DNM3f	417	TTGGAGTTGTCGTAGATCGTCGTGGTGG	chr1:170077504-170077626	123
	DNM3r	418	AAATCGCCCCACTACCGCATCCTTACTC		
	DNM3R_f	419	GCGGTTAGGTGTGGTAAAGTAGTTGGCG	chr1:170077283-170077405	123
	DNM3R_r	420	GCGCACAACCAACCTATAAACTCCGACG		
DUOX1	DUOX1f	421	GGGATTTIGTGAAGGCGGATTTG	chr15:43209229-43209307	79
	DUOX1r	422	AATATTCCG CGATACCGAAAACCCGA		
EMX1	EMX1f	423	CGGTTGGAGCGCGTTTTCGAGAAGAAT	chr2:73005041-73005163	123
	EMX1r	424	AACGCAAAACAAACCGCGACCGAAAATA		
EMX2OS	EMX2OSf	425	AGGAGAAGTCGTAGCGGGCGTC	chr10:119291932-119292032	101
	EMX2OSr	426	GACTAAACCTTCTACCGCCCACCG		
ESPN	ESPNf	427	TAGTTGCGACGGGGTGGGAAGTTACGTT	chr1:6430246-6430357	112
	ESPNr	428	AAAACCATCGCCATCCACGAAAACGACA		
EVX1	EVX1f	429	AGGAGGATGATAGTTTAGAAAGAAGAGGGT	chr7:27248900-27249019	120
	EVX1r	430	CGCGACCGCGACGATAACGATAAAAACT		
FABPS	FABP5f	431	GAAACGTGTAGGCGTCGGCGTTTATGAG	chr8:82355078-82355157	80
	FABP5r	432	CGACCTCTCGAACGCCTCCTACAAACAA		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
FBRSL1	FBRSL1f	433	GTGGAGGAGGAAGTTCGTTTC	chr12:131575948-131576052	105
	FBRSL1r	434	AACTACTACCAAACACGAAACGCA		
FLI41350	FLIf	435	GGTTAGAGTCGGTTGCGTAGTTT	chr10:102979731-102979855	125
	FLIr	436	TTTTTGTTAGGCGAAGTATAGAGAGCG		
FOXG1	FOXG1f	437	TTTTTCGATGGTCGACGGCGAGAGAG	chr14:28305617-2.8305740	124
	FOXG1r	438	TTTCCGAACTACAAACGCACACTAAAAC		
FOXL2	FOXL2f	439	GATTCGTATGGGTTTTATCGAGTTTC	chr3:140148670-140148764	95
	FOXL2r	440	ACTTAAAAATAAACTCGCCCGTACG		
FZD2	FZD2f	441	TCGTTGGTGAAGGTGTAGTGTTCGTTCG	chr17:39990814-39990938	125
	FZD2r	442	TAACGCGCGCGCTCACAAATAAAACGAC		
	FZD2R_f	443	TTTTTAGTGGTTCGAGCCGTTTGCGTTGC	chr17:39990969-39991059	91
	FZD2R_r	444	TCCGTCCTCGAAATAATTCTAACCGACGC		
HIF3A	HIF3Af	445	CGTGGTATAGTTAATCGCGCGGCGT	chr19:51492066-51492190	125
	HIF3Ar	446	TACAACCCCAACGCCATAACTCGCCAAT		
HIVEP3	HIVEP3f	447	TGTCGTCGTCGTCGGGGTTTTGTTATTT	chr1 :41901039-41901114	76
	HIVEP3r	448	ACGACGATAAACTCCCGCTAAACCCGAA		
	HIVEP3R_f	449	GAACGAGGATTTGCGTTTTTGGATCGC	chr1:41901096-41901175	80
	HIVEP3R_r	450	CCTAAACTCCTCTACATATTCCTCTACCT		
HLA-F	HLA-Ff	451	GAATGGTTGCGATATGGGGTTCGACGG	chr6:946778-946902	125
	HLA-Fr	452	CCACGATATCCGCCGCGATCCAAAAAC		
HOTAIR	HOTAIRf	453	TAAGGGTCGGTTGTTGTTTTTTTTC	chr12:52645919-52646034	116
	HOTAIRr	454	ACCGACGCCTTCCTTATAAAATACG		
HOXA10	HOXA10f	455	TGTGGGATAATTTGGCGAAGGGAGTAGA	chr7:27180403-27180526	124
	HOXA10r	456	AACTCGAAATTAACTACGAACGCCCGCC		
HOXD11	HOXD11f	457	GGCGGGGGTAGTTTTTGTATTAAGGCGA	chr2:176680987-176681111	125
	HOXD11r	458	CCTACGCTACTACTCTTCTCGACCCCCG		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
	HOXD8f	459	CGTTTCGTTCGTCGGTCGTAGCGATTG	chr2:176702636-176702749	114
	HOXD8r	460	CCGACGAAACATTTTCGCACCACAACAC		
HOXD8	HOXD8R_f	461	CGCGGTTTCGGGGTATACGGAGTTTTTG	chr2:176702549-176702668	120
	HOXD8R_r	462	GCAATTCAATCGCTACGACCGACGAACG		
HSPA12B	HSPA12Bf	463	CGTCGTAGCGGGTACGGTTAACGAGTTG	chr20:3661361-3661485	125
	HSPA12Br	464	TTTCTCCACCCGAAACGCCCGACAACC		
ISL1	ISL1f	465	CGGGGGAGAACGGTTTGAGTTTCGAGTA	chr5:50714776-50714885	110
	ISL1r	466	TCATATTTCAACCTCGCCGCCGCTAAAC		
Intergenic 1	Int1f	467	AGTAGGGATCGTCGTTCGTTGTTCGGTG	chr11:68379573-68379679	107
	Int1r	468	GACAAACGACCGAAAATACTCGCGCAAC		
	Int1R_f	469	TTTTACGGTCGGGGCGATAGTTGAAGGT	chr11:68379395-68379493	99
	Int1R_r	470	TCACGCCAATACCCGCTAATCCCTCCTA		
Intergenic 2	Int2f	471	GGGGATGGATAATTTTTAGGCGTTAAC	chr17:69460223-69460339	117
	Int2r	472	TAACCTCGTCTTTATCCCCGCG		
Intergenic 3	Int3f	473	AGTGTGTAGTCGTTTGTGGGTGAGGAGTT	chr8:95315865-95315994	130
	Int3r	474	CACCGCGAAAAACGCCCACAATCTTACC		
	Int3R_f	475	CGCGGGGGAGTTTATTTTTGAGGATTCGG	chr8:95315775-95315892	118
	Int3R_r	476	ACTCCTCACCCACAAACGACTACACACT		
Intergenic 4	Int4f	477	TAGTATTTGTACGGAGTTTTTCGGCGGTC	chr5:43054172-43054263	92
	Int4r	478	TACGACGCAACCAACGATACTATCACCAA		
Intergenic 5	Int5f	479	TAGTGATTGGTTATTTGGGCGCGGGGC	chr10:43138416-43138530	115
	Int5r	480	AAACGACATCCATCATCTCCCTCGACCC		
Intergenic 6	Int6f	481	AGGTCGCGTTTTGGTCGTGC	chr3:14827613-14827688	76
	Int6r	482	ACTTAAAAATAAACTCGCCCGTACG		
Intergenic 7	Int7f	483	ATTTTACGTAGGGTGGGGTTGAGGGCGT	chr12:52897799-52897910	112
	Int7r	484	ATCCTAACCGTCCCGCCTCAAAACCGTA		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
Intergenic 8	Int8f	485	CGTCGTAGTATTTGGCGGCGCGTTTC	chr2:236737778-236737883	106
	Int8r	486	AACGTACCTAATCCCCAAACCCACTCCT		
Intergenic 9	Int9f	487	TCGTTGTGCGCGTTTCGTTTGTTGGATTA	chr6:778755-778846	92
	Int9r	488	TCGATAATATCTCCGTCGCCTCCGCAAA		
Intergenic 10	Int10f	489	GCGCGTTTAATCGTGGGATTTTTGGGAG	chr2:174899379-174899494	116
	Int10r	490	CAAATTCGCGACACCCTACCCCAACAC		
	Int10R_f	491	GGGTGTCGCGAATTTGGGGTA	chr2:174899479-174899602	124
	Int10R_r	492	CTAAACCTCCCCCCTCCCAAATTTACCT		
Intergenic 12	Int12f	493	ATCGAGTTTTTAGCGGTTTTTGGGGCGG	chr1:119344866-119344974	109
	Int12r	494	ACTAACATCGCGCACTTAAATCTTTCCG		
Intergenic 13	Int13f	495	GGTAGCGGCGGGTAAAAAGTC	chr7:64675119-64675225	107
	Int13r	496	TACAACTTTTTACCTCCGCCGC		
Intergenic 14	Int14f	497	CGTCGATTTGCGGAATTTCGTCGTCGTT	chr1:238227938-238228045	108
	Int14r	498	ACATCCGCGTAAACTCGCCCTTTAACAC		
	Int14R_f	499	TTTCGGGATTAGGGTTTCGGAGGGTGTC	chr1:238227822-238227913	92
	Int14R_r	500	CGTATCGATCCGTCCCTCCCGCTTAAAA		
Intergenic 15	int15f	501	CGGTTTTGGTGGTAGTTTTGGTAATC	chr19:48895723-48895802	80
	Int15r	502	AAAACCTCCCGAACGACGAAATAATCCA		
	Int15R_f	503	GTAGGCGGTCGGAACGTGAAC	chr19:48895536-48895660	125
	Int15R_r	504	CGATAAAAACTACAATAACTCGACAACCA		
Intergenic 16	Int16f	505	GTTGTGAGGGTTTTCGGCGGTATC	chr1:54713046-54713165	120
	Int16r	506	CATAACAACGCGCGACCCCTA		
Intergenic 17	Int17f	507	TGATTATAAATTAGGGGGTTTGGTCGTCG	chr12:61311832-61311945	114
	Int17r	508	AAACCCTCCACCCTCGCAATACTACTCC		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
Intergenic 18	Int18f	509	TGTAGGAGATAATGGGAGTGAAGAGGGA	chr6:4971256-4971338	83
	Int18r	510	TTCCACGAAACGCGCGACTTCCTAACTA		
	Int18R_f	511	GTTGAGTTASGAGAGGCTCGATAGC	chr6:4971467-4971570	104
	Int18R_r	512	CCCGAAAACAACGACTATCGAAATCCAA		
Intergenic 19	Int19f	513	ATAAGGTTT3GTGGAAGCGTAGGAGCGT	chr6:3177175-3177289	115
	Int19r	514	ACGCCGAATAAAAATCCCGCAACCACAA		
Intergenic 20	Int20f	515	GGAGGGGAGGAGATAGCGTTATTTAGGG	chr10:118912740-118912842	103
	Int20r	516	AAACAAAACCCGAAACCCCACCTACACC		
Intergenic 21	Int21f	517	GCGTGGTAGTTGAGGATGTAGACGTGGT	chr16:45381613-45381736	124
	Int21r	518	TCCGAACTACTTAAARAATCCCCGCCGCC		
Intergenic 22	Int22f	519	TCGTTGGTTGTGATTTTTATGCGGGCGT	chr8:68037259-68037357	99
	Int22r	520	ACCTCTCCGATAAACCAAATCCTCCGCC		
	Int22R_f	521	CGGGTGAGGTTTGCTGGTTAATTTCGCGT	chr8:68037556-68037675	120
	Int22R_r	522	CTCAACCAAACTACAACGTTCCCGCCTC		
Intergenic 23	Int23f	523	AATGGAGGCGTAGATTAACGAGCGGTGT	chr5:42987147-42987254	108
	Int23r	524	ATCCTTAACAACCCCGCCGACTAACGTC		
	Int23R_f	525	ACGGGTACGGAGAAACGTCGGATTTAGT	chr5:42987852-42987946	95
	Int23R_r	526	TCCCCGCGACACTCTACCTATAACGTCC		
KCNH8	KCNH8f	527	CGTTTGGCGGGTATTGTTGTTC	chr3:19164879-19164971	93
	KCNH8r	528	CCCGACGCAAACTCCCTCTC		
KCNJ2	KCNJ2f	529	GAAGTTGTTTTTTAGGGGTTTGCGC	chr17:65676355-65676440	86
	KCNJ2r	530	ACTCAAATCTACCCTCGCTTCAACG		
KCKN4	KCNK4f	531	GCGCGGGGGTATTTTGGAGGGTTAGTTA	chr11:63816449-63816549	101
	KCNK4r	532	TCCCTACTCGCCCGCTACGACTATAACA		
KCNK17	KCNK17f	533	CGGATTTTGTTTTCGGGAGTCGTTCGGG	chr6:39390031-39390150	120
	KCNK17r	534	AACTAAACGCCTAACCCTTCCCTCCCAC		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
KIAA1751	KIAAf	535	TTCGTTTTGTTTTTCGGTTGGAGCGGGT	chr1:1925171-1925288	118
	KIAAr	536	TATAACCTAACCCTTCAACCGCGCCTCG		
	KIAA1751R_f	537	AGGCGGCGGITTTTGGCGATTGTITTTTC	chr1:1925065-1925140	76
	KIAA1751R r	538	TTCCGTTACCATAAAACTACCCGCCCC		
LASS1	LASS1f	539	GATTTCGCGTATCGTCGTGTC	chr19:18868171-18868273	103
	LASS1r	540	TAATATCCCCCGTACCCCCCG		
LOC25516 7	LOCf	541	TTTCGATAATAGCGTTTTTGCGGCGTGG	chr5:6636474-6636619	146
	LOCr	542	CAAAAACACGCGACCTACGCCCTCCTAA		
LRRC4	LRRC4f	543	CGAGTCGGA3TGAGCGTTAAGTGAGGGG	chr7:127459680-127459780	101
	LRRC4r	544	CCTATCAACGACCACCCAACTACTCCCT		
MIR155HG	MIR155HGf	545	TCGGGTTTAGCGTCGTTTGTAGTTTCGG	chr21:25856335-25856430	96
	MIR155HGr	546	AAAAACGTCTCCTTAATTCCCCGCGCTT		
NEXN	NEXNf	547	GCCGTTGGAGTAGAAGTGTTAGCGGTTAGA	chr1:78126913-78127036	124
	NEXNr	548	TCACCCTACAAAAACCGATAACCGACGA		
NKX2-1	NKX2-1f	549	AGTTGGTTATAGGCGGCGAATTGGGTTT	chr14:36057307-36057397	91
	NKX2-1r	550	TCAACACCCCCTCTCCTAACCTCTCCAA		
	NXX6-2f	551	CGGGGAAGAGTTTCGGTTCGCGTTTTAG	chr10:134449988-134450110	123
	NXX6-2r	552	CCCTCCTATAACCCCGACCTACCCGAAA		
NKX6-2	NKX6-2R_f	553	GCGCGGTAGGTGTTTTTCGGGTTGTAAA	chr10:134449796-134449892	97
	NKX6-2R_r	554	ACCTTTACCTAACTACACTCCCATCCAA		
NOTUM	NOTUMf	555	AGAGTAGGTCGTGGGGGATTC	chr17:77512836-77512922	87
	NOTUMr	556	CGCGCTAACCGCGATAAAAAC		
NRN1	NRN1f	557	AGGAGCGGGAGAGGGAAAAATAGTTAAG	chr6:5952635-5952759	125
	NRN1r	558	ACTACGCCCLAAACTCAACTACTAAAT		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
PLTP	PLTPf	559	TGGGAACGGGATAGGGACGCGTTTTAAT	chr20:43974093-43974184	92
	PLTPr	560	GAATCCCCTAAACTACCCGCCATCCCAC		
	PLTPR_f	561	TGTACGCGTATTTTTGGAGGGTGGTTTGC	chr20:43973871-43973950	80
	PLTPR_r	562	CGATCTAATCGACCACCTCCTCTCCTCC		
PRDM13	PRDM13f	563	AAGTTTCGTCGAGTTGGGGTCGTTGGTT	chr6:100168753-100168844	92
	PRDM13r	564	GACCCTTCCC GACAACCATCTCGAACA		
PRDM15	PRDM15f	565	GAAAATTGCCCGGTTGGGTTAGTAGGGG	chr21:42110148-42110259	112
	PRDM15r	566	ACCTACAAATACCGTCCCCACCCGAAAC		
PTGDR	TGDRf	567	AAGAGGGGTCTGATTCGCGAGTTTAGAT	chr14:51804089-51804198	110
	TGDRr	568	CCGCGCGCGACTCGAACGAAAAA		
RECK	RECKf	569	AAGGGTGCGATGTTTTCGTTTAGGATCG	chr9:36027398-36027485	88
	RECKr	570	TAACTAACTLAAACCGCGATAAAACGACT		
RTN4RL1	RTN4f	571	TGGTAATCGCGTAGGTGTGTGATAGGGC	chr17:1827825-1827931	107
	RTN4r	572	AAAATACAAAATACGCCCCCGACCCCGA		
	RTN4RL1R_f	573	TGAGGAGAGATTCGGAGTAGTTAGTAGA	chr17:1827743-1827851	109
	RTN4RL1R_r	574	CCCTATCACACACCTACGCGATTACCAA		
SFRPS	SFRPSf	575	TTTCGAAAAGTTGGTAGTCGGCGGTTGG	chr4:154929548-154929670	123
	SFRP5r	576	CATTCTACTCCCCCGAATCGAAACCCCC		
	SFRP5R_f	577	AAGAGGAAGAGTTCGCGCGTCGAGTTTA	chr4:154929355-154929454	100
	SFRP5R_r	578	GAAATCGCGCGCCCACGATACTACAAAA		
SHF	SHFf	579	TTATTAGTACGCGGCGTCGGGGGTT	chr15:43266978-43267127	150
	SHFr	580	CGAAAACCCCTACTCCGAAAAATCGTCCG		
	SHFR_f	581	GTTGAGATATCGAGGGGTTCGGGTTAGG	chr15:43266838-43266959	122
	SHFR_r	582	CGCCAACAACGATAAAATAAATACCGCGCC		
SHOX2	SHOX2f	583	CGTTTGTTCCATCGGGGTCGTACGAGTAT	chr3:159304063-159304162	100
	SHOX2r	584	TTTCCGCCTCCTACCTTCTAACCCGACT		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
SNCA	SNCAf	585	GGTTGGGGGAGTGGGAGGTAAATTCGTT	chr4:90977105-90977221	117
	SNCAr	586	CTAAACGCTCCCTCACGCCTTACCTTCA		
SNX32	SNX32f	587	TTGAGGGAAACGCGGTGGGAATCGTTTT	chr11:65357939-65358057	119
	SNX32r	588	CCGTAACTCGCCCGAAAAACTAACCGAA		
SP9	SP9f	589	TGATTGGTTGCGGGGTAGTTTC	chr2:174907826-174907911	86
	SP9r	590	ACACCCGCTTTAAAATACCGCTAA		
STK33	STK33f	591	GCGTTTCGGGTCGTTCGTTTTATTTCGC	chr11:8572140-8572262	123
	STK33r	592	CGACAACCTACGCCGAATATACGCACCT		
SYNGR3	SYNGR3f	593	GAAGGGATGAGGTTGAGGTTGGAGGTCG	chr16:1981075-1981195	121
	SYNGR3r	594	ACCTCCTACCCACCAATTCCGAAAAACAA		
T	Tf	595	TTACGGAGTTTTAGGCGGCGTTAC	chr6:166501979-166502099	121
	Tr	596	CATTTCCCTCTCTACGCGCGAAC		
THBS2	THBS2f	597	CGTAGGTTTTGTTGGAGCGAGAGATCGG	chr6:169395805-169395898	94
	THBS2r	598	ACATATAAAACCGCGCTACCCGAAAACCG		
TLXINB	TLX1NBf	599	TGAAAGGGGAGAGGGGAATGTTATTGTT	chr10:102871413-102871518	106
	TLX1NBr	600	AATATTCTCGCAAACCCACCGCCAAACC		
TMEM22	TMEM22f	601	AAAGAGATTCGTGTTGCGGCGGATGAAG	chr3:138021575-138021691	117
	TMEM22r	602	GATCAACACTCGAACCCGAACTTTCCGC		
TNFRSF10 D	TNFRSf	603	AAGGGAGGAGGGTGGATCGAAAGCGTTA	chr8:23077397-23077475	79
	TNFRSr	604	CGAAAACCTTTACACGCGCACAAACTACG		
TXNRD1	TXNDR1f	605	TATGGGTTGGTCGAGGGTAAGGTAGTG	chr12:103133710-103133788	79
	TXNDR1r	606	ACCATCGCCGTTCTTACCTTTCGTCTACA		
VSTM2B	VSTM2Bf	607	TTTTTAATTCGGTTCGGCGTTGATTTGT	chr19:34711435-34711559	125
	VSTM2Br	608	ACAACCGCGCGCTCCCGATAC		
ZFPM2	ZFPM2f	609	TAGCGCGGAAGTTGTGAGTTTAAGGCG	chr8:106401146-106401241	96
	ZFPM2r	610	TCCTCTAAACACCATCGAAACCCCCGAAC		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
ZNF280B	ZNF280Bf	611	AGTGGCGTTCGTTGAGATTAGGGAAGGG	chr22:21192757-21192877	121
	ZNF280Br	612	ACCGTACGCTACCGAAACGACCTTTACA		
LOC10537 8683	LOC105 Af	613	GTTTGTAATTGGTATGAGCGGC	chr1: 43023566-43023673	108
	LOC105 Ar	614	ATAACGAAACGACGCCTC		
	LOC105 Bf	615	GTAATTGGTATGAGCGGCGT	chr1: 43023570-43023660	91
	LOC105 Br	616	GCCTCCGCGAAATAAAACCAT		
	LOC105 Cf	617	AGTTAGAGTAGGTTAGGGGAT	chr1: 43023464 43023613	150
	LOC105 Cr	618	ACGCGTAACACAAACACGAC		
NPHS2	NPHS2 Af	619	GGGGGATTTTAAAGATCGTC	chr1:177811721-177811842	122
	NPHS2 Ar	620	GACGAACGCAATCCACAA		
	NPHS2 Bf	621	TGGTGGAGTTGTGGATTGCG	chr1:177811817-177811891	75
	NPHS2 Br	622	TCCCACCCAAACCTCTCTCT		
NR5A2	NR5A2 Af	623	GGTGCGTTTACGGGTTTC	chr1:198278389-198278538	150
	NR5A2 Ar	624	ACCTAATCCGATATTTCCCGA		
	NRSA2 Bf	625	GGTAGGGTTTCGGTTGCGTA	chr1:198278432-198278527	139
	+ NR5A2 Br	626	TATTTCCCGAAAACTCCACATCCA		
	NR5A2 Br	627	TCCCGAAAACTCCACATCCA		
PAX6	PAX6 Af	628	ATTTGGATGTTTCGCGTTTC		
	PAX6 Ar	629	TATCGCTACSACCCGACTAA		
	+ PAX6 Af	630	GTTAATTTGATGTTTCGCGTTTC	chr11:31783206-31783322	117
	+ PAX6 Ar	631	GTTTATCGCTACGACCCGACTAA		
	PAX6 Bf	632	AGGGGAGTCGCGTTTTTAGG	chr11:31782520-31782652	133
	PAX6 Br	633	TCCCGACCGAAACCCAAATC		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
KCNE3	KCNE3 Af	634	GAATAACGGCGTAAGTTTTTAC	chr11:73855818-73855915	98
	KCNE3 Ar	635	ATCCTCCCGAACGCAATA		
	KCNE3 Bf	636	TTGTACGTTTGTGGGTGTGGA	chr11:73855765-73855914	150
	KCNE3 Br	637	TCCTCCCGAACGCAATAATCG		
KCNA6	KCNA6 Af	638	TTAACGGTTAGGTTAGATCGC	chr12:4789322-4789421	100
	KCNA6 Ar	639	CAATCTCTAAAACGCGACAC		
	KCNA6 Bf	640	CGGGTGTCGCGTTTTAGAGAT	chr12:4789399-4789482	84
	KCNA6 Br	641	TTCTCCGATCTCATACCCCCT		
	TMEM Af	642	GAGAAAAGT TGTTTCGGTC		
	TMEM Ar	643	GCTACGTCTCTACTATCCGA		
	+ TMEM Af	644	CGGGAGAAAAGTTGTTTCGGTC	chr12:127317663-127317786	124
	+ TMEM Ar	645	CCGCTACGTCTCTACTATCCGA		
TMEM132 C	TMEM Bf	646	TTCGGGGTGAGGGTAGTC		
	TMEM Br	647	CCGACGCCCAACTAAAAA		
	+ TMEM Bf	648	GAGTTCGGGGTGAGGGTAGTC	chr12:127318043-127318179	137
	+ TMEM Br	649	GAATCCCGACGCCCAACTAAAAA		
	TMEM Cf	650	TTTTCGGGTTACGGGTCGTT	chr12:127317330-127317424	95
	TMEM Cr	651	ACGACTCCTCCGAAAATCCG		
PDX1	PDX1 Af	652	GTCGATTTTTGTTTTGAGC	chr13:27390195-27390280	86
	PDX1 Ar	653	TAAAAATAATCTACCGAATCGC		
	PDX1 Bf	654	GGCGTTAGCGGGGATTTAGA	chr13:27389563-27389694	132
	PDX1 Br	655	CGCATCAAACGAAACCCTCC		
	PDX1exp Af	656	CGGGAAGGTGTTCGTTTAATGGTTCGGT	chr13:27389489-27389590	102
	PDX1exp Ar	657	GTTTCCGCTCTAAATCCCCGCTAACGCC		
	PDX1exp Bf	658	GGAAAAAGGAGGAGGATAAGAAGCGCGG	chr13:27396588-27396685	98
	PDX1exp Br	659	CTCGCCGAAAATCACGACGCAATCCTAC		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
EPSTI1	EPSTI1 Af	660	TAGGGGAGGCGTCGAGTTC	chr13:42464253-42464369	117
	EPSTI1 Ar	661	ACTCGCTAAACGTCCCAACC		
A2BP1	A2BP1 Af	662	GAGTTTAGGGGTCGCGTC	chr16:6009425-6009564	140
	A2BP1 Ar	663	CAATACCGCCGCCTCTACTA		
	A2BP1 Bf	664	GAGAGAGTAGGAGCGGATCG	chr16:6009706-6009842	137
	A2BP1 Br	665	ACAAATCAACCCCGCCCTAA		
CRYM	CRYM Af	666	AGTGAGTGTTCGGGAGTTTC		
	CRYM Ar	667	TCATTTATTAAAAACGCGCG		
	+ CRYM Af	668	GCAGTGAGTGCTCGGGAGCCCC	chr16:21202786-21202934	149
	+ CRYM Ar	669	GGTTTTCATTTGTTAGAGGCGCGCG		
	CRYM Bf	670	CGGGTTCGCGTAGGATTAGG	chr16:21202650-21202732	83
	CRYM Br	671	ACTCCTCATCCCAACACCCT		
PRKCB	PRKCB Af	672	GTTCGTAGTTCGCGGTTTC		
	PRKCB Ar	673	CGATACTCTCCTCGCCCT		
	+ PRKCB Af	674	TCGGTTCGTAGTTCGCGGTTTC	chr16:23754928-23755052	125
	+ PRKCB Ar	675	GCACGATACTCTCCTCGCCCT		
	PRKCB Bf	676	TTGGGCGAGTGATAGTTTC	chr16:23754821-23754909	89
	PRKCB Br	677	GACCGCTACTACACCCGA		
	PRKCB Cf	678	CGGTAGAAGAACGTGTATGAGGT	chr16:23755076-23755216	141
	PRKCB Cr	679	GCTACCCTCGAAAACCCGAA		
IRF8	IRF8 Af	680	GATTTTTTTTAAGGTCGCGC	chr16:84490230-84490341	112
	+ IRF8 Af	681	TTACGATTTTTTTTAAGGTCGCGC		
	IRF8 Ar	682	ACTATACCTACCTACCGCCGTC		
	IRF8 Bf	683	ATTTCGAAGAAGGCGGGTCG	chr16:84490149-84490276	128
	IRF8 Br	684	CTCCAAACGATACGCCAACG		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
SALL3	SALL3 Af	685	TTTTGCGGGTAAGCGTTC		
	SALL3 Ar	686	CCACAACTCTCTCGACGAC		
	+ SALL3 Af	687	TGTTTTTTGCGGGTAAGCGTTC	chr18:74841456-74841551	96
	+ SALL3 Ar	688	GCCCACAACTCTCTCCACGAC		
	SALL3 Bf	689	ATTTCGGGAAAGGGTGGGTC	chr18:74840051-74840163	113
	SALL3 Br	690	ACCCTAATCCCCCTTCACCA		
	SALL3 Cf	691	TTTCGTTTCGTTTCGGTCGC	chr18:74840452-74840573	122
	SALL3 Cr	692	AACCCGCCCGAACTCAAATA		
LYPDS	LYPDS Af	693	ATTAGGAGCGTACGTTTATTC	chr19:49016646-49016788	143
	LYPDS Ar	694	TACGCACTCAAACACAA		
	LYPD5 Bf	695	CGGCGCGTTTTAAGGGTTTT	chr19:49016738-49016863	126
	LYPDS Br	696	ATTACTCTCACCTCCGCACG		
DPP10	DPP10 Af	697	GATTGCGGGAAGAAGGTAC		
	DPP10 Ar	698	AAACGAAACCAAACGACAA		
	+ DPP10 Af	699	CGGATTGCGGGAAGAAGGTAC	chr2:115635638-115635739	102
	+ DPP10 Ar	700	GACGAAACGAAACCAAACGACAA		
	DPP10 Bf	701	TTTTCGAGTTTGAAGCGTTC		
	DPP10 Br	702	CGACTCTCACCTAATCCGC		
	+ DPP10 Bf	703	CGGTTTTCGAGTTTGAAGCGTTC	chr2:115635947-115636088	142
	+ DPP10 Br	704	TACCGACTCTCACCTAATCCGC		
	DPP10 Cf	705	TTACGACGGGGAGTTCGTTC	chr2:115635821-115635943	123
	+ DPP10 Cr	706	CTTAACAACGTTCGCAAATCACGA		
	DPP10 Cr	707	ACAACGTTCGCAAATCACGA		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
C20orf56	C20orf Af	708	GTTCGTTATTTCGGAATTC	chr20:22507658-22507804	147
	C20orf Ar	709	CCGACCGATAAAATATAATTC		
	C20orf Bf	710	GGGAGGGATTTAAGCGGGAG	chr20:22507684-22507819	136
	C20orf Br	711	CCCCCTTCACTAATCCCGAC		
SOX2OT	SOX2OT Af	712	AGTGTTGAGAGTCGACGC	chr3:182919951-182920042	92
	SOX2OT Ar	713	AATAAAATAACCCGAACCGC		
	SOX2OT Bf	714	GGGTTACGGTTTCGGGTTGT	chr3:182919884-182919969	86
	SOX2OT Br	715	CGCGTCGACTCTCAACACTA		
CDKL2	CDKL2 Af	716	GGTCGAGTCGAGTCGTTAC		
	CDKL2 Ar	717	AAAACGCCTCCTAACGAA		
	+ CDKL2 Af	718	ATTGGTCGAGTCGAGTCGTTAC	chr4:76774785-76774935	151
	+ CDKL2 Ar	719	ACAAAAAAACGCCTCCTAACGAA		
	CDKL2 Bf	720	TATTTTTGGGCGAAGGCGTTG	chr4:76774698-76774806	109
	CDKL2 Br	721	GTAACGACTCGACTCGACCA		
MARCH11	MARCH11 Af	722	TCGGCGTTTTCGTTTTTC	chr5:16232623-16232697	75
	MARCH11 Ar	723	CGACGACACAACCATAAACTTT		
	MARCH11 Bf	724	AAGGTTTTGTAGTTGCGGCG	chr5:16232839-16232935	97
	MARCH11 Br	725	TCTCACGCGCAACCGAAT		
CCL28	CCL28 Af	726	GTGGAGTTTTAGGTAGCGC		
	CCL28 Ar	727	ACCCGCGATAAACTAAACC		
	+ CCL28 Af	728	AGGGTGGAGTTTTAGGTAGCGC	chr5:43433001-43433128	128
	+ CCL28 Ar	729	AACAACCCGCGATAAACTAAACC		
	CCL28 Bf	730	TGTAGTCGTGGTTGTCGTGG	chr5:43432695-43432834	140
	CCL28 Br	731	CCAAATAAACGACGTCCCGC		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
AP3B1	AP3B1 Af	732	ATTTTATAGTCGCGTTAAAAGC	chr5:77304383-77304519	137
	AP3B1 Ar	733	ACTTTTATTACTCGCGATCC		
	AP3B1 Bf	734	GGTAGGGTGAGTTTGGTCGG	chr5:77304339-77304484	146
	AP3B1 Br	735	CGCCGAACCACGTAAAAACT		
CARD11	CARD11 Af	736	ATTTGGGGCGTTTATGTTTC	chr7:3049825-3049944	120
	CARD11 Ar	737	CCCTCGAAAAACGACTCC		
	CARD11 Bf	738	AGGGGTTGTAGGGTCGGG		
	+ CARD11 Bf	739	TTTAGGGGTTGTAGGGTCGGG	chr7:3049955-3050087	133
	CARD11Br	740	ATTTTACATTTCCCTCCCCCGC		
BLACE	BLACE Af	741	AGAATAAAAGTAGGCGGC	chr7:154859246-154859384	139
	BLACE Ar	742	TCTCGAAACCAAAATAAACG		
	BLACE Bf	743	AGTAGGCGGCGGATTTGTAG	chr7:154859254-154859357	104
	BLACE Br	744	CCGAAAATACGCGAAATCAACC		
PTPRN2	PTPRN2 Af	745	GAGGAGATAAAGGTGTCGC		
	PTPRN2 Ar	746	AACGTACCTAACCCGAAAAC		
	+ PTPRN2 Af	747	TCGGAGGAGATAAAGGTGTCGC	chr7:157176188-157176342	155
	+ PTPRN2 Ar	748	CCAACGTACCTAACCCGAAAAC		
	PTPRN2 Bf	749	GACGGTTTCGGTAGGGTC		
	PTPRN2 Br	750	CCGAACCGAATATAAAACGA		
	+ PTPRN2 Bf	751	CGGACGGTTTCGGTAGGGTC	chr7:157176379-157176463	85
	+ PTPRN2 Br	752	GCGCCGAACCGAATATAAAACGA		
RUNX1T1	RUNX1T1 Af	753	TTAGGTTCGTAAAGAGGGC	chr8:93183286-93183401	116
	RUNX1T1 Ar	754	TTAAAACCACGTCCGAATA		
	RUNX1T1 Bf	755	TTTCGGGCGGGAGTTATAGG	chr8:93183412-93183529	118
	RUNX1T1 Br	756	ACGCGCTCTAAACTCAACCG		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
L1TD1	L1 TD1 Af	757	GCGCGTGGGGTTCGTAGCGTTTTAAG	chr1:62433357-62433465	109
	L1 TD1 Ar	758	TTACCCGAAACACCCCGCGCCCTTC		
PPFIA3	PPFIA3 Af	759	AGATACGGAGATTTAGCGCGAGATCGGT	chr19:54337953-54338094	143
	PPFIA3 Ar	760	AAATTAACCGCCGAACACTCACAATACG		
FILIP1L	FILIPIL Af	761	TTGTAGTGTCGCGTTGCGAGTCGATTGT	chr3:101077651-101077753	103
	FILIP1L Ar	762	ACAATAACGTAACGCCCATAAACCGAACG		
NUDT16P	NUDT Af	763	GAGGACGGGTGAATCGTGGTTTGTTGG	chr3:132563775-132563858	84
	NUDT Ar	764	ACTACGATAATCAAAACGCTCCACGCGA		
TOP2P1	TOP Af	765	GTGCGCGTTTAGTAGGGCGAGAATGG	chr6:28283268-28283417	150
	TOP Ar	766	CGAAAACCAAATCCGAACCACCGTCTCC		
	TOP Bf	767	TGATTTGGGTGGATGTAGAGGTTGTGGT	chr6:28283447-28283568	122
	TOP Br	768	TTTCGAATAACGCTACTCCGAACCGCGA		
UNKWN1	UNKWN1 Af	769	TTGAGAGTAGGGATTGTGGTGCGTCGTC	chr5:72634694-72634838	145
	UNKWN1 Ar	770	CTAACTCCCGAACGCTACATTCGCTCCA		
GALR3	GALR3 Af	771	GGTTGTGGTGAGTTTGGTTTACGGGCG	chr22:36550907-36551049	143
	GALR3 Ar	772	CGTAAAACGCGACCACCGCCAACATA		
PRSS27	PRSS Af	773	GGGAGGTTATTCGTAGGATTTGGCGCGG	chr16:270S610-2705748	139
	PRSS Ar	774	ATCCTAACGACTACGCACTACTTCCGCA		
SLC7A4	SLC Af	775	GAGTTCGTTTAGTTCGTCGGCGTC	chr22:19716858-19717005	148
	SLC Ar	776	AACCCCGATAAACTCCGATAACGACCT		
LEF1	LEF1 Af	777	AGAGTTGGGGGCGGTATAGTTAGGGTGT	chr4:109307444-109307547	104
	LEF1 Ar	778	TTCAATCCCTACGACCCCAACGCCTAAA		
NFIC	NFIC Af	779	CGTGGATACGAGTTTTGGCGGCGATTAT	chr19:3386117-3386219	103
	NFIC Ar	780	GCCACCAACCCTACCTCCTTCCATATCC		
	NFIC Bf	781	TTTTTCGGTTTGAGTTATCGTGGCGGGA	chr19:3386234-3386379	146
	NFIC Br	782	CCGAACCGTACTTCCAACCAAACGCAACT		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
TMEM90B	TMEM90 Af	783	TAGGAAGGGGTCGATGTTGGTTTGGGTT	chr20:24398648-24398747	100
	TMEM90 Ar	784	TCTCACCAACTCCCATCGAATTCGCACA		
	TMEM90 Bf	785	GTTTTGGTTTCGTTTCGGAGCGCGTAGA	chr20:24398510-24398642	133
	TMEM90 Br	786	TTTCTCTACCGACTCAACTCCCCCTCCC		
UBD	UBD Af	787	TCGGTTGCGTAAATCGCGTTTTTGGTTG	chr6:29629437-29629564	128
	UBD Ar	788	TTCTCGATAATATCTCCGTCGCCTCCGC		
GIPC2	GIPC Af	789	GTTTAGGGGTGGAGGTCGGGGTTTTGA	chr1:78284199-78284289	91
	GIPC Ar	790	CCGAACCCCGCGCAAATAAAAACAACCT		
EFNA4	ERNA Af	791	GGGGCGCGTTTTTATGGAAAGTTAGGGT	chr1:153310423-153310549	127
	ERNA Ar	792	CTACGCCCTAAAACACGCCTCGACTTCT		
	ERNA Bf	793	TGTGCGAAAGAGACGCGGGGTTTAGTTA	chr1:153310139-153310288	150
	ERNA Br	794	CCCGTAATCGCTAAAACATCCGCCCTTA		
DRD4	DRD4 Af	795	CGTCGGGCGATGCTTGGTTTGTTCGTG	chr11:627035-627175	141
	DRD4 Ar	796	GCGACGCTCCACCGTAAACCCAATATTTA		
TCTEX1D1	TCTEX Af	797	CGGGGAGGGTCGAGGGTTTTGTTTGAG	chr1:66990668-66990782	101
	TCTEX Ar	798	GCGTCCCAAACTTCATTCAACCGACGAC		
PHOX28	PHOX Af	799	GCGGACGTAGTAATGGATTAAACGGGGA	chr4:41447111-41447255	145
	PHOX Ar	800	AAATCCGACTCCCTACACTCCCGACTTT		
TSPAN33	TSPAN Af	801	GGGGGTTGTGTTAGTTCTTTGTTTAGCGA	chr7:128596487-128596593	107
	TSPAN Ar	802	CGAAACTATTTCCCGCCAAACCGAACCC		
CA9	CA9 Af	803	TTTCGGGCGGGAGTATCGGGTTTTGTAG	chr9:35666101-35666239	139
	CA9 Ar	804	GCTCCTTTACCCCTTCTCGACCAACTCC		
UNKWN2	UNKWN2 Af	805	TTACGGATTTTATTTGTATTCGGAATCGTA	chr10:102409232-102409335	104
	UNKWN2 Ar	806	ACGCATCAAACTCGACACAAAATTTCATC		
WT1	WT1 Af	807	GGTGTTTTCGTAAGACGGGGTAGTGGGT	chr11:32406776-32406869	94
	WT1 Ar	808	TTCTCCTCCGCTAARAAATCCGAATACGA		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
OTX2	OTX2 Af	809	AGGGATTGTATTTCGAGGTGGTCGAGGT	chr14:56331673-56331781	109
	OTX2 Ar	810	CCGACABRATCGAAACCTTCGCCCGAAAC		
HOXB13	HOXB13 Af	811	TCGCGGGTTATAAATATTTGGTTGCGGC	chr17:44157793-44157885	93
	HOXB13 Ar	812	GACCGCCACTACCTCGAAAACATTTCCC		
BRCAL	BRCA1 Af	813	GGTAACGGAAAAGCGCGGGAATTATAGA	chr17:38530874-38530968	95
	BRCA1 Ar	814	CCCACAACCATCCCCCGTCCAAAAA		
ITPRIPL1	ITPRIPL1f	815	TTTTGTACGTTGGGTTACGGGGGTTTGG	chr2:96354715-96354857	143
	ITPRIPL1r	816	TAAACGCGATAAACCCCTACGACCCCCA		
HES5	HES5-F	817	TATCGGTTTTCGTAGTTGCGGGAGGAGG	Chr1:2451323-2451386	118
	HESS-R	818	CCGAATAAACACCAAACTCGCCCGACGC		
CSRP1/LOC 376693	CSRP1/LOC376693-F	819	CGGGTAGAGGGGAGGTAGGAATTGGAGA	Chr1:199775889-199775914	80
	CSRP1/LOC376693-R	820	CCGAATAAACGTCACCCCTACACACCGC		
ALOX5	ALOX5-F	821	TTTTGCGGTTAGGTGAAGGCGTAGAGGT	Chr10:45234681-45234732	106
	ALOXS-R	822	GACCGAATACCCCGCTTTCTCTCTCGAC		
PPM1H/M ON2	PPM1H/MON2-F	823	AGGAGTAGTATTGCGAGGGTGGAGGGT	Chr12:61311943-61312001	112
	PPM1H/MON2-R	824	TAAACCCGAAAAACAACGCCAATCCCGC		
KIAA0984	KIAA0984-F	825	GGGGATTTGTTGTAGAGTCGTAGGAGAA	Chr12:63515983-63516043	62
	KIAA0984-R	826	CCGCATCCCACCCTTTAAAACTCTA		
TXNRD1	TXNRD1-F	827	TATGGGTTGCGTCGAGGGTAAGGTAGTG	Chr12:103133737-103133768	86
	TXNRD1-R	828	TACGACGACCATCGCCGTTCTTACCTTT		
CHST11	CHST11-F	829	AAATTTGGATTGCGGGAGGGACGAGGTT	Chr12:103376469-103376538	124
	CHST11-R	830	CTTCGCAACCGAACTACTCACCCCCGAC		
EFS	EFS-F	831	GGTCGTTGGAGTGGTCGTTTCGGTTTAG	Chr14:22904743-22904785	98
	EFS-R	832	CCTCAAACCCCCGAACGCGCTAAATAAA		
ANXA2	ANXA2-F	833	GTTCGGGGAGGGAGGGAGATTCGTTTTG	Chr15:58478046-58478098	107
	ANXA2-R	834	AACTCCCGACTTTAACCTCCCAACCCAA		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
RHCG	RHCG-F	835	GTTGTAGGGGTGTTTGGTCGGGTTGGTA	Chr15:87840807-87840869	118
	RHCG-R	836	ATCAACTACTCCGTACCCCACGTAACCG		
RARA	RARA-F	837	AGTCGGGGTGGTTGGTCGAAGAGG	Chr17:35718896-35718981	137
	RARA-R	838	CCCTCTCAACTCGATTCAAAATTCCCCC		
PTRF	PTRF-F	839	AAAGTAATAAGTGGTTTCGGGCGGAGTC	Chr17:37827277-37827326	104
	PTRF-R	840	ACCCCGCATACCTACGAAAACGAAAACC		
RND2	RND2-F	841	CGGGATTATGGAGGGGTAGAGCGGTCG	Chr17:38430910-38430955	99
	RND2-R	842	ACGTCCTTAACGAACACCTACAACAACG		
TMP4	TMP4-F	843	AGGTTTTGTAGTAGTAGGCGGACGAGGC	Chr19:16048446-16048512	121
	TMP4-R	844	ACGAATACGAAACCCGAAACCGAAACGC		
HIF3A	HIF3A-F	845	CGTGGTATAGTTAATCGCGCGGCGT	Chr19:51492259-51492376	118
	HIF3A-R	846	TACAACCCCAACGCCATAACTCGCCAAT		
KLKS	KLK4-F	847	TAGCGGGGATTTATTAGGGGAGAGGTGG	Chr19:56107959-56108027	123
	KLK4-R	848	ATCACCTACGAACACTATCCCTCACCCG		
AMOTL2	AMOTL2-F	849	GCGGAATAGTCGCGGTTTTGGAATGTT	Chr3:135565786-135565856	125
	AMOTL2-R	850	AAACGTTTCCGCTCCCCGAAAAACGAAT		
SCGB3A1	SCGB3A1-F	851	GGAGATAGTTTTGAGAGGGGGAGGTCGC	Chr5:179950858-179950923	120
	SCGB3A1-R	852	CGCTACCTACGCCGATCGTAAATCCCAA		
HLA-F	HLA-F-F	853	GAATGGTTGCGATATGGGGTTCGACGGA	Chr6:29799978-29800035	112
	HLA-F-R	854	CGCGATCCAAAAACGCAAATCCTCGTTC		
HLA-J-1	HLA-J,NCRNA00171-1-F	855	GGTTTTGGTCGAGATTTGGGCGGGTGAG	Chr6:30082430-30082476	101
	HLA-J,NCRNA00171-1-R	856	CCCGAATCCTACGCCCCAACCAAATAAA		
HLA-J-3	HLA-J,NCRNA00171-2-F	857	TGAGTGATTTCGGTTCGGGGCGTAGATT	Chr6:30083115-30083168	125
	HLA-J,NCRNA00171-2-R	858	CGAAAATCTCTACAAATCCCGCAACCTCG		
PON3	PON3-F	859	ATGGTTTCGGGGTGTTTAGCGGCGATTG	Chr7:94863624-94863674	105
	PON3-R	860	AACGAAACCGAACGAACCCCAATCCGTA		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
LRRC4/SN D1	LRRC4-F	861	GAGTCGGAGCCGAGCGTTAAGTGAGGGG	Chr7:127459707-127459730	77
	LRRC4-R	862	TCCCTCCGACCGACCCAAAATAACTACG		
PAH	PAH-F	863	TTCGTTGTTCGTTTTGGGTAAAGGGAAG	Chr12:101835348-101835409	116
	PAH-R	864	AAACTCGCTCCCCAAACTTCTAAAAATC		
EPSTI1	EPSTI1-F	865	GGGGAGGCGTCGAGTTCGGAGTTTATTA	Chr13:42464282-42464345	117
	EPSTI1-R	866	AAAACTCGCTAAACGTCCCAACCGCATC		
ADCY4	ADCY4-F	867	CGGGTATTGTTGGTTTAGGTTGTAGTAGGT	Chr14:23873644-23873710	123
	ADCY4-R	868	CGACCCTAACCAACCCCGAAACTCGAAA		
HAPLN3	HAPLN3-F	869	AGGGTAGAAAGGAAGCGGTAGTAGAAAA	Chr15:87239811-87239872	116
	HAPLN3-R	870	ACAACAACTCCTCCCTTCGAACCCAACC		
HSF4	HSF4-F	871	TGTGGGAGGGAAGGGAAATCGAGATTGG	Chr16:65762053-65762164	113
	HSF4-R	872	ACGACAAAACGAAACCCACAATCCTACCC		
NBR1/TME M106A	NBR1/TMEM106A-F	873	ATTCGGATTGGTTAGTTTTTGCGGAAGT	Chr17:38719260-38719296	91
	NBR1/TMEM106A-R	874	TTCGCCACGCAACAACCTAAAACGCTAC		
HAAO	HAAO-F	875	GGTTGCGGCGTTTATTTAGCGGGAAGTC	Chr2:42873761-42873822	114
	HAAO-R	876	CTCGCCGAACCCGCGACGAAATCTAC		
RARB	RARB-F	877	TAGAGGAATTTAAAGTGTGGGTTGGGGG	Chr3:25444371-25444441	12.5
	RARB-R	878	ACCAACTTCTCTCCCTTTACGCCTTTTT		
ALDH1L1	ALDH1L1-F	879	TGGGTTAAGTATTTGTTATGTGTTACGGA	Chr3:127382511-127382580	121
	ALDH1L1-R	880	CGCTATCCACCCGAATACGCAACT		
HIST1H3G	HIST1H3G-F	881	GCGCGGCGTTTTGTTATCGGTGGATT	Chr6:26379588-26379647	60
	HIST1H3G-R	882	TCTAAAATAACCCGCACCAAACAAACTACA		
ZSCAN12	ZSCAN 12-F	883	TTATAAAGGTCGGAAGCGGTTACGGGGG	Chr6:28475534-28475572	93
	ZSCAN12-R	884	AACCCCTTTCGCTCCCTTCCTAAAACGA		
HCG4P6	HCG4P6-F	885	GTATGGTTGCGATTTGGGGTTGGAAGGG	Chr6:30002983-30003042	114
	HCG4P6-R	886	GCCGCGATCCAAAAACGCAAATCCTAAT		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
HLA-J-3	HLA-J,NCRNA00171-3-F	887	TAGGGAATGTTTGGTTGCGATTTGGGG	Chr6:30083115-30083168	80
	HLA-J,NCRNA00171-3-R	888	TCCTTACCGTCGTAAACATACTACTCAT		
EYA4	EYA4-F	889	GCGTAAGTGCGAGGTTGTCGGTAGC	Chr6:133604154-133604229	125
	EYA4-R	890	TTTCCCGCAACTCTTTCCCCCTCTCT		
HOXA7	HOXA7-F	891	TGCGGTTAAAGAATTCGTTCGCGTTCGG	Chr7:27162955-27162982	82
	HOXA7-R	892	CTAAACGCTCCCGCGAAACCTCCAAATC		
USP44	USP44/p-F	893	TTCGGGTATTTTGAGGTTGTCGTCGGGA	Chr12:94466379-94466481	103
	USP44/p-R	894	GACGACGACGCGTCCGACGAATTTTA		
CYP27A1	CYP27A1/p-F	895	GTTTTGGTCGGGGCGTCGTGGATATTTT	Chr2:219354932-219355042	111
	CYP27A1/p-R	896	AAAAACCAACTAAACCCCTTCCCGCTCG		
PRSS3	PRSS3/p-F	897	GTGTGGAAAGGGTTTGGCGGTTGTTAGG	Chr9:33740574-33740686	113
	PRSS3/p-R	898	CTCGCCAAATACGTCCACCCAAAAACGA		
C18orf62	C18orf62/p-F	899	TAGGAGGGGACGTAGAGTTTACGGCGAA	Chr18:71296729-71296833	105
	C18orf62/p-R	900	GAATACCCGACCCGACCCATCCATCAC		
SFRP2	SFRP2/p-1-F	901	TGCGTTTGTAGGAGAAGTCGGGTTGGTT	Chr4:154929326-154929408	83
	SFRP2/p-1-R	902	ACTCTTCCTCGCCTCGCACTACTACCTA		
	SFRP2/p-2-F	903	GTGCGATTCGGGGTTTCGAAAAGTTGGT	Chr4:154929535-154929641	107
	SFRP2/p-2-R	904	GAAACTACGCGCGAACTTACAACGCCTC		
SLCO4C1	SLCO4C1/p-F	905	GAGCGTAGAGCGTTGAGCGGGG	ChrS:101660047-101660169	123
	SLCO4C1/p-R	906	CGCCGCCGAATAACACGCCCAC		
COR01C	CORO1C/p-1-F	907	AGCGGGGATTTTCGGAGTTGGAGAGTTT	Chr12:107686622-107686733	112
	CORO1C/p-1-R	908	CTCCATCCGCCCGACCTAACCCTAAAAA		
	CORO1C/p-2-F	909	GGGAAGTGGCGTAGTGGGCGTTTGTATC	Chr12:107686752-107686848	97
	CORO1C/p-2-R	910	TACCTCCAACGACCACGCCCACAAAATA		
KJ904227	KJ904227/p-F	911	TGGAGCGTTGAGTCGAAGTTTTGATTTT	Chr3:127489474-127489582	109
	KJ904227/p-R	912	TCTTACCCGAACTTTAACCCCAACCGCT		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
C6orf141	C6orf141/p-1-F	913	GGTTGGGAGTTCGGAGTTGTAGTAGAGG	Chr6:49626357-49626455	99
	C6orf141/p-1-R	914	CTTTAACCGATTCAAACAACAAACGCCT		
	C6orf141/p-2-F	915	GTAGGGCGC3GGGTTTCGTTAGTTTC	Chr6:49626570-49626668	99
	C6orf141/p-2-R	916	ATCTACCGTTCTATCCTCGTAACCGCCG		
BC030768	BC030768/p-F	917	TCGTTTGGGAGGGATCGTTTTTGGGAGA	Chr1:26424688-26424767	80
	BC030768/p-R	918	AACCCGAATACTATCCAACTACCGCCGC		
DMRTA2	DMRTA2/p-F	919	CGAGCGTGGGTATTAAGTCGGTAGTGGA	Chr1:50657067-50657169	103
	DMRTA2/p-R	920	GACCTCAACCCCCTACGCCTAACCTACT		
HFE	HFE/p-1-F	921	GTAGATCGCGGTTTTGTAGGGGCGTTTG	Chr6:26195692-26195783	92
	HFE/p-1-R	922	CTAATTTCGATTTTTCCACCCCCGCCGC		
	HFE/p-2-F	923	GAGTGTTTGTCGAGAAGGTTGAGTAAAT	Chr6:26196140-26196221	82
	HFE/p-2-R	924	CACCGCCCAACGCATTCGTTCTAAAATA		
CADPS2	CADPS2/p-F	925	ATAAAAGTGGGGTGGGTGGCGGAGGG	Chr7:121744063-121744166	104
	CADPS2/p-R	926	GCGCCGAAATAACAACCCAACCTACCAA		
CYTH4	CYTH4/p-F	927	TTTATCGGGGAAGTTTTCGAGGGTGGGC	Chr22:36050993-36051112	120
	CYTH4/p-R	928	TCCCAACTACCTCCTACGCACGAACGAT		
Intergenic (Chr4)	Chr4/p-1-F	929	ATGAAATGTGGTTCGTGGAAGGTGTTTGT	Chr4:186174475-186174549	75
	Chr4/p-1-R	930	ACGACCCGAACGTTAATCCTCTTACTAC		
NHLH2	NHLH2/p-F	931	ACGTAGTTTTCGAGTTAGTGTCGTTAGAA	Chr1:116172677-116172793	117
	NHLH2/p-R	932	GACAAACGCCTCAAACCCGACCG		
NRN1	NRN1/p-F	933	AGGAGCGGGAGAGGGAAAAATAGTTAAG	Chr6:5952635-5952767	133
	NRN1/p-R	934	CGCTCCAAACTACGCCCAAAACTCAA		
HMGCLL1	HMGCLL1/p-F	935	ATTAGAGTTGTTTTGCGTATTGCGGCGG	Chr6:55551934-55552030	97
	HMGCLL1/p-R	936	CAAATACCCCGTACACCCGCTACCCCAA		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
Me3	Me3/p-1-F	937	GGGAGTTGAGGTTTACGCGGTTTCGTTG	Chr11:86061026-86061124	99
	Me3/p-1-R	938	GACCGCCAACGCGATCCACCCATTAAC		
	Me3/p-2-F	939	AGTTTTGGAAGTAGATTCGGTGCGGGTG	Chr11:86060867-86060948	82
	Me3/p-2-R	940	GCCGCGCAATCGCCTCTTTTTCAC		
Intergenic (Chr3)	Chr3/p-1-F	941	AGACGATAGATGGCGGGTAGGAAGGGAG	Chr3:135608250-135608374	125
	Chr3/p-1-R	942	GCCGCCTACAACCGACGAACTACAAATC		
Intergenic (Chr8)	Chr8/p-1-F	943	TCGCGGGTGAGGTTTGTGGTTAATTTCG	Chr8:68037553-68037676	124
	Chr8/p-1-R	944	GCTCAACCAAACTACAACGTTCCCGCCT		
NBPF1	NBPF1/p-F	945	TGAGAGGCGTATTTTGTTGGTTACGGTT	Chr1:146219493-146219574	82
	NBPF1/p-R	946	CGAAAACCATTCCGCTACCCTTCCAACT		
Intergenic (Chr10)	Chr10/p-1-F	947	GGGGCGTTGGGTTATGGAGATTACGTTTT	Chr10:42748953-42749053	101
	Chr10/p-1-R	948	GTCCCGCGCTTAACGAATTCTACGAACG		
ASAP1	ASAP1/p-F	949	GTTCGGGTAGGGGTCGGGGGTC	Chr8:131524437-131524546	110
	ASAP1/p-R	950	CCCGAAACGACGTACTTAACGACCCGAA		
intergenic (Chr1)	Chr1/p-1-F	951	GGGAGGTTTGAGCGTCGAAGTTTTCGTT	Chr1:119352428-119352549	122
	Chr1/p-1-R	952	GCCCACTACCCCGCGAAACCTTATCAAC		
PPP2R5C	PPP2RSC/p-F	953	AGTCGTTAGGTTGTTAAGGCGCGTTGTG	Chr14:101317476-101317534	59
	PPP2R5C/p-R	954	ACAAAAATAAAATCGAACCTAACCCCACG		
Intergenic (Chr2)	Chr2/p-1-F	955	CGTATTAAGGGTTAAGCGGCGCGGT	Chr22:44883312-44883404	93
	Chr2/p-1-R	956	AACTTTCTCGAACGACTCGATAAACCTAA		
KRT78	KRT78/p-F	957	AGGTTTTGGGAATTTGGAAGTTCGCGGG	Chr12:51554274-51554370	97
	KRT78/p-R	958	AAAAACGCTCGAACCCAACCAATCGACG		
LINC240	LINC240/p-1-F	959	AAAGGAAGATCGTGGGTAGTTCGTGCG	Chr6:27167780-27167859	80
	LINC240/p-1-R	960	ACTACAACTCACGTTTCCCCTCCAACAC		
	LINC240/p-2-F	961	AGGTTTATTTGACGTTTTAGGTCGATAGT	Chr6:27172709-27172830	122
	LINC240/p-2-R	962	CGATCTCTCCCTTTCTTCCGCTTCCTAA		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
Intergenic (Chr16)	Chr16/p-1-F	963	GGCGTCGGTTGCGGTTTTAGAT	Chr16:53648145-53648269	125
	Chr16/p-1-R	964	ACGCGAAAATCTACCTTTTAATTACGAACC		
HIST1H3G/ 1H2BI	HIST1H3G/1H2BI/p-F	965	TCGTCGGTGGTCGGCGCGTTTTT	Chr6:26379488-26379589	102
	HIST1H3G/1H2BI/p-R	966	AACCCGCACCAAACAAACTACACGCAAA		
PPM1H	PPM1H/p-1-F	967	GAATGGTAGCGAGAGGTTGCGGGTTAGG	Chr12:61312222-61312310	89
	PPM1H/p-1-R	968	CTCTACCCTCAAAATCGCGACGCAAACG		
	PPM1H/p-2-F	969	AGGAGTAGTATTGCGAGGGTGGAGGGTT	Chr12:61311917-61312012	96
	PPM1H/p-2-R	970	CGCCAATCCCGCTCCGACACTATAACAA		
TUBB2B	TUBB2B/p-F	971	ATAAGGTTTGGTGGAAGCGTAGGAGCGT	Chr12:3177175-3177262	88
	TUBB2B/p-R	972	ACGATATTCTAACCTCCGCCGCGAAACT		
C2CD4A	C2CSF	973	GGTAGAGGGATAGGGAAGAGTTTGGCGT	Chr15:60146378-60146528	150
	C2C5R	974	ATTCAAAACGCGCGCGACGAAATTCAAC		
COL19A1	COL2F	975	GCGGAGTGGGAGGGTTATATTGGCGAGAG	Chr6:70633134-70633240	106
	COL2R	976	CCGAACAAAACTACGACACCGCCGAAAA		
DCDC2	DCDSF	977	ACGACGGGTTGAGATAGGTGGTTGGATT	Chr6:24465938-24466027	90
	DCDSR	978	CCCGACGCGAAACAACGAACTAAAACGA		
DHRS3	DGR2F	979	TTTTTGTACGTTTTCGGGGTCGGAGGAG	Chr1:12601840-12601942	102
	DHR2R	980	AATCGCCGTCTAAACAAATCGCGAACTA		
GALNT3	GAL1F	981	CGGCGGTCGCGGTTTGTAGTTTAGAATTG	Chr2:166358281-166358431	150
	GAL1R	982	ACGCGCTTCCACTCCGACTAACAAATTA		
	GAL3F	983	GGCGTCGTTCGGGTTAAGTTTGGTTGT	Chr2:166359152-166359230	78
	GAL3R	984	CACAACTTACGCGAAACAACAACCTCGC		

(continued)

Gene	Probe	SEQ ID NO.	- 3' Primer Sequence (Bisulfite)	Chr: Location	PCR Product Length
HESS	HES1F	985	TGGGTTGCTGTCGCGCGAATTTTTGTTT	Chr1:2451234-2451350	116
	HES1R	986	CCTCCTCCCGCAACTACGAAAACCGATA		
	HES3F	987	GTTGGGGGTTATGTTTGGCGCGGAATAG	Chr1:2451478-2451622	144
	HES3R	988	CGCCTATATAAAACGTCGACGCGCGAAA		
	HES4F	989	GTTCGGGCGTCGCGGTCGTTTTTATATT	Chr1:2453144-2453266	122
	HES4R	990	AAAACGCCCATTATACCCGCGCCAATTC		
KILLIN	KILSF	991	TAAGAATCG3CGGTAGTTAGTAGGCGGG	Chr10:89611638-89611783	145
	KIL5R	992	TCCTACGCCGCGACGAAAACAAAAACTC		
	KIL6F	993	AGGTGGGGCGCGTTTATTAGTTTAGGGG	Chr10:89611428-89611578	150
	KIL6R	994	ACCTCTCCATCGCTAATACCCTACCGCT		
MUC21	MUC2F	995	GAGTGTTTCGAGGGTAGGAGGTTGTCGG	Chr6:31031426-31031559	133
	MUC2R	996	CAAAAACCGCCCGCAAAACGAAACCTAA		
NR2E1/OS TM1	OST3F	997	ACGGATCGATCGCGGTTTTGGTAAGGAT	Chr6:108542828-108542915	87
	OST3R	998	CGCAAAAACGAAAAACTACGTACGCGCT		
	OST4F	999	GTTGTTTGAGGACGGGTCGTTTAGCGG	Chr6:108543090-108543189	99
	OST4R	1000	ACCCCTATCCTACAACCCTACGAACGCA		
PAMR1	PAM4F	1001	TTTCGGGAGGTGTGGTTACGTTTGGAGA	Chr11:35503958-35504077	119
	PAM4R	1002	CCCCTCCTCCCAACACCCAACACTAAAA		
SCRN1	SCR2F	1003	GGTTGTGGTTTTTAAAAGGGAAAATTCGGG	Chr7:29996282-29996388	106
	SCR2R	1004	TAAACGCCGAAACCCGAACGTAACAACC		
SEZ6	SEZ3F	1005	AGGTGATTAGAAGGGAGAGGGGGAGGTT	Chr17:24371083-24371180	97
	SEZ3R	1006	TCATTATACACGACGCGCCCCTCCAAAT		
	SEZSF	1007	TACGTGGGTGTAGGTTAGGTCGGGTTGA	Chr17:24371224-24371345	121
	SEZ5R	1008	ACCACGCGACTACCGTATAAACAACCGAA		

EQUIVALENTS

[0215] The above-described embodiments are intended to be examples only. Alterations, modifications and variations can be effected to the particular embodiments by those of skill in the art.

REFERENCES

[0216]

1. How KA, Nielsen HM, Tost J. DNA methylation based biomarkers: practical considerations and applications. Biochimie 2012; 94: 2314-37.
2. Mikeska T, Craig JM. DNA methylation biomarkers: cancer and beyond. Genes (Basel) 2014; 5: 821-64.
3. Noehammer C, Pulverer W, Hassler MR, Hofner M, Wielscher M, Vierlinger K, et al. Strategies for validation and testing of DNA methylation biomarkers. Epigenomics. 2014; 6: 603-22.
4. Warton K, Samimi G. Methylation of cell-free circulating DNA in the diagnosis of cancer. Front Mol.Biosci. 2015; 2: 13.
5. Wittenberger T, Sleigh S, Reisel D, Zikan M, Wahl B, Alunni-Fabbroni M, et al. DNA methylation markers for early detection of women's cancer: promise and challenges. Epigenomics. 2014; 6: 311-27.
6. Usadel H, Brabender J, Danenberg KD, Jeronimo C, Harden S, Engles J, et al. Quantitative adenomatous polyposis coli promoter methylation analysis in tumour tissue, serum, and plasma DNA of patients with lung cancer. Cancer Res. 2002; 62: 371-5.
7. Esteller M, Sanchez-Cespedes M, Rosell R, Sidransky D, Baylin SB, Herman JG. Detection of aberrant promoter hypermethylation of tumour suppressor genes in serum DNA from non-small cell lung cancer patients. Cancer Res. 1999; 59: 67-70.
8. Mazurek A, Pierzyna M, Giglok M, Dworzecka U, Suwinski R, Ma UE. Quantification of concentration and assessment of EGFR mutation in circulating DNA. Cancer Biomark. 2015; 15: 515-24.
9. Ostrow KL, Hoque MO, Loyo M, Brait M, Greenberg A, Siegfried JM, et al. Molecular analysis of plasma DNA for the early detection of lung cancer by quantitative methylation-specific PCR. Clin.Cancer Res. 2010; 16: 3463-72.
10. Powrozek T, Krawczyk P, Kucharczyk T, Milanowski J. Septin 9 promoter region methylation in free circulating DNA-potential role in noninvasive diagnosis of lung cancer: preliminary report. Med.Oncol. 2014; 31: 917.
11. Lin PC, Lin JK, Lin CH, Lin HH, Yang SH, Jiang JK, et al. Clinical Relevance of Plasma DNA Methylation in Colorectal Cancer Patients Identified by Using a Genome-Wide High-Resolution Array. Ann.Surg.Oncol. 2014.
12. Philipp AB, Nagel D, Stieber P, Lamerz R, Thalhammer I, Herbst A, et al. Circulating cell-free methylated DNA and lactate dehydrogenase release in colorectal cancer. BMC.Cancer 2014; 14: 245.
13. Chimonidou M, Strati A, Malamos N, Georgoulias V, Lianidou ES. SOX17 promoter methylation in circulating tumour cells and matched cell-free DNA isolated from plasma of patients with breast cancer. Clin.Chem. 2013; 59: 270-9.
14. Chimonidou M, Tzitzira A, Strati A, Sotiropoulou G, Sfikas C, Malamos N, et al. CST6 promoter methylation in circulating cell-free DNA of breast cancer patients. Clin.Biochem. 2013; 46: 235-40.
15. Martinez-Galan J, Torres-Torres B, Nunez MI, Lopez-Penalver J, Del MR, Ruiz De Almodovar JM, et al. ESR1 gene promoter region methylation in free circulating DNA and its correlation with estrogen receptor protein expression in tumour tissue in breast cancer patients. BMC.Cancer 2014; 14: 59.
16. Matuschek C, Bolke E, Lammering G, Gerber PA, Peiper M, Budach W, et al. Methylated APC and GSTP1 genes in serum DNA correlate with the presence of circulating blood tumour cells and are associated with a more aggressive and advanced breast cancer disease. Eur.J.Med.Res. 2010; 15: 277-86.
17. Fackler MJ, Lopez BZ, Umbricht C, Teo WW, Cho S, Zhang Z, et al. Novel methylated biomarkers and a robust assay to detect circulating tumour DNA in metastatic breast cancer. Cancer Res. 2014; 74: 2160-70.
18. Avraham A, Uhlmann R, Shperber A, Birnbaum M, Sandbank J, Sella A, et al. Serum DNA methylation for monitoring response to neoadjuvant chemotherapy in breast cancer patients. Int.J.Cancer 2012; 131: E1166-E1172.
19. Sharma G, Mirza S, Parshad R, Gupta SD, Ralhan R. DNA methylation of circulating DNA: a marker for monitoring efficacy of neoadjuvant chemotherapy in breast cancer patients. Tumour.Biol. 2012; 33: 1837-43.
20. Legendre C, Gooden GC, Johnson K, Martinez RA, Liang WS, Salhia B. Whole-genome bisulfite sequencing of cell-free DNA identifies signature associated with metastatic breast cancer. Clin.Epigenetics. 2015; 7: 100.
21. Jones PA. Functions of DNA methylation: islands, start sites, gene bodies and beyond. Nat.Rev.Genet. 2012; 13: 484-92.
22. Cope LM, Fackler MJ, Lopez-Bujanda Z, Wolff AC, Visvanathan K, Gray JW, et al. Do breast cancer cell lines provide a relevant model of the patient tumour methylome? PLoS.One. 2014; 9: e105545.
23. Becker D, Lutsik P, Ebert P, Bock C, Lengauer T, Walter J. BiQ Analyzer HiMod: an interactive software tool for

high-throughput locus-specific analysis of 5-methylcytosine and its oxidized derivatives. Nucleic Acids Res. 2014; 42: W501-W507
24. Lutsik P, Feuerbach L, Arand J, Lengauer T, Walter J, Bock C. BiQ Analyzer HT: locus-specific analysis of DNA methylation by high-throughput bisulfite sequencing. Nucleic Acids Res. 2011; 39: W551-W556.
25. Soreide K. Receiver-operating characteristic curve analysis in diagnostic, prognostic and predictive biomarker research. J.Clin.Pathol. 2009; 62: 1-5.
26. Madic, J. et al. Pyrophosphorolysis-activated polymerization detects circulating tumor DNA in metastatic uveal melanoma. Clinical Cancer Research: an official journal of the American Association for Cancer Research. 2012; 18: 3934-3941.
27. Bidard, F. C. et al. Detection rate and prognostic value of circulating tumor cells and circulating tumor DNA in metastatic uveal melanoma. International Journal of Cancer 2014; 134: 1207-1213.
28. The Molecular Taxonomy of Primary Prostate Cancer. Cell. 2015; 163(4): 1011-25.

Claims

1. A method for detecting a tumour, comprising:

extracting DNA from a cell-free sample obtained from a human subject; bisulphite converting the DNA;
amplifying multiple regions of the bisulphite converted DNA present in the sample using a plurality of PCR primers, wherein

(i) amplifying with at least one primer set designed to amplify at least one methylation site having a methylation value at or below -0.3 in normal tissue; and

detecting signals wherein at least one signal comprises methylation of at least two adjacent methylation sites within at least one region of the multiple regions;
wherein the multiple regions are methylated in at least 50% of the tumours in a population of samples for a selected tumour type or tumour subtype and are not methylated in tissues and blood of subjects without tumours;
wherein individual primers of the plurality of PCR primers are designed based on methylated DNA within individual regions of the multiple regions;
wherein the presence of a tumour in the subject is indicated when signals comprising methylation of at least two adjacent methylation sites within the at least one region of the multiple regions are detected.

2. The method of claim 1, wherein

(ii) the at least one primer set designed to amplify at least one methylation site has a difference between the average methylation value in the cancer and the normal tissue of greater than 0.3; and/or
(iii) the at least one primer set comprising primer pairs designed to amplify at least one methylation site having at least one adjacent methylation site within 200 base pairs that also has:

a methylation value below -0.3 in normal tissue; and
a difference between the average methylation value in the cancer and the normal tissue of greater than 0.3.

3. The method of claims 1 or 2, wherein the at least one methylation site has a methylation value below -0.3 in normal tissue; and/or
wherein the at least one methylation site has a difference between the average methylation value in the cancer and the normal tissue of greater than 0.3.

4. The method of any of claims 1-3, wherein the multiple regions of methylated DNA are determined by:

obtaining data for the population of samples comprising a plurality of genomic methylation data sets, each of said genomic methylation data sets associated with biological information for a corresponding sample;
segregating the methylation data sets into a first group corresponding to one tissue or cell type exhibiting a tumour characteristic, and a second group corresponding to a plurality of tissue or cell types not possessing the tumour characteristic;
matching methylation data from the first group to methylation data from the second group on a site-by-site basis across the genome;

identifying a set of CpG sites that meet a predetermined threshold for establishing differential methylation between the first group and second groups;
identifying, using the set of CpG sites, target genomic regions comprising at least two differentially methylated CpGs with 300bp that meet said predetermined criteria;
extending the target genomic regions to encompass at least one adjacent differentially methylated CpG site that does not meet the predetermined criteria;
wherein the extended target genomic regions provide the methylation signature indicative of the tumour characteristic.

5. The method of claim 4, wherein:

identifying further comprises limiting the set of CpG sites to CpG sites that further exhibit differential methylation with peripheral blood cells from control samples; or
the predetermined threshold is indicative of methylation in the first group and nonmethylation in the second group; or
the predetermined threshold is met in at least 50% methylation of the samples in the first group; or
the predetermined threshold is a difference in average methylation between the first group and second group of 0.3 or greater.

6. The method of claim 4 or 5, wherein the tumour characteristic comprises one or more of:

malignancy;
a cancer type;
a cancer classification;
a molecular subtype classification;
a cancer grade;
a histological classification;
a metabolic profile;
a disease-associated mutation;
a clinical outcome; and
a drug response.

7. The method of any one of claims 1 to 6, further comprising determining sites of hydroxymethylation.

8. The method of any one of claims 1 to 7, wherein detecting comprises amplifying with primers designed to anneal sequences having at least one methylated site therein.

9. The method of claim 8, comprising one or more of:

the primers are designed without preference of location of the at least one methylated site within target sequences;
the primers are designed to amplify DNA fragments 75 to 150 bp in length;
the primers are designed to amplify DNA fragments comprising 3 to 12 CpG methylation sites;
each of the regions is amplified in sections using multiple primer pairs;
the tumour signals comprise more than two adjacent methylation sites within the single sequencing read.

10. The method of any one of claims 1 to 9, wherein the detection of at least one signal is indicative of a tumour during one or more of:

determining response to treatment;
monitoring tumour load;
detecting residual tumour post-surgery;
detecting relapse;
use as a secondary screen;
use as a primary screen;
monitoring cancer development; and
monitoring cancer risk.

11. The method of any one of claims 1 to 10, further comprising determining a distribution of tumour signals across the multiple regions; and:

comparing the distribution to at least one pattern associated with a cancer, wherein similarity between the distribution and the pattern is indicative of the cancer; or
comparing the distribution to a plurality of patterns, each one associated with a cancer type, wherein similarity between the distribution and one of the plurality of patterns is indicative of the associated cancer type.

12. The method of any one of claims 1 to 11, wherein the tumour is a breast cancer tumour, a prostate cancer tumour or a subtype thereof, a colon cancer tumour or a subtype thereof, a lung cancer tumour or a subtype thereof, or a uveal melanoma cancer tumour or a subtype thereof.

13. The method of claim 12, wherein the regions comprise C2CD4A, COL19A1, DCDC2, DHRS3, GALNT3, HESS, KILLIN, MUC21, NR2E1/OSTM1, PAMR1, SCRN1, and SEZ6, and wherein the tumour is uveal melanoma.

14. The method of claim 12, wherein the regions comprise ADCY4, ALDH1L1, ALOX5, AMOTL2, ANXA2, CHST11, EFS, EPSTI1, EYA4, HAAO, HAPLN3, HCG4P6, HESS, HIF3A, HLA-F, HLA-J, HOXA7, HSF4, KLK4, LOC376693, LRRC4, NBR1, PAH, PON3, PPM1H, PTRF, RARA, RARB, RHCG, RND2,TMP4, TXNRD1, and ZSCAN12, and wherein the tumour is prostate cancer.

15. The method of claim 12, wherein the regions comprise ASAP1, BC030768, C18orf62, C6orf141, CADPS2, CORO1C, CYP27A1, CYTH4, DMRTA2, EMX1, HFE, HIST1H3G/1H2BI, HMGCLL1, KCNK4, KJ904227, KRT78, LINC240, Me3, MIR1292, NBPF1, NHLH2, NRN1, PPM1H, PPP2R5C, PRSS3, SFRP2, SLC04C1, SOX2OT, TUBB2B, USP44, Intergenic (Chr1), Intergenic (Chr2), Intergenic (Chr3), Intergenic (Chr4), Intergenic (Chr8), and Intergenic (Chr10), and wherein the tumour is aggressive prostate cancer.

16. The method of claim 12, wherein the regions comprise ALX1, ACVRL1, BRCA1,C1orf114, CA9, CARD11, CCL28, CD38, CDKL2, CHST11, CRYM, DMBX1, DPP10, DRD4, EFNA4, EPSTI1, EVX1, FABP5, FOXA3, GALR3, GIPC2, HINF1B, HOXA9, HOXB13, Intergenic5, Intergenic 8, IRF8, ITPRIPL1, LEF1, LOC641518, MAST1, BARHL2, BOLL, C5orf39, DDAH2, DMRTA2, GABRA4, ID4, IRF4, NT5E, SIM1, TBX15, NFIC, NPHS2, NR5A2, OTX2, PAX6, GNG4, SCAND3, TAL1, PDX1, PHOX2B, POU4F1, PFIA3, PRDM13, PRKCB, PRSS27, PTGDR, PTPRN2, SALL3, SLC7A4, SOX2OT, SPAG6, TCTEX1D1, TMEM132C, TMEM90B, TNFRSF10D, TOP2P1, TSPAN33, TTBK1, UDB, and VWC2, and wherein the tumour is triple negative breast cancer (TNBC).

17. The method according to any one of claim 1 to 16 wherein each region to be amplified is amplified with primer pairs listed for the respective region in Table 15.

18. A kit for detecting a tumour in DNA from a sample obtained from a human subject, comprising:

reagents for carrying out the method of any one of claims 1 to 13 and 17; and
instructions for detecting tumour signals;
wherein detecting includes detecting multiple regions methylated in cancer from the DNA;
wherein the multiple regions are methylated in at least 50% of the tumours in a population of samples for a selected tumour type or tumour subtype and are not methylated in normal tissues and normal blood cells;
wherein the reagents comprise a plurality of PCR primers, wherein individual primers are designed based on the methylated DNA sequences of individual regions of the multiple regions and wherein the primers include a DNA sequence such that individual primers anneal to a methylated DNA sequence of individual regions of the multiple regions;
wherein the reagents comprise two or more primers pairs identified in Table 15 and selected from the group consisting of SEQ ID NOs: 973-1008.

Patentansprüche

1. Verfahren zum Nachweisen eines Tumors, umfassend:

Extrahieren von DNA aus einer zellfreien Probe, die aus einem menschlichen Probanden erhalten wurde;
Bisulfit-Umwandeln der DNA;

Vervielfältigen mehrerer Regionen der in der Probe vorhandenen bisulfitumgewandelten DNA unter Verwendung einer Vielzahl von PCR-Primern, wobei

(i) Amplifizieren mit mindestens einem Primersatz, der entworfen ist, mindestens eine Methylierungsstelle mit einem Methylierungswert von -0,3 oder weniger in normalem Gewebe zu vervielfältigen; und

Nachweisen von Signalen, wobei mindestens ein Signal eine Methylierung von mindestens zwei benachbarten Methylierungsstellen innerhalb mindestens einer Region der mehreren Regionen umfasst;
wobei die mehreren Regionen in mindestens 50% der Tumoren in einer Population von Proben für einen ausgewählten Tumortyp oder Tumorsubtyp methyliert sind, und in Geweben und Blut von Probanden ohne Tumoren nicht methyliert sind;
wobei einzelne Primer der Vielzahl von PCR-Primern auf der Grundlage von methylierter DNA innerhalb einzelner Regionen der mehreren Regionen entworfen sind;
wobei das Vorhandensein eines Tumors im Probanden angezeigt wird, wenn Signale nachgewiesen werden, die eine Methylierung von mindestens zwei benachbarten Methylierungsstellen innerhalb der mindestens einen Region der mehreren Regionen umfassen.

2. Verfahren nach Anspruch 1, wobei

(ii) der mindestens eine Primersatz, der entworfen ist, mindestens eine Methylierungsstelle zu vervielfältigen, einen Unterschied zwischen dem durchschnittlichen Methylierungswert im Krebs und dem normalen Gewebe von mehr als 0,3 aufweist; und/oder
(iii) der mindestens eine Primersatz, der Primerpaare umfasst, die entworfen sind, mindestens eine Methylierungsstelle mit mindestens einer benachbarten Methylierungsstelle innerhalb von 200 Basenpaaren zu vervielfältigen, auch aufweist:

einen Methylierungswert unter -0,3 im normalen Gewebe; und
einen Unterschied zwischen dem durchschnittlichen Methylierungswert im Krebs und dem normalen Gewebe von mehr als 0,3.

3. Verfahren nach Ansprüchen 1 oder 2, wobei die mindestens eine Methylierungsstelle einen Methylierungswert von unter -0,3 in normalem Gewebe aufweist; und/oder
wobei die mindestens eine Methylierungsstelle einen Unterschied zwischen dem durchschnittlichen Methylierungswert im Krebs und dem normalen Gewebe von mehr als 0,3 aufweist.

4. Verfahren nach einem beliebigen der Ansprüche 1-3, wobei die mehreren Regionen von methylierter DNA bestimmt werden durch:

Erhalten von Daten für die Population von Proben, die eine Vielzahl von genomischen Methylierungsdatensätzen umfassen, wobei jeder der genomischen Methylierungsdatensätze mit biologischer Information für eine entsprechende Probe assoziiert ist;
Segregation der Methylierungsdatensätze in eine erste Gruppe, die einem Gewebe- oder Zelltyp entspricht, der eine Tumoreigenschaft aufweist, und eine zweite Gruppe, die einer Vielzahl von Gewebe- oder Zelltypen entspricht, die die Tumoreigenschaft nicht besitzen;
Abgleich der Methylierungsdaten aus der ersten Gruppe mit den Methylierungsdaten aus der zweiten Gruppe auf einer Stelle-für-Stelle-Basis über das Genom hinweg;
Identifizieren einer Gruppe von CpG-Stellen, die eine vorbestimmte Schwelle für das Feststellen einer unterschiedlichen Methylierung zwischen der ersten Gruppe und der zweiten Gruppe erreichen;
Identifizieren von Zielgenomregionen, die mindestens zwei unterschiedlich methylierte CpGs mit 300bp umfassen, die die vorbestimmten Kriterien erfüllen, unter Verwendung des Satzes von CpG-Stellen;
Erweitern der Zielgenomregionen, mindestens eine benachbarte unterschiedlich methylierte CpG-Stelle zu umfassen, die die vorbestimmten Kriterien nicht erfüllt;
wobei die erweiterten Zielgenomregionen die Methylierungssignatur liefern, die auf die Tumoreigenschaft hinweist.

5. Verfahren nach Anspruch 4, wobei:

Identifizieren des Weiteren ein Beschränken des Satzes von CpG-Stellen auf CpG-Stellen umfasst, die weiter

eine unterschiedliche Methylierung mit peripheren Blutzellen aus Kontrollproben aufweisen; oder
die vorbestimmte Schwelle auf eine Methylierung in der ersten Gruppe und eine Nichtmethylierung in der zweiten Gruppe hinweist; oder
die vorbestimmte Schwelle bei mindestens 50% Methylierung der Proben in der ersten Gruppe erreicht wird; oder
die vorbestimmte Schwelle ein Unterschied in der durchschnittlichen Methylierung zwischen der ersten Gruppe und der zweiten Gruppe von 0,3 oder mehr ist.

6. Verfahren nach Anspruch 4 oder 5, wobei die Tumoreigenschaft eines oder mehrere umfasst von:

Malignität;
einer Krebsart;
einer Krebsklassifizierung;
einer molekularen Subtypklassifizierung;
einem Krebsgrad;
einer histologischen Klassifizierung;
einem Stoffwechselprofil;
einer krankheitsassoziierten Mutation;
einem klinischen Ergebnis; und
einem Ansprechen auf Medikamente.

7. Verfahren nach einem beliebigen der Ansprüche 1 bis 6, das des Weiteren ein Bestimmen von Hydroxymethylierungsstellen umfasst.

8. Verfahren nach einem beliebigen der Ansprüche 1 bis 7, wobei ein Nachweisen ein Vervielfältigen mit Primern umfasst, die entworfen sind, sich an Sequenzen mit mindestens einer methylierten Stelle darin anzulagern.

9. Verfahren nach Anspruch 8, umfassend eines oder mehrere von:

die Primer sind ohne Präferenz für die Position der mindestens einen methylierten Stelle innerhalb der Zielsequenzen entworfen;
die Primer sind entworfen, DNA-Fragmente von 75 bis 150 bp Länge zu vervielfältigen;
die Primer sind entworfen, DNA-Fragmente umfassend 3 bis 12 CpG-Methylierungsstellen zu vervielfältigen;
jede der Regionen wird in Abschnitten unter Verwendung mehrerer Primerpaare vervielfältigt;
die Tumorsignale umfassen mehr als zwei benachbarte Methylierungsstellen innerhalb des einzelnen Sequenzierungs-Reads.

10. Verfahren nach einem beliebigen der Ansprüche 1 bis 9, wobei der Nachweis mindestens eines Signals auf einen Tumor hinweist während eines oder mehrerer von:

Bestimmen eines Ansprechens auf eine Behandlung;
Überwachen der Tumorlast;
Nachweisen von Resttumor nach einer Operation;
Nachweisen eines Rückfalls;
Verwendung als sekundäres Screening;
Verwendung als primäres Screening;
Überwachen von Krebsentwicklung; und
Überwachen von Krebsrisiko.

11. Verfahren nach einem beliebigen der Ansprüche 1 bis 10, das des Weiteren Bestimmen einer Verteilung von Tumorsignalen über die mehreren Regionen umfasst; und:

Vergleichen der Verteilung mit mindestens einem Muster, das mit einem Krebs assoziiert ist,
wobei eine Ähnlichkeit zwischen der Verteilung und dem Muster auf den Krebs hinweist; oder
Vergleichen der Verteilung mit einer Vielzahl von Mustern, von denen jedes mit einem Krebstyp assoziiert ist,
wobei eine Ähnlichkeit zwischen der Verteilung und einem der Vielzahl von Mustern auf den assoziierten Krebstyp hinweist.

12. Verfahren nach einem beliebigen der Ansprüche 1 bis 11, wobei der Tumor ein Brustkrebstumor, ein Prostata-

krebstumor oder ein Subtyp davon, ein Darmkrebstumor oder ein Subtyp davon, ein Lungenkrebstumor oder ein Subtyp davon, oder ein Aderhautmelanom-Krebstumor oder ein Subtyp davon ist.

13. Verfahren nach Anspruch 12, wobei die Regionen C2CD4A, COL19A1, DCDC2, DHRS3, GALNT3, HES5, KILLIN, MUC21, NR2E1/OSTM1, PAMR1, SCRN1 und SEZ6 umfassen, und wobei der Tumor ein Aderhautmelanom ist.

14. Verfahren nach Anspruch 12, wobei die Regionen ADCY4, ALDH1L1, ALOX5, AMOTL2, ANXA2, CHST11, EFS, EPSTI1, EYA4, HAAO, HAPLN3, HCG4P6, HES5, HIF3A, HLA-F, HLA-J, HOXA7, HSF4, KLK4, LOC376693, LRRC4, NBR1, PAH, PON3, PPM1H, PTRF, RARA, RARB, RHCG, RND2, TMP4, TXNRD1 und ZSCAN12 umfassen, und wobei der Tumor Prostatakrebs ist.

15. Verfahren nach Anspruch 12, wobei die Regionen ASAP1, BC030768, C18orf62, C6orf141, CADPS2, CORO1C, CYP27A1, CYTH4, DMRTA2, EMX1, HFE, HIST1H3G/1H2BI, HMGCLL1, KCNK4, KJ904227, KRT78, LINC240, Me3, MIR1292, NBPF1, NHLH2, NRN1, PPM1H, PPP2R5C, PRSS3, SFRP2, SLC04C1, SOX2OT, TUBB2B, USP44, Intergenic (Chr1), Intergenic (Chr2), Intergenic (Chr3), Intergenic (Chr4), Intergenic (Chr8) und Intergenic (Chr10) umfassen, und wobei der Tumor aggressiver Prostatakrebs ist.

16. Verfahren nach Anspruch 12, wobei die Regionen ALX1, ACVRL1, BRCA1, C1orf114, CA9, CARD11, CCL28, CD38, CDKL2, CHST11, CRYM, DMBX1, DPP10, DRD4, EFNA4, EPSTI1, EVX1, FABP5, FOXA3, GALR3, GIPC2, HINF1B, HOXA9, HOXB13, Intergenic5, Intergenic 8, IRF8, ITPRIPL1, LEF1, LOC641518, MAST1, BARHL2, BOLL, C5orf39, DDAH2, DMRTA2, GABRA4, ID4, IRF4, NT5E, SIM1, TBX15, NFIC, NPHS2, NR5A2, OTX2, PAX6, GNG4, SCAND3, TAL1, PDX1, PHOX2B, POU4F1, PFIA3, PRDM13, PRKCB, PRSS27, PTGDR, PTPRN2, SALL3, SLC7A4, SOX2OT, SPAG6, TCTEX1D1, TMEM132C, TMEM90B, TNFRSF10D, TOP2P1, TSPAN33, TTBKI, UDB und VWC2 umfassen, und wobei der Tumor ein dreifach negativer Brustkrebs (TNBC) ist.

17. Verfahren nach einem beliebigen der Ansprüche 1 bis 16, wobei jede zu vervielfältigende Region mit Primerpaaren vervielfältigt wird, die für die jeweilige Region in Tabelle 15 aufgeführt sind.

18. Kit zum Nachweisen eines Tumors in DNA aus einer aus einem menschlichen Probanden erhaltenen Probe, umfassend:

Reagenzien zum Durchführen des Verfahrens gemäß einem beliebigen der Ansprüche 1 bis 13 und 17; und Anweisungen zum Nachweisen von Tumorsignalen;
wobei das Nachweisen ein Nachweisen mehrerer bei Krebs methylierter Regionen aus der DNA einschließt;
wobei die mehreren Regionen in mindestens 50% der Tumoren in einer Population von Proben für einen ausgewählten Tumortyp oder Tumorsubtyp methyliert sind, und in normalen Geweben und normalen Blutzellen nicht methyliert sind;
wobei die Reagenzien eine Vielzahl von PCR-Primern umfassen, wobei einzelne Primer auf der Grundlage der methylierten DNA-Sequenzen einzelner Regionen der mehreren Regionen entwickelt wurden, und wobei die Primer eine DNA-Sequenz einschließen, so dass einzelne Primer sich an eine methylierte DNA-Sequenz einzelner Regionen der mehreren Regionen anlagern;
wobei die Reagenzien zwei oder mehr Primerpaare umfassen, die in Tabelle 15 identifiziert sind, und aus der Gruppe bestehend aus SEQ ID NOs: 973-1008 ausgewählt sind.

Revendications

1. Procédé de détection d'une tumeur, comprenant :

l'extraction d'ADN à partir d'un échantillon acellulaire prélevé sur un sujet humain ;
la conversion de l'ADN au bisulfite ;
l'amplification de plusieurs régions de l'ADN converti au bisulfite présent dans l'échantillon à l'aide de plusieurs amorces de PCR, dans lequel

(i) l'amplification est réalisée avec au moins un ensemble d'amorces conçu pour amplifier au moins un site de méthylation ayant une valeur de méthylation inférieure ou égale à -0,3 dans un tissu normal ; et

la détection de signaux, dans lequel au moins un signal comprend la méthylation d'au moins deux sites de

méthylation adjacents dans au moins une région parmi les multiples régions ;
dans lequel les multiples régions sont méthylées dans au moins 50 % des tumeurs dans une population d'échantillons pour un type tumoral ou un sous-type tumoral sélectionné et sont non méthylées dans les tissus et le sang de sujets indemnes de tumeurs ;
dans lequel les amorces individuelles de la pluralité d'amorces de PCR sont conçues sur la base de l'ADN méthylé dans des régions individuelles parmi les multiples régions ;
dans lequel la présence d'une tumeur chez le sujet est indiquée lorsque sont détectés des signaux comprenant la méthylation d'au moins deux sites de méthylation adjacents dans ladite au moins une région parmi les multiples régions.

2. Procédé selon la revendication 1, dans lequel

(ii) l'au moins un ensemble d'amorces conçues pour amplifier au moins un site de méthylation présente une différence entre la valeur moyenne de méthylation dans le tissu cancéreux et dans le tissu normal supérieure à 0,3 ; et/ou
(iii) l'au moins un ensemble d'amorces comprenant des paires d'amorces conçues pour amplifier au moins un site de méthylation ayant au moins un site de méthylation adjacent au sein de 200 paires de bases qui présente également :

une valeur de méthylation inférieure à -0,3 dans le tissu normal ; et
une différence entre la valeur moyenne de méthylation dans le tissu cancéreux et dans le tissu normal supérieure à 0,3.

3. Procédé selon les revendications 1 ou 2, dans lequel l'au moins un site de méthylation présente une valeur de méthylation inférieure à -0,3 dans le tissu normal ; et/ou
dans lequel l'au moins un site de méthylation présente une différence entre la valeur moyenne de méthylation dans le tissu cancéreux et dans le tissu normal supérieure à 0,3.

4. Procédé selon l'une quelconque des revendications 1 à 3, dans lequel les multiples régions d'ADN méthylé sont déterminées par :

l'obtention de données pour la population d'échantillons comprenant une pluralité d'ensembles de données de méthylation génomique, chacun desdits ensembles de données de méthylation génomique étant associé à des informations biologiques pour un échantillon correspondant ;
la séparation des ensembles de données de méthylation en un premier groupe correspondant à un type de tissu ou de cellule présentant une caractéristique tumorale, et un second groupe correspondant à une pluralité de types de tissus ou de cellules ne possédant pas la caractéristique tumorale ;
la mise en correspondance des données de méthylation provenant du premier groupe avec les données de méthylation provenant du second groupe, site par site, sur l'ensemble du génome ;
l'identification d'un ensemble de sites CpG qui atteignent un seuil prédéterminé pour établir une méthylation différentielle entre le premier groupe et le second groupe ;
l'identification, au moyen de l'ensemble de sites CpG, de régions génomiques cibles comprenant au moins deux CpG différentiellement méthylés de 300 pb qui répondent auxdits critères prédéterminés ;
l'extension des régions génomiques cibles pour englober au moins un site CpG différentiellement méthylé adjacent qui ne répond pas aux critères prédéterminés ;
dans lequel les régions génomiques cibles étendues fournissent la signature de méthylation indicative de la caractéristique tumorale.

5. Procédé selon la revendication 4, dans lequel :

l'identification comprend en outre la limitation de l'ensemble de sites CpG aux sites CpG qui présentent en outre une méthylation différentielle avec les cellules du sang périphérique des échantillons témoins ; ou
le seuil prédéterminé indique une méthylation dans le premier groupe et une non-méthylation dans le second groupe ; ou
le seuil prédéterminé est atteint dans au moins 50 % de méthylation des échantillons dans le premier groupe ; ou
le seuil prédéterminé est une différence de méthylation moyenne entre le premier groupe et le second groupe de 0,3 ou plus.

6. Procédé selon la revendication 4 ou 5, dans lequel la caractéristique tumorale comprend une ou plusieurs caractéristiques parmi :

la malignité ;
un type de cancer ;
une classification du cancer ;
une classification de sous-types moléculaires ;
un grade du cancer ;
une classification histologique ;
un profil métabolique ;
une mutation associée à la maladie ;
une issue clinique ; et
une réponse à un médicament.

7. Procédé selon l'une quelconque des revendications 1 à 6, comprenant en outre la détermination de sites d'hydroxyméthylation.

8. Procédé selon l'une quelconque des revendications 1 à 7, dans lequel la détection comprend l'amplification avec des amorces conçues pour s'hybrider à des séquences comportant au moins un site méthylé dans celles-ci.

9. Procédé selon la revendication 8, comprenant un ou plusieurs éléments parmi :

les amorces conçues sans préférence quant à l'emplacement de l'au moins un site méthylé au sein des séquences cibles ;
les amorces conçues pour amplifier des fragments d'ADN de 75 à 150 pb de long ;
les amorces conçues pour amplifier des fragments d'ADN comprenant de 3 à 12 sites de méthylation CpG ;
chacune des régions étant amplifiée en sections à l'aide de plusieurs paires d'amorces ;
les signaux tumoraux comprenant plus de deux sites de méthylation adjacents dans la lecture de séquençage unique.

10. Procédé selon l'une quelconque des revendications 1 à 9, dans lequel la détection d'au moins un signal est indicative d'une tumeur lors d'une ou plusieurs parmi :

la détermination de la réponse à un traitement ;
la surveillance de la charge tumorale ;
la détection d'une tumeur résiduelle postopératoire ;
la détection d'une rechute ;
l'utilisation comme dépistage secondaire ;
l'utilisation comme dépistage primaire ;
la surveillance de l'évolution du cancer ; et
la surveillance du risque de cancer.

11. Procédé selon l'une quelconque des revendications 1 à 10, comprenant en outre la détermination d'une distribution des signaux tumoraux dans les multiples régions ; et :

la comparaison de la distribution à au moins un profil associé à un cancer, dans lequel la similarité entre la distribution et le profil est indicative du cancer ; ou
la comparaison de la distribution à une pluralité de profils, chacun étant associé à un type de cancer, dans lequel la similarité entre la distribution et l'un de la pluralité de profils est indicative du type de cancer associé.

12. Procédé selon l'une quelconque des revendications 1 à 11, dans lequel la tumeur est une tumeur de cancer du sein, une tumeur de cancer de la prostate ou un sous-type de celui-ci, une tumeur de cancer du côlon ou un sous-type de celui-ci, une tumeur de cancer du poumon ou un sous-type de celui-ci, ou une tumeur de mélanome uvéal ou un sous-type de celui-ci.

13. Procédé selon la revendication 12, dans lequel les régions comprennent C2CD4A, COL19A1, DCDC2, DHRS3, GALNT3, HESS, KILLIN, MUC21, NR2E1/OSTM1, PAMR1, SCRN1 et SEZ6, et dans lequel la tumeur est un mélanome uvéal.

14. Procédé selon la revendication 12, dans lequel les régions comprennent ADCY4, ALDH1L1, ALOX5, AMOTL2, ANXA2, CHST11, EFS, EPSTI1, EYA4, HAAO, HAPLN3, HCG4P6, HESS, HIF3A, HLA-F, HLA-J, HOXA7, HSF4, KLK4, LOC376693, LRRC4, NBR1, PAH, PON3, PPM1H, PTRF, RARA, RARB, RHCG, RND2, TMP4, TXNRD1 et ZSCAN12, et dans lequel la tumeur est un cancer de la prostate.

15. Procédé selon la revendication 12, dans lequel les régions comprennent ASAP1, BC030768, C18orf62, C6orf141, CADPS2, CORO1C, CYP27A1, CYTH4, DMRTA2, EMX1, HFE, HIST1H3G/1H2BI, HMGCLL1, KCNK4, KJ904227, KRT78, LINC240, Me3, MIR1292, NBPF1, NHLH2, NRN1, PPM1H, PPP2R5C, PRSS3, SFRP2, SLC04C1, SOX2OT, TUBB2B, USP44, Intergénique (Chr1), Intergénique (Chr2), Intergénique (Chr3), Intergénique (Chr4), Intergénique (Chr8) et Intergénique (Chr10), et dans lequel la tumeur est un cancer agressif de la prostate.

16. Procédé selon la revendication 12, dans lequel les régions comprennent ALX1, ACVRL1, BRCA1, C1orf114, CA9, CARD11, CCL28, CD38, CDKL2, CHST11, CRYM, DMBX1, DPP10, DRD4, EFNA4, EPSTI1, EVX1, FABP5, FOXA3, GALR3, GIPC2, HINF1B, HOXA9, HOXB13, Intergénique 5, Intergénique 8, IRF8, ITPRIPL1, LEF1, LOC641518, MAST1, BARHL2, BOLL, C5orf39, DDAH2, DMRTA2, GABRA4, ID4, IRF4, NTSE, SIM1, TBX15, NFIC, NPHS2, NR5A2, OTX2, PAX6, GNG4, SCAND3, TAL1, PDX1, PHOX2B, POU4F1, PFIA3, PRDM13, PRKCB, PRSS27, PTGDR, PTPRN2, SALL3, SLC7A4, SOX2OT, SPAG6, TCTEX1D1, TMEM132C, TMEM90B, TNFRSF10D, TOP2P1, TSPAN33, TTBK1, UDB et VWC2, et dans lequel la tumeur est un cancer du sein triple négatif (CSTN).

17. Procédé selon l'une quelconque des revendications 1 à 16, dans lequel chaque région à amplifier est amplifiée avec des paires d'amorces répertoriées pour la région respective dans le tableau 15.

18. Kit de détection d'une tumeur dans l'ADN provenant d'un échantillon prélevé sur un sujet humain, comprenant :

des réactifs pour la mise en œuvre du procédé selon l'une quelconque des revendications 1 à 13 et 17 ; et
des instructions pour la détection de signaux tumoraux ;
dans lequel la détection comprend la détection de multiples régions méthylées dans le cancer à partir de l'ADN ;
dans lequel les multiples régions sont méthylées dans au moins 50 % des tumeurs dans une population d'échantillons pour un type tumoral ou un sous-type tumoral sélectionné et ne sont pas méthylées dans les tissus normaux et les cellules sanguines normales ;
dans lequel les réactifs comprennent une pluralité d'amorces de PCR, dans lequel les amorces individuelles sont conçues sur la base des séquences d'ADN méthylées de régions individuelles parmi les multiples régions et dans lequel les amorces comprennent une séquence d'ADN telle que les amorces individuelles s'hybrident à une séquence d'ADN méthylée de régions individuelles parmi les multiples régions ;
dans lequel les réactifs comprennent deux paires d'amorces ou plus identifiées dans le tableau 15 et sélectionnées dans le groupe constitué de SEQ ID NO : 973 à 1008.

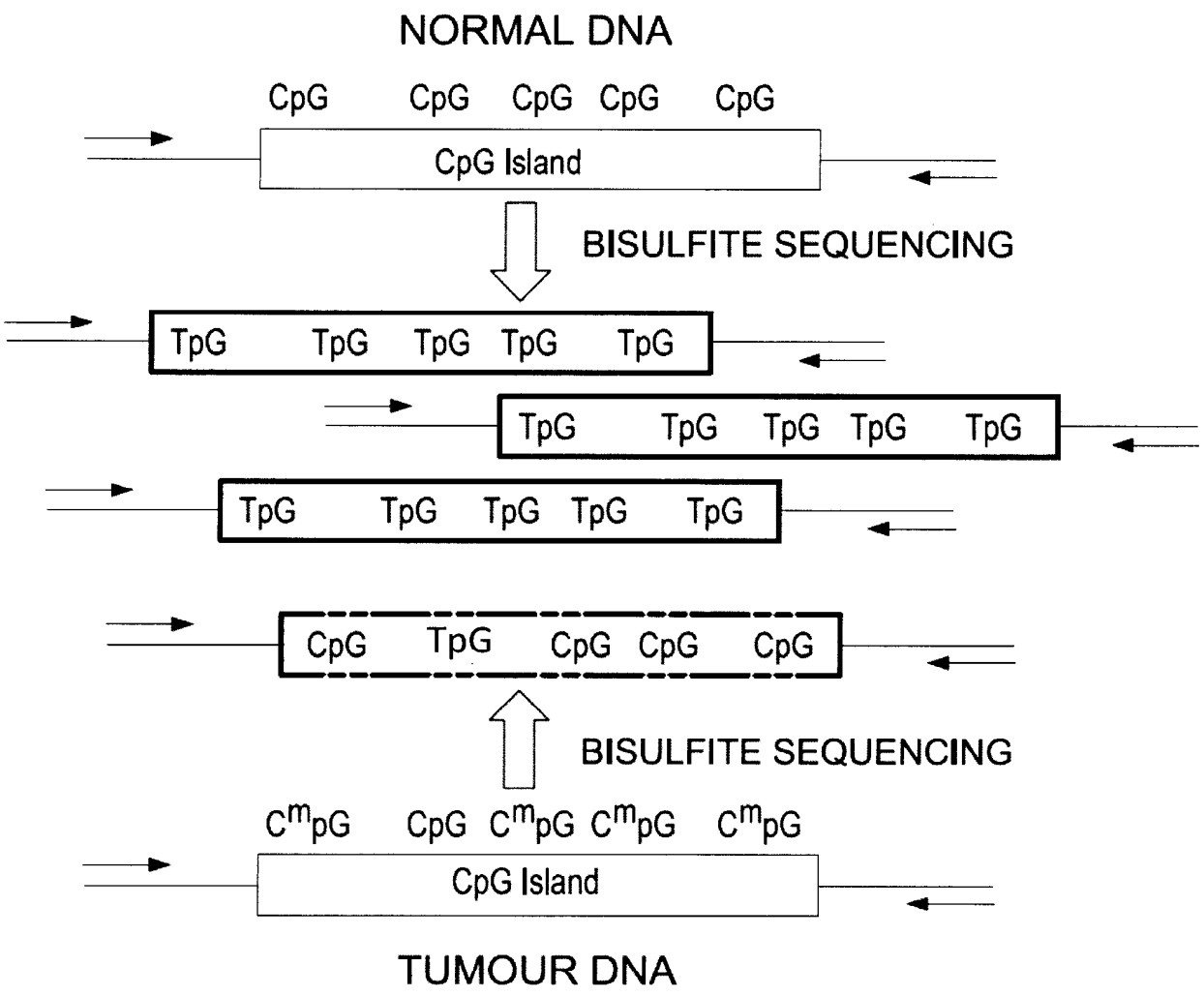

NORMAL DNA
CpG CpG CpG CpG CpG
CpG Island
BISULFITE SEQUENCING
TpG TpG TpG TpG TpG
TpG TpG TpG TpG TpG
TpG TpG TpG TpG TpG
CpG TpG CpG CpG CpG
BISULFITE SEQUENCING
CmpG CpG CmpG CmpG CmpG
CpG Island
TUMOUR DNA

Fig. 1

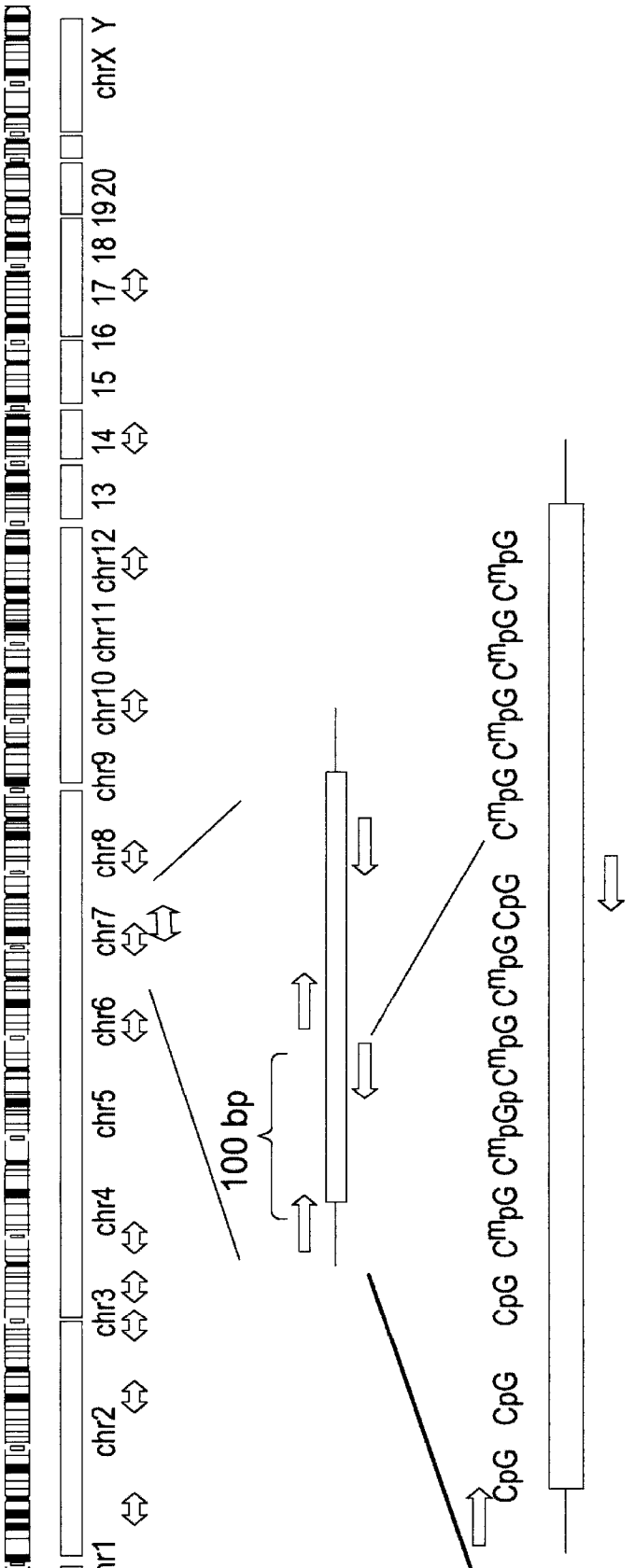

chr1
chr2
chr3
chr4
chr5
chr6
chr7
chr8
chr9
chr10
chr11
chr12
13
14
15
16
17
18
19
20
chrX
Y
100 bp
CpG
CpG
CpG
CmpG
CmpGp
CmpG
CmpG
CpG
CmpG
CmpG
CmpG
CmpG

Fig. 2

	Area	Std. Error	95% CI	P value
cg03257575	0.8773	0.01278	0.8523 to 0.9024	< 0.0001
cg25764893	0.95	0.008397	0.9335 to 0.9664	< 0.0001
cg25191623	0.9261	0.01008	0.9064 to 0.9459	< 0.0001
cg22336004	0.9098	0.01053	0.8892 to 0.9305	< 0.0001
cg12071883	0.9168	0.01006	0.8971 to 0.9365	< 0.0001
cg04550737	0.9012	0.01089	0.8799 to 0.9225	< 0.0001
cg22877503	0.8984	0.01146	0.8759 to 0.9209	< 0.0001
cg17816394	0.9136	0.01019	0.8936 to 0.9336	< 0.0001
cg05099503	0.9182	0.01046	0.8977 to 0.9387	< 0.0001
cg22831607	0.8664	0.01502	0.8369 to 0.8958	< 0.0001
cg21384402	0.8963	0.01125	0.8742 to 0.9183	< 0.0001
cg15556502	0.9087	0.01014	0.8888 to 0.9286	< 0.0001
cg19897940	0.8473	0.01317	0.8214 to 0.8731	< 0.0001
cg13691247	0.8976	0.01212	0.8738 to 0.9213	< 0.0001
cg13879483	0.9415	0.008398	0.9250 to 0.9579	< 0.0001
cg01940855	0.8454	0.0144	0.8171 to 0.8736	< 0.0001
cg27398263	0.865	0.01322	0.8391 to 0.8909	< 0.0001
cg13631572	0.8556	0.01321	0.8297 to 0.8815	< 0.0001
cg04368094	0.8967	0.01107	0.8750 to 0.9184	< 0.0001
cg05527869	0.8242	0.01534	0.7941 to 0.8543	< 0.0001
cg03348973	0.8991	0.01093	0.8776 to 0.9205	< 0.0001
cg20945565	0.8479	0.01321	0.8220 to 0.8738	< 0.0001
cg15146853	0.9161	0.01115	0.8942 to 0.9379	< 0.0001
cg06537894	0.9244	0.00991	0.9050 to 0.9439	< 0.0001
cg22473620	0.8525	0.01338	0.8263 to 0.8787	< 0.0001
cg00778995	0.8738	0.01229	0.8497 to 0.8979	< 0.0001
cg13356895	0.8268	0.01579	0.7959 to 0.8578	< 0.0001
cg23448584	0.8714	0.01422	0.8435 to 0.8993	< 0.0001
cg08260891	0.9309	0.00953	0.9122 to 0.9495	< 0.0001
cg19127283	0.8919	0.01179	0.8688 to 0.9150	< 0.0001
cg00442112	0.8498	0.014	0.8223 to 0.8772	< 0.0001
cg14866200	0.8071	0.01626	0.7753 to 0.8390	< 0.0001
cg24154839	0.958	0.00663	0.9450 to 0.9710	< 0.0001
cg03205103	0.9322	0.009127	0.9143 to 0.9501	< 0.0001
cg18148997	0.9154	0.01079	0.8942 to 0.9365	< 0.0001
cg08516515	0.9184	0.01039	0.8980 to 0.9387	< 0.0001
cg05766140	0.908	0.01147	0.8855 to 0.9305	< 0.0001
cg14151259	1	0	1.000 to 1.000	< 0.0001
cg00124375	0.8762	0.01189	0.8529 to 0.8995	< 0.0001
cg25459553	0.8939	0.01131	0.8718 to 0.9161	< 0.0001
cg27363327	0.8514	0.01377	0.8244 to 0.8784	< 0.0001
cg13315970	0.8697	0.01282	0.8446 to 0.8948	< 0.0001
cg21684012	0.8854	0.01139	0.8631 to 0.9078	< 0.0001
cg16924337	0.8455	0.01437	0.8173 to 0.8736	< 0.0001
cg14045872	0.8401	0.01494	0.8108 to 0.8694	< 0.0001
cg00158523	0.8997	0.01093	0.8782 to 0.9211	< 0.0001
cg25832771	0.8646	0.01259	0.8399 to 0.8893	< 0.0001

Fig. 3

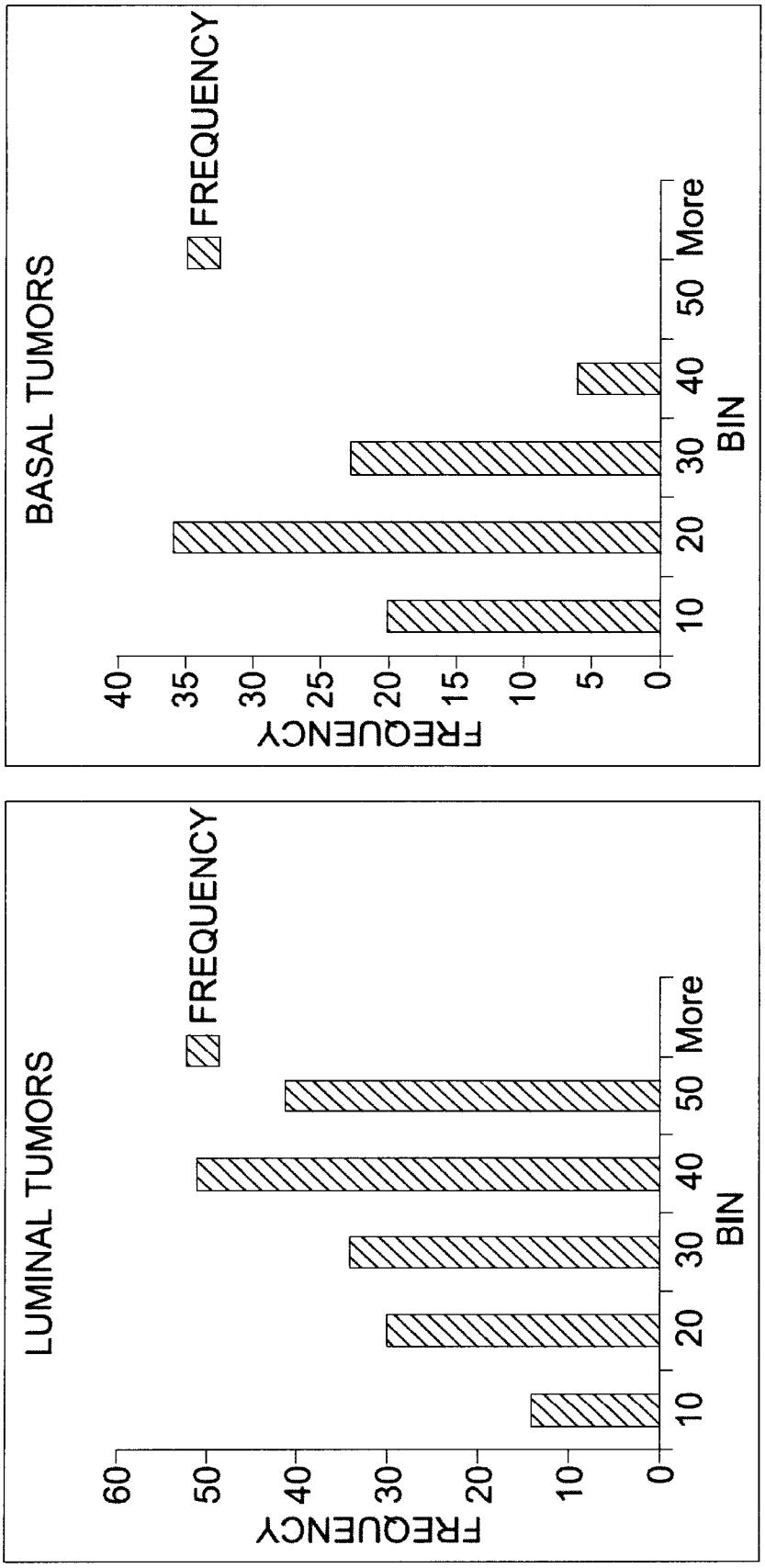

LUMINAL TUMORS
FREQUENCY
FREQUENCY
BIN
0
10
20
30
40
50
More
0
10
20
30
40
50
60
PANEL A
BASAL TUMORS
FREQUENCY
FREQUENCY
BIN
0
10
20
30
40
50
More
0
5
10
15
20
25
30
35
40
PANEL B

Fig. 4

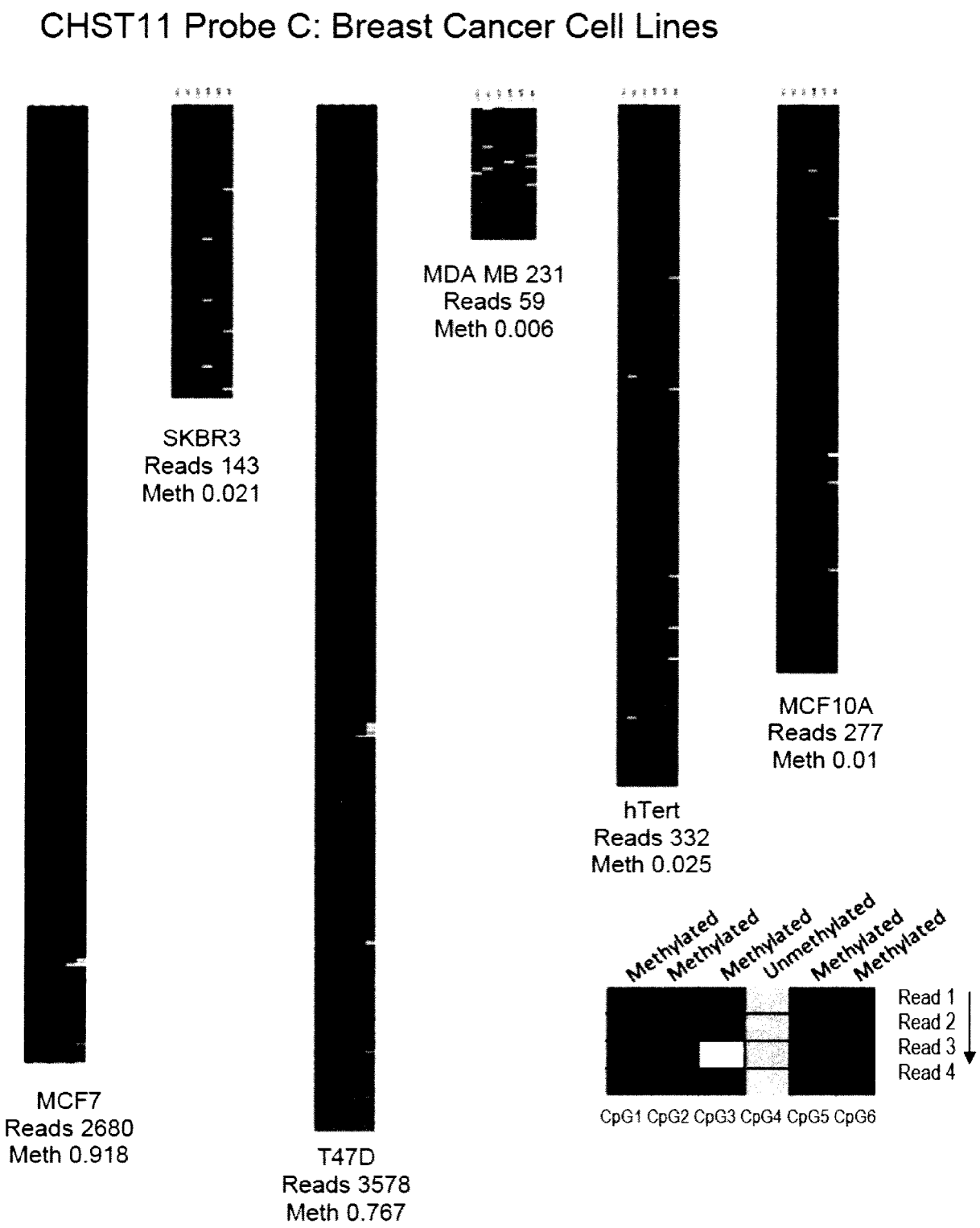

CHST11 Probe C: Breast Cancer Cell Lines
MCF7
Reads 2680
Meth 0.918
SKBR3
Reads 143
Meth 0.021
T47D
Reads 3578
Meth 0.767
MDA MB 231
Reads 59
Meth 0.006
hTert
Reads 332
Meth 0.025
MCF10A
Reads 277
Meth 0.01
Methylated
Methylated
Methylated
Unmethylated
Methylated
Methylated
Read 1
Read 2
Read 3
Read 4
CpG1 CpG2 CpG3 CpG4 CpG5 CpG6

Fig. 5

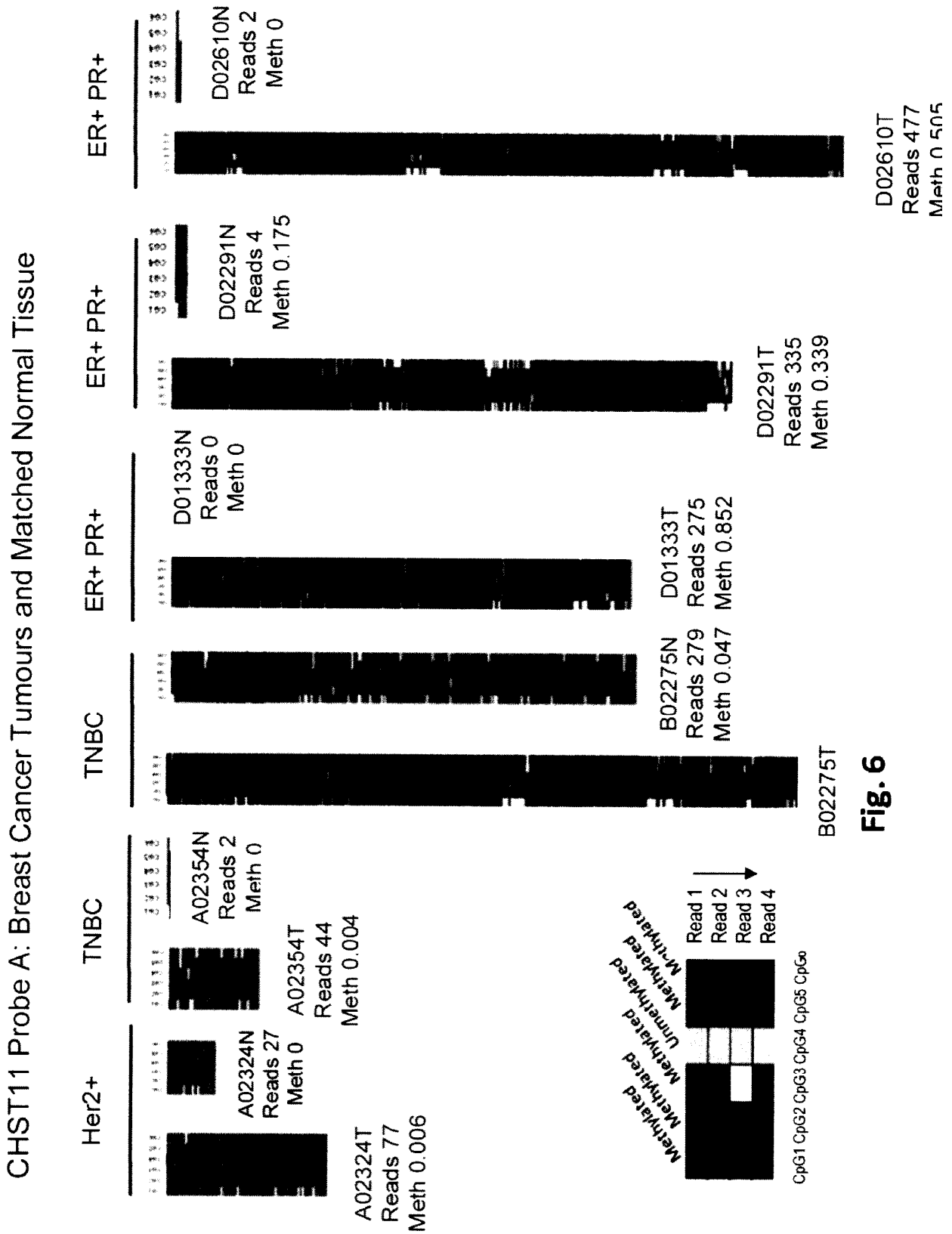

CHST11 Probe A: Breast Cancer Tumours and Matched Normal Tissue
Her2+
TNBC
TNBC
ER+ PR+
ER+ PR+
ER+ PR+
A02324N
Reads 27
Meth 0
A02354N
Reads 2
Meth 0
D01333N
Reads 0
Meth 0
D02291N
Reads 4
Meth 0.175
D02610N
Reads 2
Meth 0
A02324T
Reads 77
Meth 0.006
A02354T
Reads 44
Meth 0.004
B02275N
Reads 279
Meth 0.047
D01333T
Reads 275
Meth 0.852
D02291T
Reads 335
Meth 0.339
D02610T
Reads 477
Meth 0.505
B02275T
Methylated
Methylated
Methylated
Unmethylated
Methylated
Methylated
Read 1
Read 2
Read 3
Read 4
CpG1 CpG2 CpG3 CpG4 CpG5 CpG6

Fig. 6

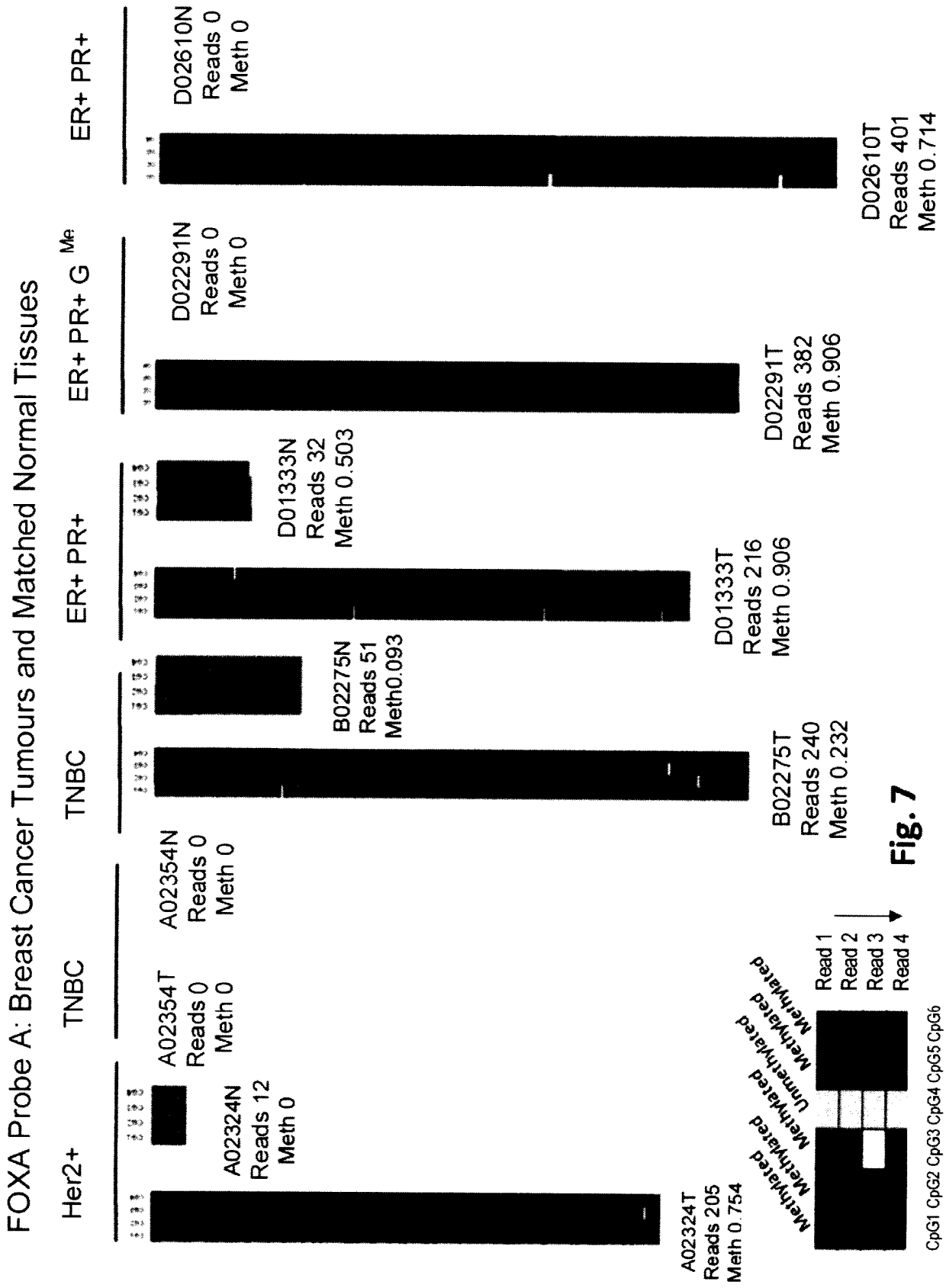

FOXA Probe A: Breast Cancer Tumours and Matched Normal Tissues
Her2+
TNBC
TNBC
ER+ PR+
ER+ PR+ G Me
ER+ PR+
A02324N
Reads 12
Meth 0
A02354T
Reads 0
Meth 0
A02354N
Reads 0
Meth 0
B02275N
Reads 51
Meth0.093
D01333N
Reads 32
Meth 0.503
D02291N
Reads 0
Meth 0
D02610N
Reads 0
Meth 0
A02324T
Reads 205
Meth 0.754
B02275T
Reads 240
Meth 0.232
D01333T
Reads 216
Meth 0.906
D02291T
Reads 382
Meth 0.906
D02610T
Reads 401
Meth 0.714
Methylated
Methylated
Methylated
Unmethylated
Methylated
Methylated
Read 1
Read 2
Read 3
Read 4
CpG1 CpG2 CpG3 CpG4 CpG5 CpG6

Fig. 7

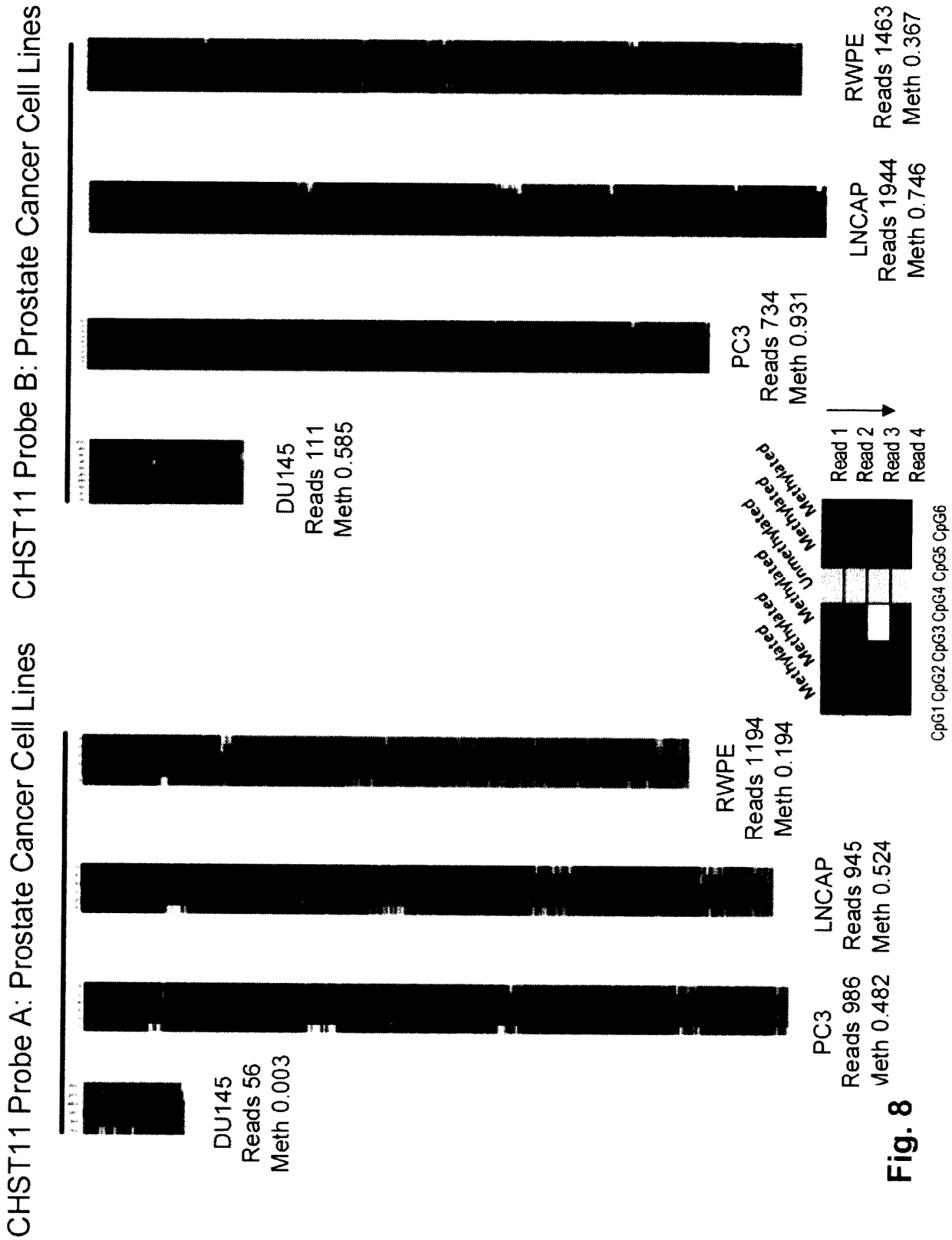

CHST11 Probe A: Prostate Cancer Cell Lines
DU145
Reads 56
Meth 0.003
PC3
Reads 986
Meth 0.482
LNCAP
Reads 945
Meth 0.524
RWPE
Reads 1194
Meth 0.194
CHST11 Probe B: Prostate Cancer Cell Lines
DU145
Reads 111
Meth 0.585
PC3
Reads 734
Meth 0.931
LNCAP
Reads 1944
Meth 0.746
RWPE
Reads 1463
Meth 0.367
Methylated
Methylated
Unmethylated
Methylated
Methylated
Read 1
Read 2
Read 3
Read 4
CpG1 CpG2 CpG3 CpG4 CpG5 CpG6

Fig. 8

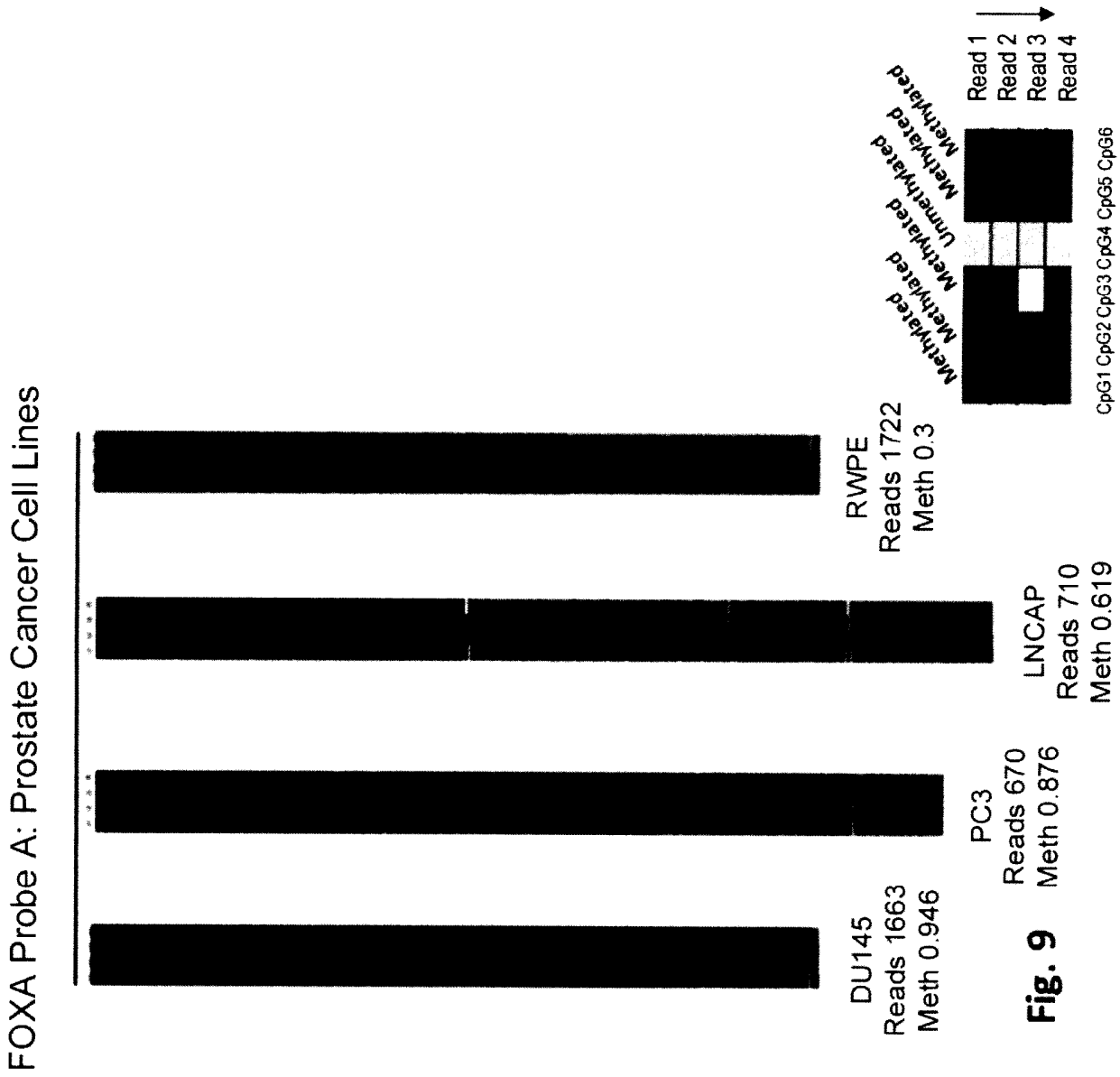

FOXA Probe A: Prostate Cancer Cell Lines
DU145
Reads 1663
Meth 0.946
PC3
Reads 670
Meth 0.876
LNCAP
Reads 710
Meth 0.619
RWPE
Reads 1722
Meth 0.3
Methylated
Methylated
Methylated
Unmethylated
Methylated
Methylated
Read 1
Read 2
Read 3
Read 4
CpG1 CpG2 CpG3 CpG4 CpG5 CpG6

Fig. 9

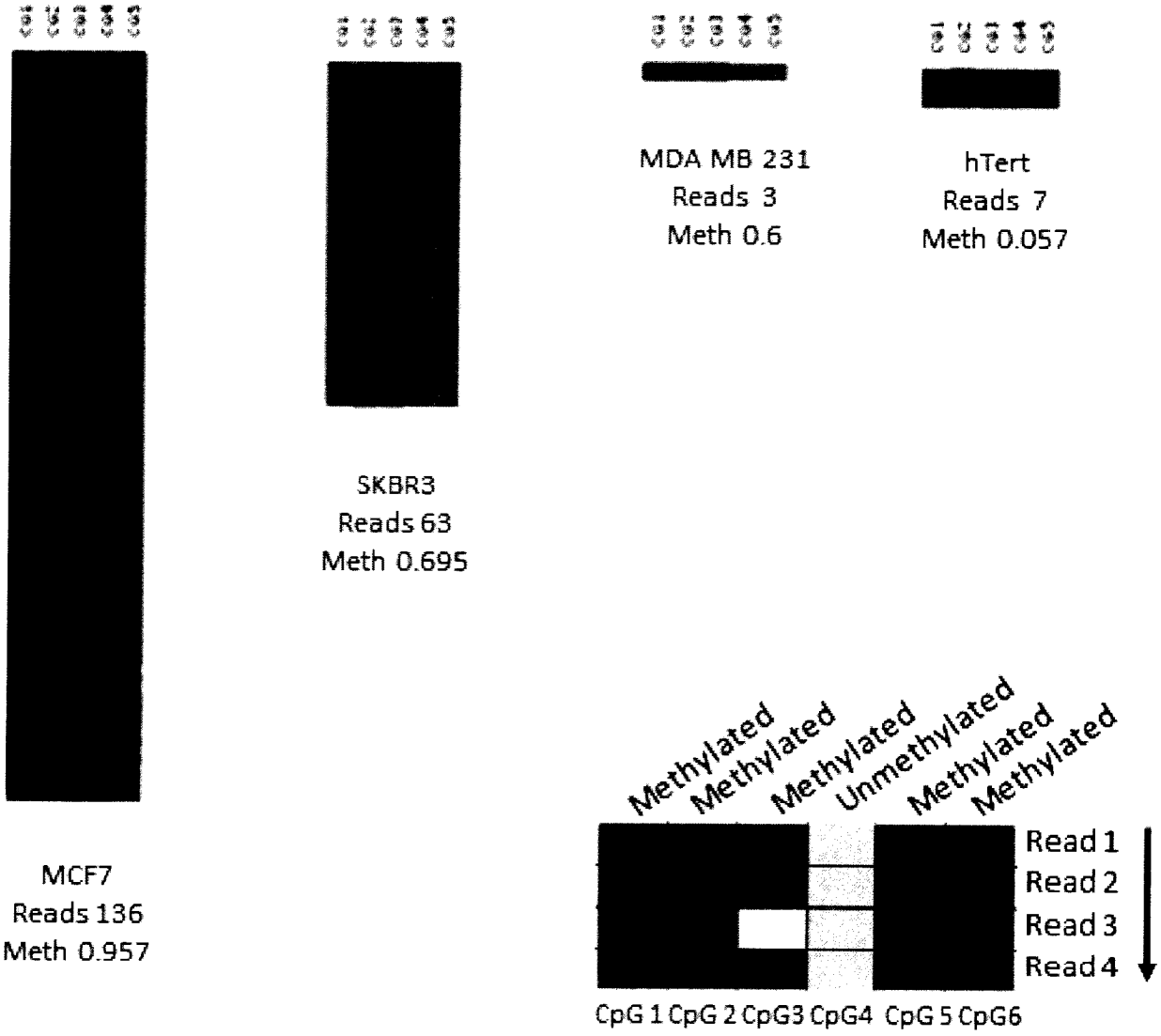

NT5 Probe E: Breast Cancer Cell Lines
MCF7
Reads 136
Meth 0.957
SKBR3
Reads 63
Meth 0.695
MDA MB 231
Reads 3
Meth 0.6
hTert
Reads 7
Meth 0.057
Methylated
Methylated
Methylated
Unmethylated
Methylated
Methylated
Read 1
Read 2
Read 3
Read 4
CpG 1 CpG 2 CpG3 CpG4 CpG 5 CpG6

Fig. 10

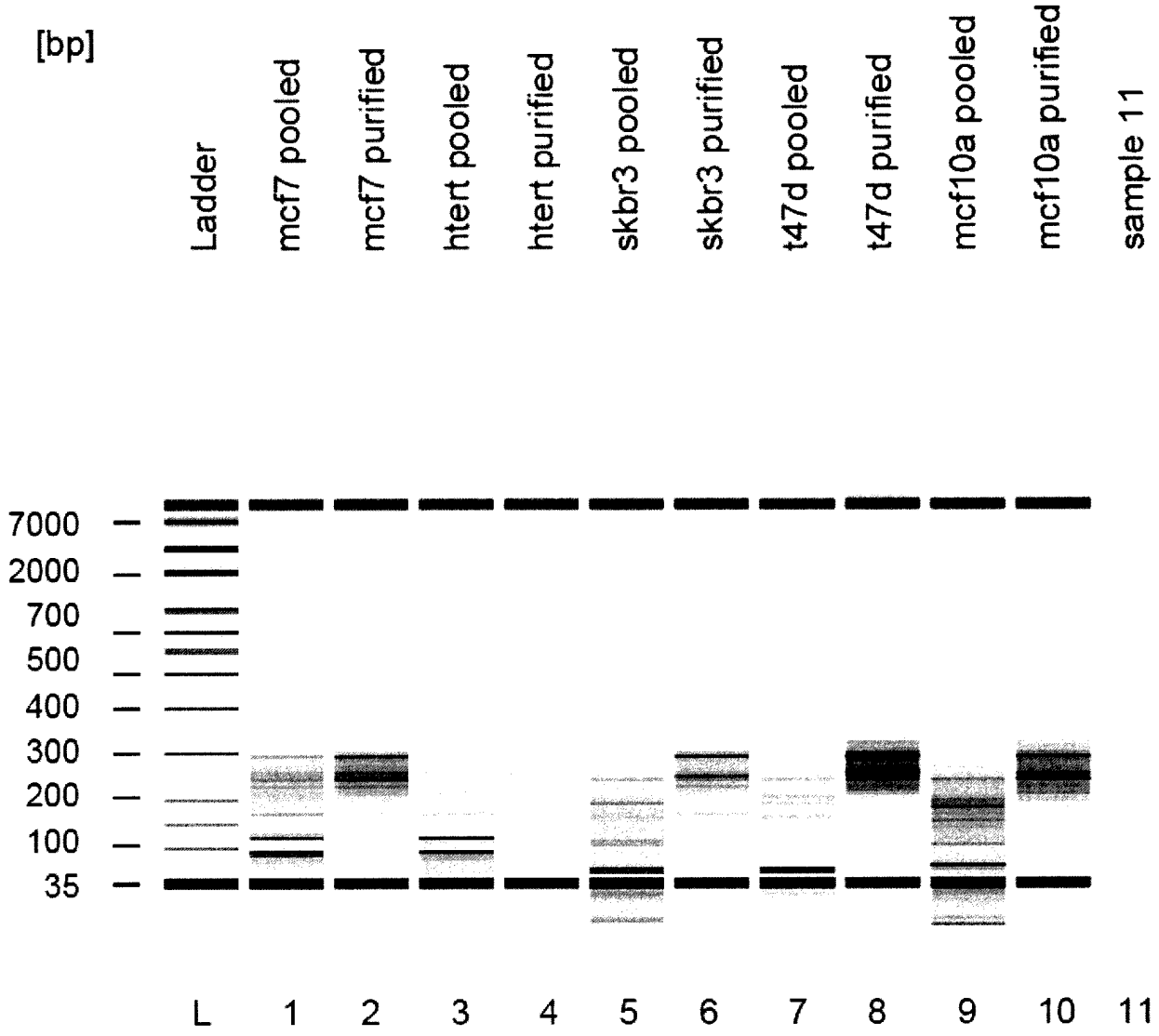

[bp]
Ladder
mcf7 pooled
mcf7 purified
htert pooled
htert purified
skbr3 pooled
skbr3 purified
t47d pooled
t47d purified
mcf10a pooled
mcf10a purified
sample 11
7000
2000
700
500
400
300
200
100
35
L
1
2
3
4
5
6
7
8
9
10
11

Fig. 11

Reference	MCF7 # Reads	MCF7 Mean Me	SKBR3 # Reads	SKBR3 Mean Me	T47D # Reads	T47D Mean Me	MDA MB 231 # Reads	MDA MB 231 Mean Me	MCF10A # Reads	MCF10A Mean Me	hTert # Reads	hTert Mean Me
ADCYFtrim	204	0.968	351	0.975	334	0.965	40	0.781	603	0.475	175	0.548
ADCYGtrim	121	0.975	219	0.981	218	0.967	30	0.862	427	0.522	121	0.627
ADCYHtrim	0	0	0	0	0	0	0	0	0	0	0	0
C1Dtrim	1597	0.942	1184	0.922	1047	0.832	418	0.916	215	0.575	104	0.933
C1Etrim	1423	0.784	1976	0.68	726	0.762	746	0.999	2289	0.675	118	0.149
C1Ftrim	1545	0.937	1156	0.919	995	0.8	418	0.916	214	0.758	100	0.917
C1Gtrim	1477	0.615	1980	0.646	890	0.529	568	0.928	700	0.501	158	0.032
MIRBtrim	300	0.783	383	0.024	315	0.711	311	0.78	507	0.251	458	0.073
MIRCtrim	374	0.916	448	0.565	471	0.884	118	0.559	858	0.49	929	0.643
MIRDtrim	145	0.954	6	0.148	258	0.856	118	0.989	134	0.189	10	0.126
MIREtrim	2078	0.976	524	0.614	1295	0.965	536	0.946	1818	0.484	172	0.305
MIRFtrim	170	0.895	68	0.196	115	0.798	14	0.801	144	0.279	167	0.397
VWCJtrim	635	0.915	195	0.239	1329	0.633	205	0.903	261	0.622	102	0.281
VWCKtrim	44	0.975	23	0.279	98	0.764	7	0.971	114	0.599	15	0.603
VWCLtrim	1786	0.909	747	0.472	2805	0.733	125	0.896	4493	0.806	2253	0.566
VWCMtrim	952	0.964	632	0.275	943	0.784	1051	0.788	1408	0.767	63	0.67
VWCNtrim	1062	0.934	220	0.254	1764	0.603	291	0.855	326	0.659	111	0.39
CHSAtrim	223	0.914	66	0.012	259	0.485	7	0.048	191	0.009	52	0.007
CHSBtrim	1387	0.928	5	0.02	1840	0.776	0	0	2	0	30	0
CHSCtrim	1847	0.918	108	0.016	2421	0.772	59	0.006	205	0.007	220	0.021
CHSDtrim	1357	0.928	5	0.02	1761	0.778	0	0	3	0	30	0
DMBAtrim	0	0	0	0	2	0.269	0	0	0	0	0	0
DMBBtrim	1120	0.965	113	0.098	1117	0.956	885	0.955	1792	0.803	2976	0.515
DMBCtrim	795	0.971	105	0.162	874	0.959	814	0.964	1404	0.799	2324	0.553
FOXAtrim	481	0.871	417	0.636	362	0.741	169	0.001	280	0.512	50	0.143
FOXBtrim	30	0.878	0	0	123	0.79	39	0.043	4	0.167	49	0.418
FOXCtrim	0	0	0	0	0	0	1	0	0	0	0	0
FOXDtrim	316	0.97	321	0.9	496	0.885	450	0.329	259	0.696	627	0.783
FOXEtrim	636	0.98	1040	0.951	938	0.956	800	0.88	588	0.881	1128	0.906
HOXAAtrim	1969	0.949	1561	0.088	2319	0.726	796	0.902	1639	0.678	1911	0.734
HOXABtrim	2006	0.958	1703	0.305	2460	0.802	780	0.924	1676	0.714	1943	0.775
HOXACtrim	13	0.85	48	0.246	81	0.749	135	0.841	79	0.744	44	0.934
HOXADtrim	469	0.935	1108	0.395	701	0.829	173	0.911	543	0.295	497	0.368
SFRAtrim	594	0.901	220	0.123	667	0.884	662	0.978	641	0.565	491	0.252
SFRBtrim	1061	0.959	0	0	1046	0.946	325	0.997	778	0.719	629	0.4
SFRCtrim	1256	0.984	173	0.306	1254	0.968	1292	0.998	853	0.689	458	0.633
SFRDtrim	643	0.867	231	0.023	728	0.823	731	0.926	666	0.49	503	0.219
SFREtrim	1195	0.955	0	0	1170	0.936	307	0.997	875	0.62	815	0.137
TTBAtrim	419	0.968	100	0.04	674	0.77	400	0.766	205	0.206	478	0.343
TTBBtrim	381	0.946	0	0	457	0.74	242	0.933	9	0.4	383	0.134
TTBCtrim	198	0.967	1	1	199	0.64	59	0.844	15	0.4	42	0.291
TTBDtrim	656	0.974	4	0.2	557	0.866	377	0.957	19	0.649	25	0.874
4th Generation												
mbBARHL2 Trim	73	0.968	50	0.515			76	0.961	811	0.883	159	0.976
mbBOLL Trim	46	0.974	53	0.954			16	0.935	234	0.759	54	0.873
mbC5orf Trim	26	0.832	14	0.842			15	0.886	1077	0.983	661	0.745
mbCDO Trim	44	0.95	59	0.556			48	0.988	523	0.517	373	0.95
mbCOL1 Trim	297	0.966	522	0.857			496	0.984	917	0.841	348	0.552
mbCYTL Trim	23	0.804	3	0.778			4	0.25	251	0.561	118	0.164
mbDDAH Trim	5	0.4	0	0			0	0	59	0.571	1	0.4
mbDMRTA Trim	48	0.964	59	0.199			106	0.907	517	0.811	346	0.714
mbEGFLAM Trim	76	0.936	11	0			66	0.867	826	0.632	15	0.35
mbGABRA A Trim	1	0.909	0	0			11	0.873	15	0.713	4	0.907
mbGABRA B Trim	55	0.786	26	0.433			54	0.854	557	0.682	78	0.451
mbGNG Trim	67	0.971	45	0.859			44	0.966	222	0.931	131	0.974
mbID4 Trim	40	0.967	111	0.81			42	0.607	264	0.909	91	0.6
mbIRF Trim	151	0.953	0	0			112	0.895	593	0.731	81	0.318
mbNT5E Trim	136	0.957	63	0.695			3	0.6	502	0.757	0	0
mbPDE4 Trim	58	0.922	25	0.485			40	1	503	0.691	93	0.625
mbPOU3F Trim	178	0.776	122	0.432			132	0.59	1439	0.695	382	0.656
mbRUSC Trim	18	0.902	71	0.902			27	0.984	916	0.946	136	0.514
mbSCAND Trim	111	0.014	116	0.022			249	0.808	463	0.044	238	0.039

Fig. 12A

Reference	MCF7 Reads Exp.	MCF7 Mn. Meth.	SKBR3 Reads Exp.	SKBR3 Mn. Meth.	T47D Reads Exp.	T47D Mn. Meth.	MDA MB 231 Reads Exp.	MDA MB 231 Mn. Meth.	MCF10A Reads Exp.	MCF10A Mn. Meth.	hTert Reads Exp.	hTert Mn. Meth.
mbSHISA Trim	208	0.958	0	0			0	0	648	0.786	7	0.167
mbSIM A Trim	144	0.966	219	0.928			53	0.997	374	0.848	228	0.742
mbSIM B Trim	45	0.978	103	0.728			113	0.997	293	0.944	334	0.652
mbSLC Trim	236	0.96	38	0.23			8	0	740	0.938	52	0.976
mbTAL Trim	300	0.939	115	0.467			84	0.699	351	0.664	411	0.772
mbTBX15 Trim	51	0.954	9	0.358			41	0.897	144	0.516	76	0.505
mbTRIM A Trim	21	0.939	40	0.619			23	0.664	77	0.608	65	0.422
mbTRIM B Trim	118	0.97	167	0.892			95	0.915	298	0.735	133	0.619
pbBARHL Trim	55	0.932	69	0.222			94	0.83	515	0.762	138	0.779
pbBOLL Trim	35	0.942	18	0.719			18	0.681	151	0.803	336	0.66
pbCDO Trim	56	0.852	81	0.149			62	0.824	176	0.244	147	0.569
pbCOL1 Trim	302	0.965	522	0.867			496	0.985	890	0.863	348	0.615
pbCYTL Trim	39	0.842	29	0.913			7	0.25	207	0.239	60	0.104
pbCorf Trim	26	0.86	21	0.838			13	0.904	1171	0.979	785	0.687
pbDDAH Trim	5	0.4	0	0			0	0	60	0.585	1	0
pbDMRTA Trim	61	0.956	31	0.276			46	0.977	83	0.583	64	0.732
pbEGFLAM Trim	43	0.944	0	0			40	0.879	256	0.664	13	0.528
pbGABRA Trim	161	0.717	68	0.222			114	0.849	1092	0.686	156	0.417
pbGNG Trim	125	0.952	116	0.869			48	1	359	0.794	107	0.988
pbID4 A Trim	44	0.96	104	0.911			36	0.847	562	0.944	242	0.805
pbID4 B Trim	70	0.929	159	0.909			4	0.663	318	0.977	43	0.902
pbIRF4 Trim	79	0.96	0	0			36	0.961	392	0.847	211	0.703
pbPDE4 Trim	33	0.735	20	0			22	0.659	720	0.638	149	0.675
pbPOU3F Trim	196	0.696	135	0.056			144	0.184	1591	0.241	427	0.289
pbRUSC Trim	19	0.885	73	0.907			23	0.981	1131	0.942	172	0.526
pbSCAND Trim	108	0.012	117	0.019			248	0.809	461	0.044	236	0.037
pbSHISA Trim	205	0.945	0	0			0	0	611	0.868	8	0
pbSIM A Trim	210	0.97	330	0.943			58	1	699	0.969	520	0.726
pbSIM B Trim	55	0.919	75	0.673			35	0.932	261	0.897	128	0.879
pbSIM C Trim	165	0.953	261	0.89			49	0.995	936	0.582	701	0.512
pbSLC Trim	70	0.952	38	0.553			0	0	297	0.76	226	0.965
pbTAL Trim	100	0.952	8	0			78	0.752	326	0.513	138	0.443
pbTBX15 Trim	1	0.875	0	0			0	0	4	0.975	0	0
pbTRIM Trim	133	0.967	200	0.957			112	0.907	689	0.822	221	0.603

Fig. 12B

Reference	A02324 T HER2 + # Reads	A02324 T HER2 + Mean Me	A02324 N HER2 + # Reads	A02324 N HER2 + Mean Me	A02354 T TRIPLE - # Reads	A02354 T TRIPLE - Mean Me	A02354 N TRIPLE - # Reads	A02354 N TRIPLE - Mean Me	B02275 T TRIPLE - GR + # Reads	B02275 T TRIPLE - GR + Mean Me	B02275 N TRIPLE - GR + Mean Me	B02275 N TRIPLE - GR + Mean Me
ADCYFtrim	16	0.991	0	0	0	0	4	1	125	0.791	251	0.529
ADCYGtrim	10	0.987	0	0	0	0	3	1	96	0.832	210	0.62
ADCYHtrim	0	0	0	0	0	0	0	0	0	0	0	0
C1Dtrim	344	0.843	45	0.776	0	0	0	0	955	0.912	556	0.549
C1Etrim	393	0.347	728	0.036	193	0.007	1	0	663	0.778	753	0.359
C1Ftrim	331	0.741	51	0.912	0	0	0	0	913	0.889	550	0.501
C1Gtrim	378	0.439	73	1	0	0	0	0	504	0.892	246	0.581
MIRBtrim	557	0.099	485	0.031	136	0.001	499	0.027	253	0.471	247	0.054
MIRCtrim	23	0.64	286	0.084	175	0.094	9	0	130	0.735	328	0.242
MIRDtrim	0	0	0	0	63	0	31	0	213	0.868	144	0.287
MIREtrim	248	0.996	80	1	0	0	191	0.026	475	0.944	685	0.355
MIRFtrim	88	0.214	92	0.012	112	0.044	603	0.082	30	0.383	39	0.061
VWCJtrim	1	0	78	0.55	32	0.071	2	0.5	604	0.879	351	0.3
VWCKtrim	0	0	9	0.269	0	0	0	0	248	0.915	39	0.62
VWCLtrim	0	0	3	0	339	0.011	0	0	697	0.925	476	0.47
VWCMtrim	0	0	0	0	92	0.153	0	0	454	0.981	234	0.673
VWCNtrim	1	0.2	93	0.658	34	0.273	2	0.6	809	0.912	403	0.425
CHSAtrim	77	0.006	27	0	44	0.004	2	0	378	0.748	279	0.047
CHSBtrim	0	0	0	0	0	0	0	0	1039	0.946	240	0.34
CHSCtrim	125	0.256	46	0	36	0.046	583	0.748	1396	0.921	337	0.176
CHSDtrim	0	0	0	0	0	0	0	0	1037	0.946	241	0.339
DMBAtrim	0	0	0	0	0	0	0	0	0	0	0	0
DMBBtrim	1078	0.876	586	0.386	559	0.935	219	0.486	1078	0.887	544	0.373
DMBCtrim	1000	0.891	564	0.399	530	0.939	202	0.461	1014	0.889	506	0.399
FOXAtrim	205	0.745	12	0	0	0	0	0	240	0.232	51	0.093
FOXBtrim	155	0.355	32	0.078	54	0.09	2363	0.159	6	0.056	12	0.069
FOXCtrim	0	0	0	0	0	0	21	0.179	0	0	0	0
FOXDtrim	806	0.918	0	0	0	0	0	0	44	0.023	38	0
FOXEtrim	1139	0.956	0	0	185	0.072	173	0.888	341	0.157	268	0.173
HOXAAtrim	905	0.824	536	0.376	1899	0.696	781	0.589	1076	0.199	1163	0.178
HOXABtrim	896	0.824	529	0.466	1877	0.764	770	0.7	1079	0.225	1153	0.265
HOXACtrim	31	0.987	78	0.379	190	0.668	67	0.973	107	0.055	118	0.469
HOXADtrim	189	0.181	189	0.056	299	0.294	121	0.671	750	0.124	639	0.026
SFRAtrim	353	0.351	798	0.094	410	0.104	192	0.089	520	0.61	403	0.339
SFRBtrim	836	0.667	0	0	0	0	583	0.998	591	0.94	168	0.72
SFRCtrim	331	0.633	700	0.277	370	0.282	1830	0.937	1006	0.671	468	0.557
SFRDtrim	375	0.232	881	0.027	458	0.028	212	0.018	553	0.636	435	0.326
SFREtrim	831	0.508	0	0	0	0	595	0.999	578	0.927	169	0.58
TTBAtrim	35	0	12	0.15	157	0.276	163	0.79	434	0.691	150	0.098
TTBBtrim	1	0.4	0	0	0	0	125	0.95	131	0.937	62	0.21
TTBCtrim	0	0	0	0	1	0.333	1	0.25	69	0.782	8	0.588
TTBDtrim	0	0	0	0	0	0	0	0	361	0.888	126	0.484

Fig. 13A

Reference	D01333 T ER+ PR+ # Reads	D01333 T ER+ PR+ Mean Me	D01333 N ER+ PR+ # Reads	D01333 N ER+ PR+ Mean Me	D02291 T ER+ PR+ GR+ # Reads	D02291 T ER+ PR+ GR+ Mean Me	D02291 N ER+ PR+ GR+ # Reads	D02291 N ER+ PR+ GR+ Mean Me	D02610 T ER+ PR+ # Reads	D02610 T ER+ PR+ Mean Me	D02610 N ER+ PR+ # Reads	D02610 N ER+ PR+ Mean Me
ADCYFtrim	33	0.896	5	0.92	30	0.966	0	0	216	0.776	13	0
ADCYGtrim	21	0.905	4	1	23	0.98	0	0	131	0.84	14	0.217
ADCYHtrim	0	0	0	0	0	0	0	0	0	0	0	0
C1Dtrim	170	0.923	14	0.99	342	0.967	27	0.425	1009	0.968	35	0.095
C1Etrim	355	0.802	77	0.148	529	0.943	82	0.032	808	0.906	506	0.621
C1Ftrim	165	0.926	13	0.981	326	0.964	26	0.506	986	0.97	40	0
C1Gtrim	218	0.578	14	0.381	332	0.981	0	0	409	0.882	0	0
MIRBtrim	103	0.611	435	0.121	156	0.061	8	0	530	0.077	65	0.005
MIRCtrim	103	0.724	47	0.049	87	0.918	28	0.257	231	0.621	5	0
MIRDtrim	91	0.782	10	0.017	52	0.775	0	0	159	0.31	70	0.149
MIREtrim	203	0.924	5	0	208	0.832	0	0	393	0.925	184	0.836
MIRFtrim	27	0.446	36	0.052	3	0.167	24	0.063	112	0.04	56	0
VWCJtrim	56	0.969	3	0.051	184	0.758	0	0	48	0.174	6	0.013
VWCKtrim	30	0.98	0	0	29	0.914	0	0	0	0	0	0
VWCLtrim	399	0.976	0	0	422	0.901	0	0	245	0.766	0	0
VWCMtrim	70	0.977	3	0.267	243	0.65	0	0	50	0.242	6	0.217
VWCNtrim	275	0.852	0	0	335	0.339	4	0.175	477	0.505	2	0
CHSAtrim	486	0.973	0	0	588	0.739	0	0	125	0.802	0	0
CHSBtrim	572	0.968	3	0	702	0.763	0	0	330	0.751	4	0
CHSCtrim	483	0.972	0	0	589	0.737	0	0	125	0.802	0	0
CHSDtrim	0	0	0	0	0	0	0	0	0	0	0	0
DMBAtrim	500	0.896	175	0.65	424	0.97	4	0	1359	0.745	0	0
DMBBtrim	470	0.909	147	0.61	400	0.976	4	0	1291	0.771	0	0
DMBCtrim	216	0.906	32	0.503	382	0.906	0	0	401	0.714	0	0
FOXAtrim	4	0.333	70	0.19	12	0.417	35	0.01	53	0.305	1	0
FOXBtrim	0	0	1	0	0	0	0	0	0	0	0	0
FOXCtrim	100	0.96	0	0	371	0.91	0	0	499	0.878	0	0
FOXDtrim	213	0.978	9	0.667	511	0.966	0	0	908	0.93	0	0
FOXEtrim	291	0.704	277	0.205	748	0.776	3	0.133	1055	0.118	67	0.051
HOXAAtrim	292	0.753	277	0.423	746	0.796	3	0.25	1052	0.25	66	0.078
HOXABtrim	43	0.738	19	0.189	194	0.61	0	0	296	0.453	0	0
HOXACtrim	106	0.619	34	0.306	245	0.614	3	0.293	518	0.224	60	0.003
HOXADtrim	308	0.685	104	0.078	447	0.649	48	0.082	594	0.339	0	0
SFRAtrim	320	0.967	79	0.881	438	0.742	0	0	534	0.825	0	0
SFRBtrim	407	0.811	87	0.238	726	0.907	45	0.237	1050	0.682	0	0
SFRCtrim	328	0.627	117	0.016	485	0.613	52	0.017	617	0.278	0	0
SFRDtrim	314	0.966	80	0.83	428	0.665	0	0	524	0.755	0	0
SFREtrim	221	0.553	54	0.119	240	0.408	1	0	178	0.036	15	0
TTBAtrim	60	0.81	0	0	138	0.527	0	0	39	0.046	0	0
TTBBtrim	116	0.355	0	0	79	0.417	0	0	1	0.25	0	0
TTBCtrim	106	0.751	0	0	155	0.5	0	0	0	0	0	0
TTBDtrim	341	0.963	0	0	356	0.995	0	0	0	0	0	0

Fig. 13B

	DU145	DU145	PC3	PC3	LNCAP	LNCAP	RWPE	RWPE
Reference	# Reads	Mean Me	# Reads	Mean Me	# Reads	Mean Me	# Reads	Mean Me
ADCYFtrim	216	0.916	213	0.459	199	0.903	71	0.297
ADCYGtrim	146	0.917	188	0.611	161	0.921	46	0.416
ADCYHtrim	4	0.847	0	0	5	0.795	0	0
C1Dtrim	548	0.916	616	0.95	941	0.954	58	0.063
C1Etrim	621	0.74	585	0.943	2458	0.956	292	0.023
C1Ftrim	526	0.93	591	0.936	872	0.942	61	0.049
C1Gtrim	736	0.78	523	0.721	1583	0.786	0	0
MIRBtrim	187	0.096	745	0.824	994	0.169	775	0.032
MIRCtrim	265	0.387	461	0.876	369	0.564	81	0.019
MIRDtrim	155	0.152	0	0	289	0.092	24	0.071
MIREtrim	566	0.377	1110	0.97	1190	0.547	31	0.032
MIRFtrim	56	0.1	173	0.873	62	0.095	200	0.021
VWCJtrim	494	0.948	891	0.676	766	0.836	379	0.103
VWCKtrim	208	0.956	44	0.911	36	0.877	8	1
VWCLtrim	1041	0.923	746	0.911	1419	0.851	675	0.405
VWCMtrim	1071	0.922	917	0.867	1083	0.88	1	0
VWCNtrim	657	0.938	1120	0.736	992	0.853	425	0.268
CHSAtrim	56	0.003	986	0.482	945	0.524	1194	0.194
CHSBtrim	111	0.585	734	0.931	1944	0.746	1463	0.367
CHSCtrim	345	0.246	1074	0.927	2539	0.762	1983	0.218
CHSDtrim	113	0.592	730	0.93	1933	0.745	1467	0.367
DMBAtrim	0	0	0	0	0	0	0	0
DMBBtrim	1046	0.977	1388	0.967	2759	0.892	3164	0.175
DMBCtrim	1026	0.98	1282	0.974	2597	0.911	3049	0.218
FOXAtrim	1663	0.946	670	0.876	710	0.619	1722	0.3
FOXBtrim	7	1	4	0.667	70	0.607	90	0.259
FOXCtrim	0	0	0	0	0	0	0	0
FOXDtrim	387	0.873	510	0.958	964	0.919	1974	0.438
FOXEtrim	1547	0.975	1336	0.964	1619	0.903	3207	0.709
HOXAAtrim	2289	0.965	2522	0.056	1952	0.46	2234	0.135
HOXABtrim	2298	0.972	2505	0.142	1939	0.598	2216	0.199
HOXACtrim	302	0.906	38	0	286	0.355	116	0.016
HOXADtrim	710	0.971	1741	0.134	603	0.607	1313	0.051
SFRAtrim	452	0.357	374	0.872	562	0.092	920	0.101
SFRBtrim	330	0.784	531	0.855	0	0	855	0.609
SFRCtrim	817	0.672	933	0.932	506	0.246	1400	0.133
SFRDtrim	484	0.283	412	0.832	611	0.021	1006	0.081
SFREtrim	321	0.719	514	0.896	18	0.993	846	0.428
TTBAtrim	823	0.673	351	0.63	1197	0.6	1262	0.111
TTBBtrim	301	0.752	374	0.818	343	0.822	295	0.723
TTBCtrim	161	0.678	29	0.548	453	0.483	51	0.634
TTBDtrim	890	0.693	428	0.854	845	0.799	508	0.809

Fig. 14

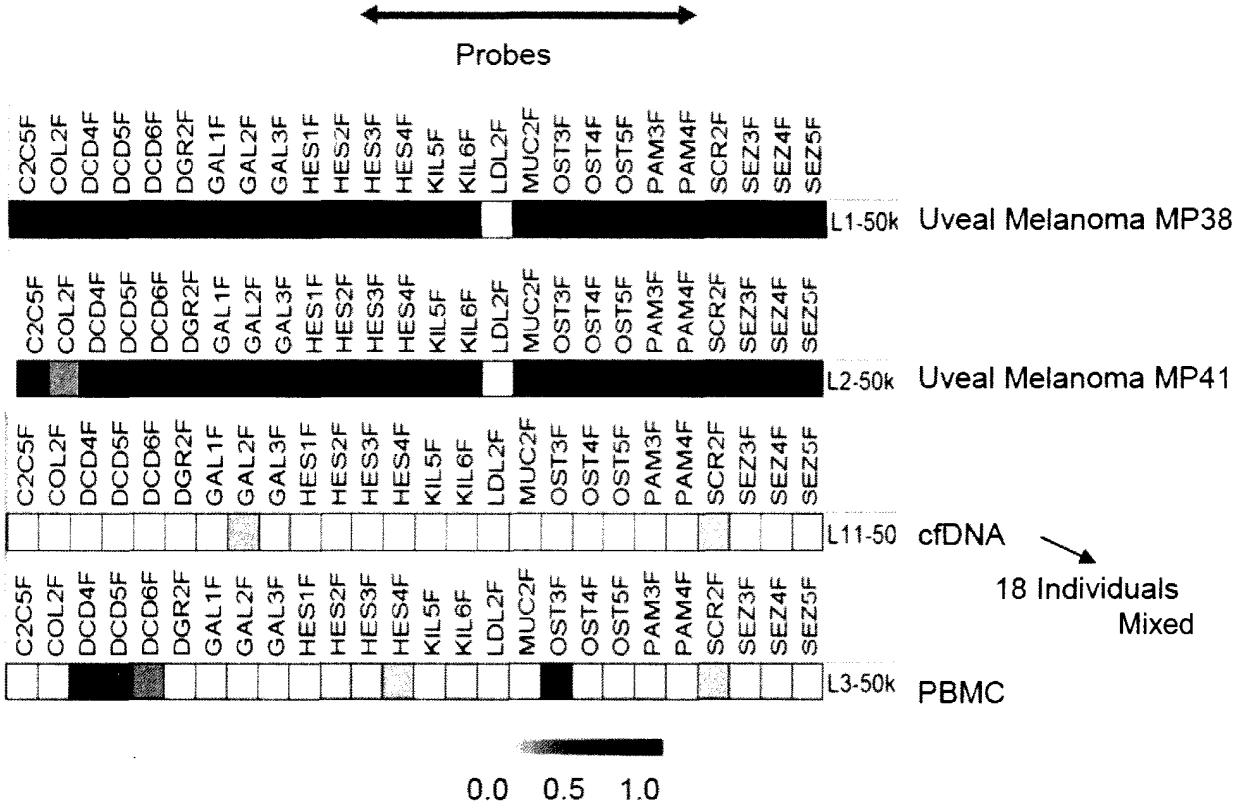

Probes
C2C5F COL2F DCD4F DCD5F DCD6F DGR2F GAL1F GAL2F GAL3F HES1F HES2F HES3F HES4F KIL5F KIL6F LDL2F MUC2F OST3F OST4F OST5F PAM3F PAM4F SCR2F SEZ3F SEZ4F SEZ5F
L1-50k
Uveal Melanoma MP38
L2-50k
Uveal Melanoma MP41
L11-50
cfDNA
18 Individuals
Mixed
L3-50k
PBMC
0.0 0.5 1.0

Fig. 15

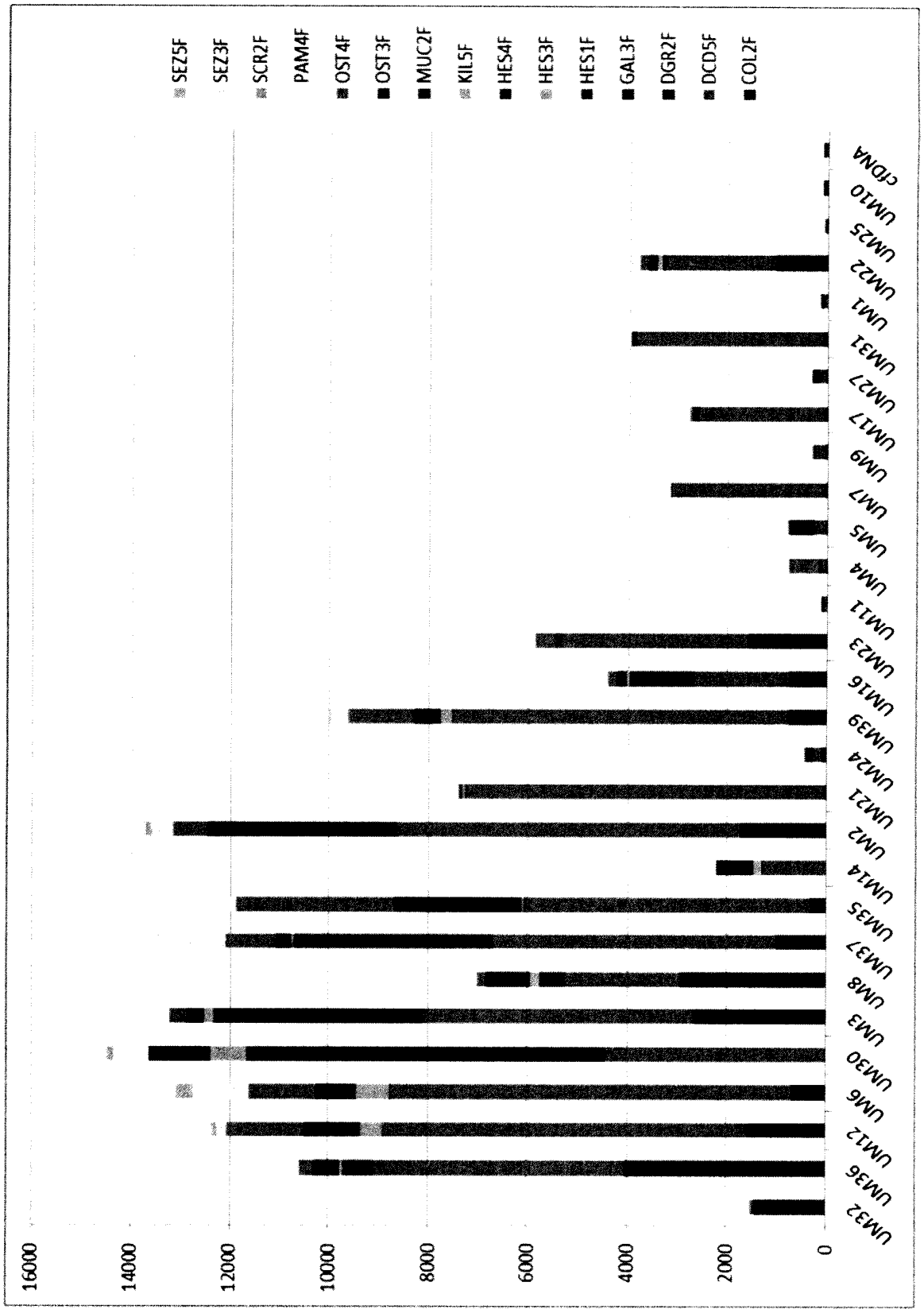
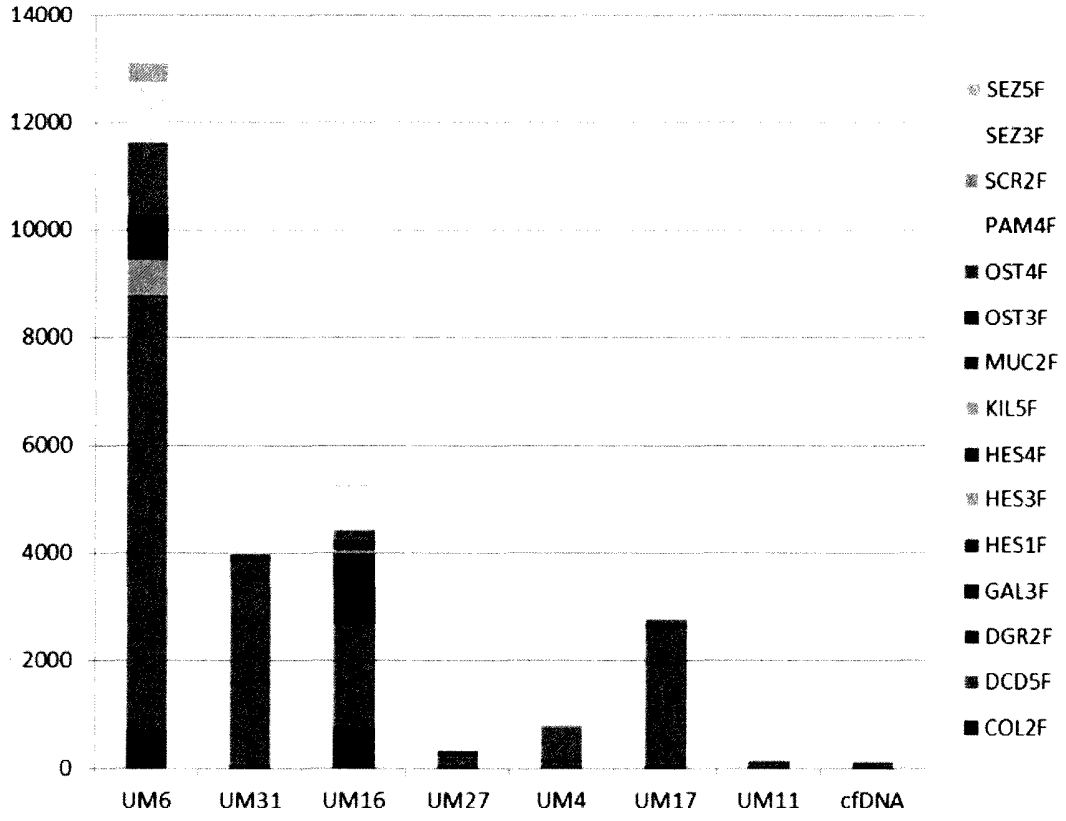
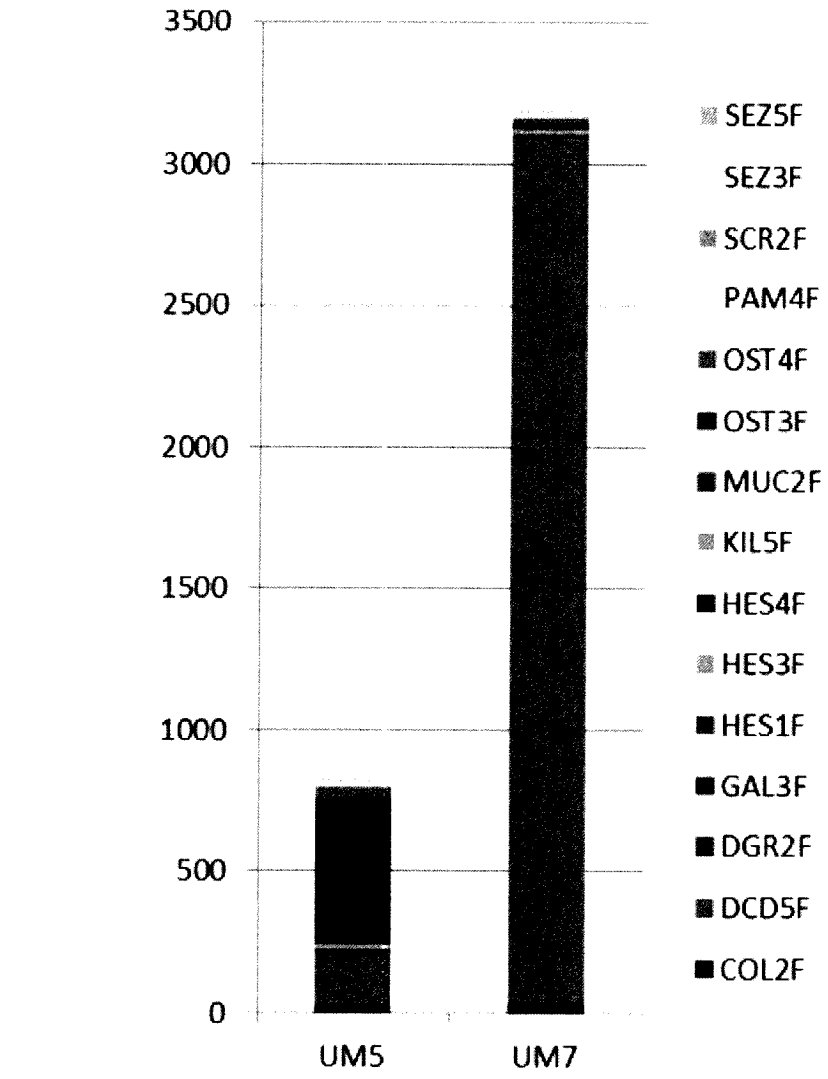

SEZ5F
SEZ3F
SCR2F
PAM4F
OST4F
OST3F
MUC2F
KIL5F
HES4F
HES3F
HES1F
GAL3F
DGR2F
DCD5F
COL2F
cDNA
UM10
UM25
UM22
UM1
UM31
UM27
UM17
UM9
UM7
UM5
UM4
UM11
UM23
UM16
UM39
UM24
UM21
UM2
UM14
UM35
UM37
UM8
UM3
UM30
UM6
UM12
UM36
UM32
16000
14000
12000
10000
8000
6000
4000
2000
0

Fig. 16



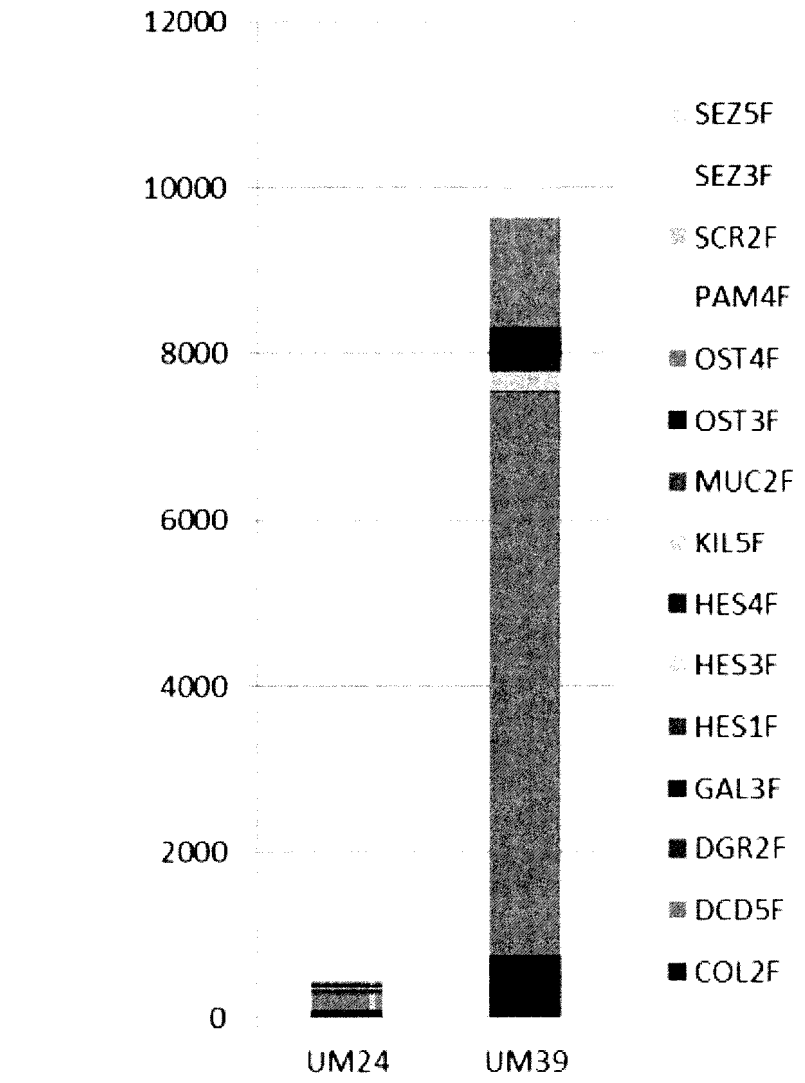
	UM6	UM31	UM16	UM27	UM4	UM17	UM11	cfDNA
PAP Copies	9423	<5	38.6	<5	<5	<5	6.3	
Tumour Volume cm^3	16.7	16	14.8	6.4	5.6	0.6	0.6	

Fig. 17



Tumour Volume Unknown 0.5 cm^3

Fig. 18A



Tumour Volume 483 cm^3 Unknown

Fig. 18B

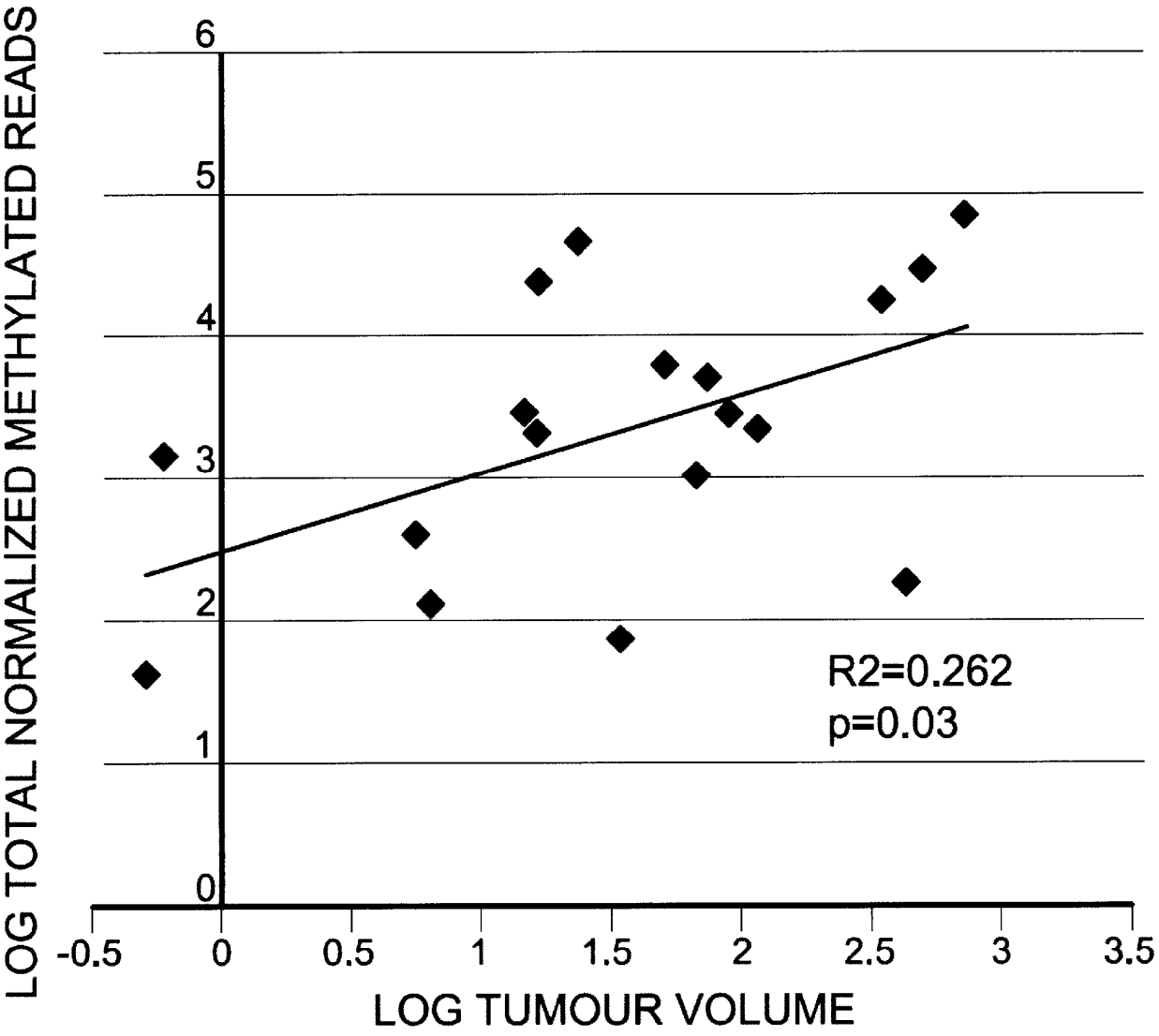

LOG TOTAL NORMALIZED METHYLATED READS
6
5
4
3
2
1
0
-0.5
0
0.5
1
1.5
2
2.5
3
3.5
LOG TUMOUR VOLUME
R2=0.262
p=0.03

Fig. 19

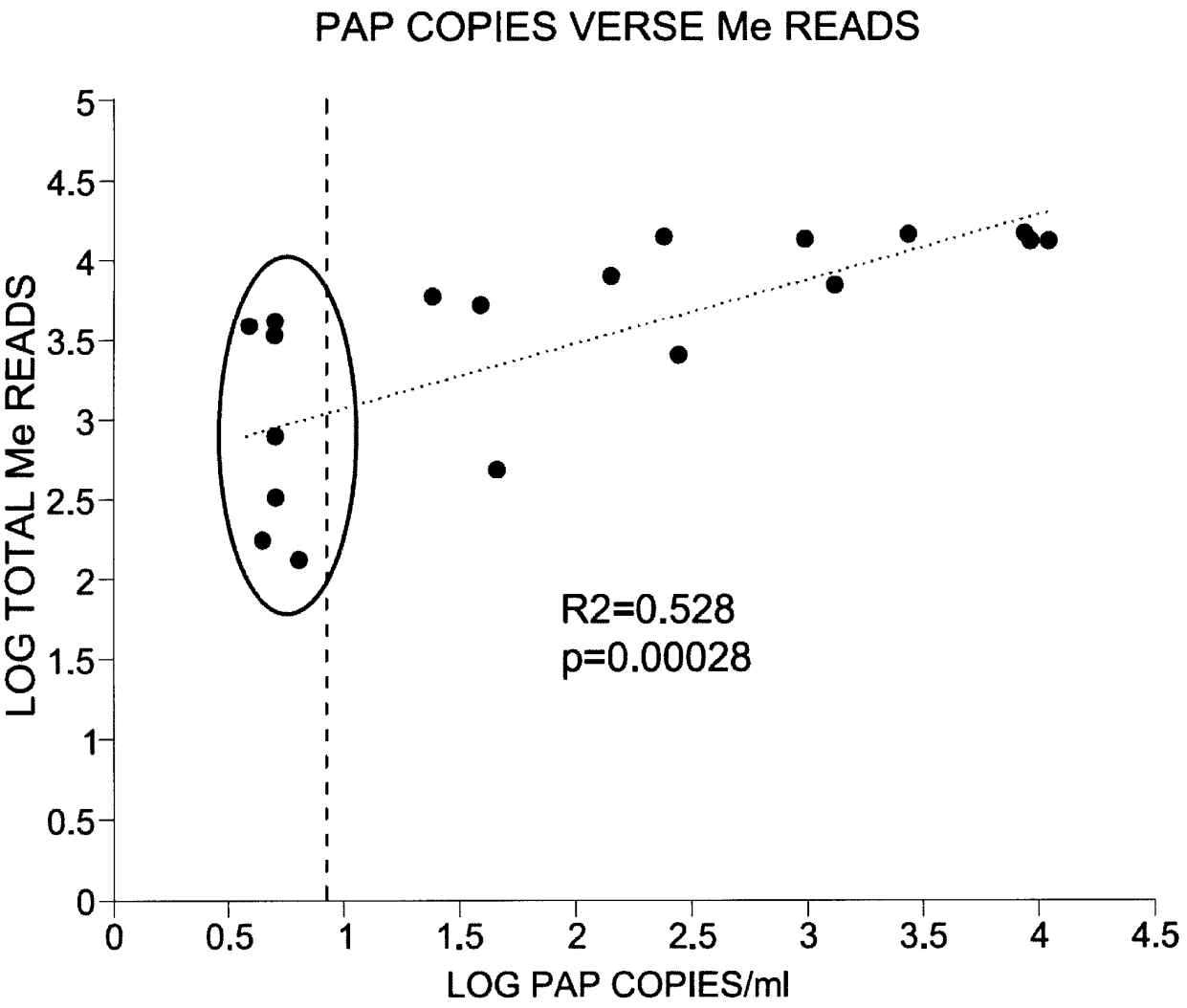

PAP COPIES VERSE Me READS
LOG TOTAL Me READS
LOG PAP COPIES/ml
R2=0.528
p=0.00028
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5

Fig. 20

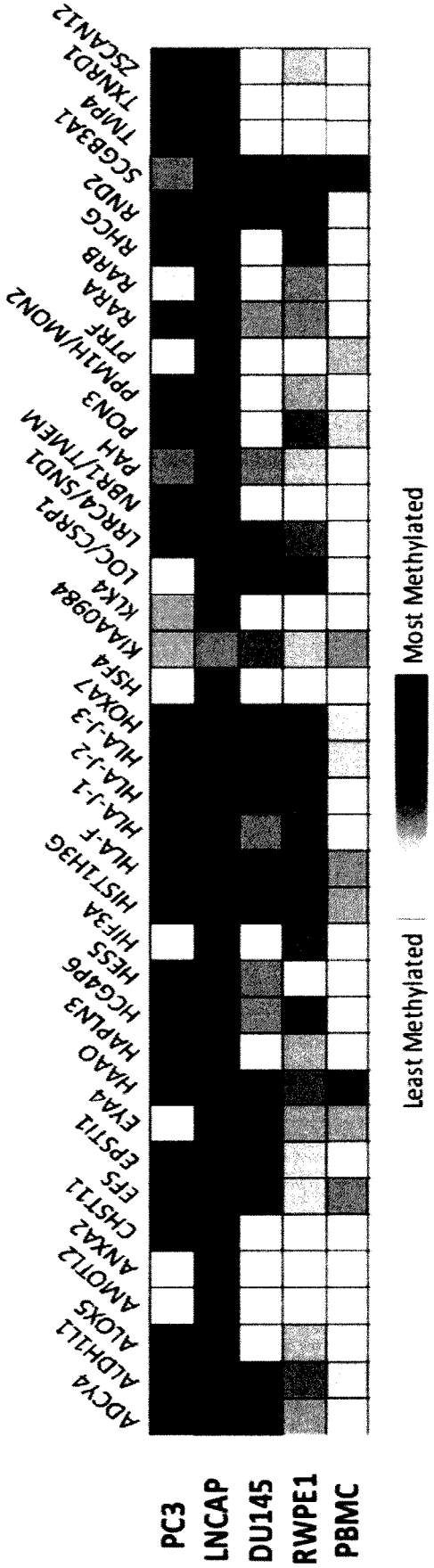

PC3
LNCAP
DU145
RWPE1
PBMC
ADCY4
ALDH1L1
ALOX5
AMOTL2
ANXA2
CHST11
EFS
EPST11
EYA4
HAAO
HAPLN3
HCG4P6
HES5
HIF3A
HIST1H3G
HLA-F
HLA-J-1
HLA-J-2
HLA-J-3
HOXA7
HSF4
KIAA0984
KLK4
LOC/CSRP1
LRRC4/SND1
NBR1/TMEM
PAH
PON3
PPM1H/MON2
PTRF
RARA
RARB
RHCG
RND2
SCGB3A1
TM4
TXNRD1
ZSCAN12
Least Methylated
Most Methylated

Fig. 21

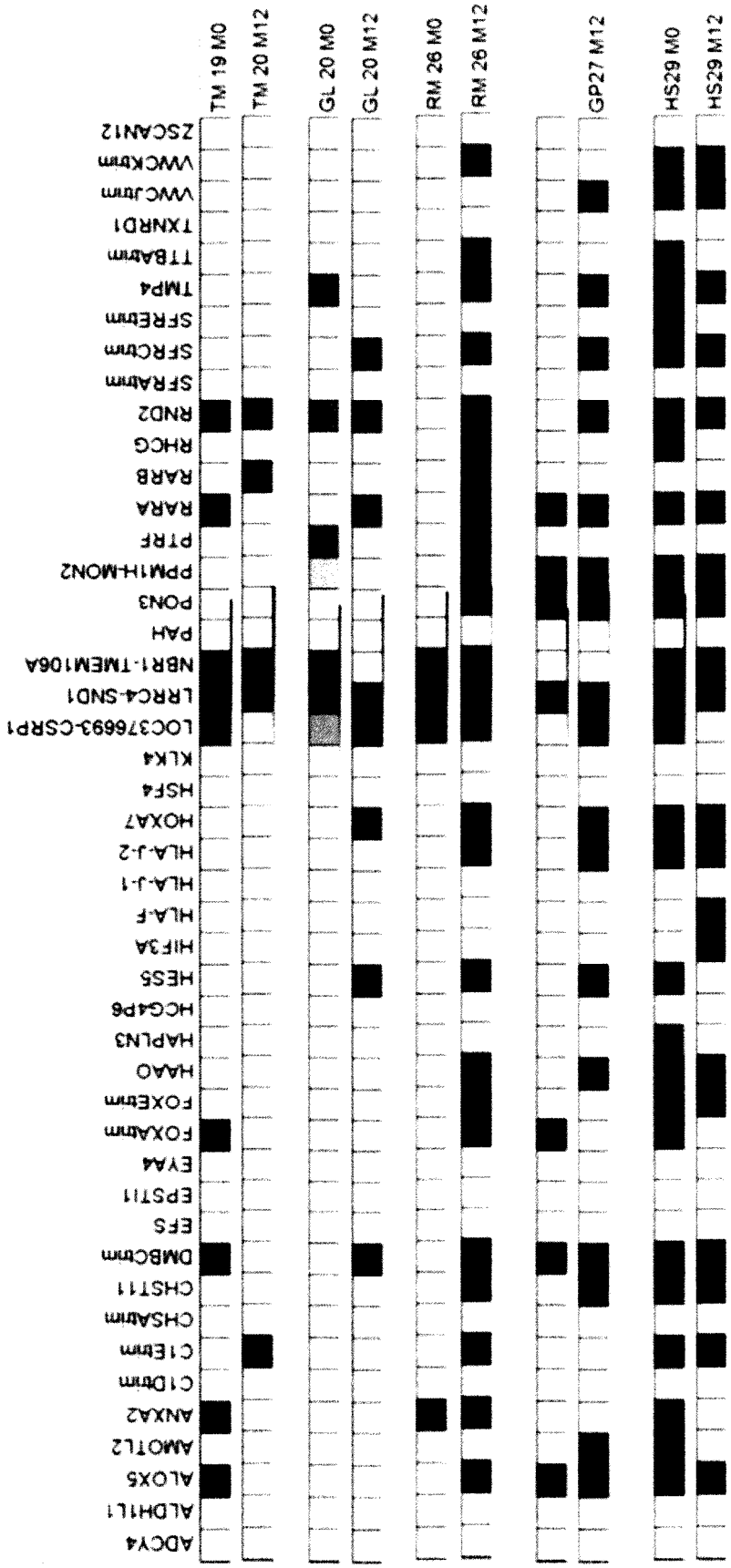

TM 19 M0
TM 20 M12
GL 20 M0
GL 20 M12
RM 26 M0
RM 26 M12
GP27 M12
HS29 M0
HS29 M12
ADCY4
ALDH1L1
ALOX5
AMOTL2
ANXA2
C1Dtrm
C1Etrm
CHSAtrm
CHST11
DMBCtrm
EFS
EPST11
EYA4
FOXAtrm
FOXEtrm
HAAO
HAPLN3
HCG4P6
HES5
HIF3A
HLA-F
HLA-J-1
HLA-J-2
HOXA7
HSF4
KLK4
LOC376693-CSRP1
LRRC4-SND1
NBR1-TMEM106A
PAH
PON3
PPM1H-MON2
PTRF
RARA
RARB
RHCG
RND2
SFRAtrm
SFRCtrm
SFREtrm
TMP4
TTBAtrm
TXNRD1
VWCJtrm
VWCKtrm
ZSCAN12

Fig. 22

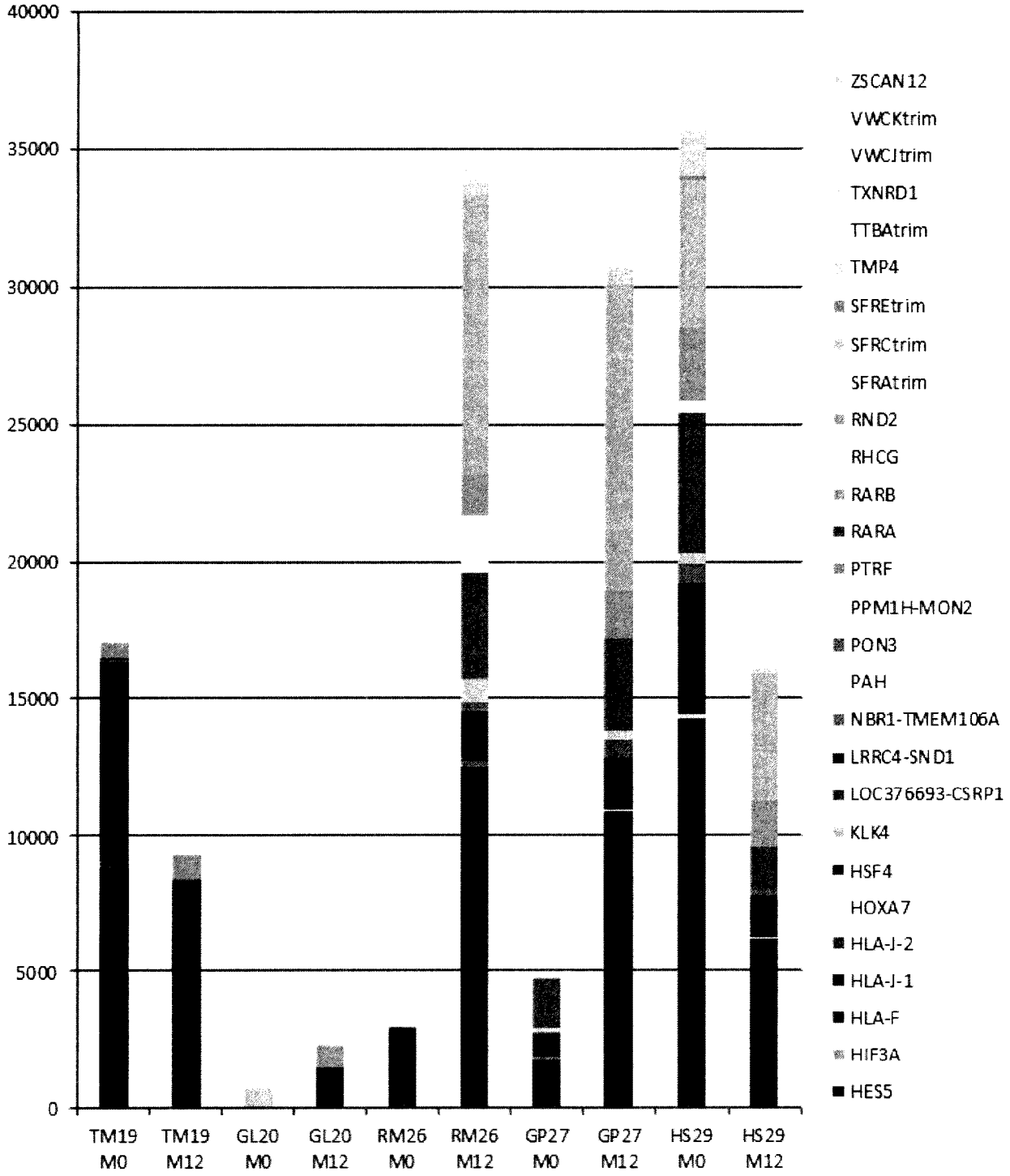

40000
35000
30000
25000
20000
15000
10000
5000
0
TM19 M0
TM19 M12
GL20 M0
GL20 M12
RM26 M0
RM26 M12
GP27 M0
GP27 M12
HS29 M0
HS29 M12
ZSCAN12
VWCKtrim
VWCJtrim
TXNRD1
TTBAtrim
TMP4
SFREtrim
SFRCtrim
SFRAtrim
RND2
RHCG
RARB
RARA
PTRF
PPM1H-MON2
PON3
PAH
NBR1-TMEM106A
LRRC4-SND1
LOC376693-CSRP1
KLK4
HSF4
HOXA7
HLA-J-2
HLA-J-1
HLA-F
HIF3A
HES5

Fig. 23

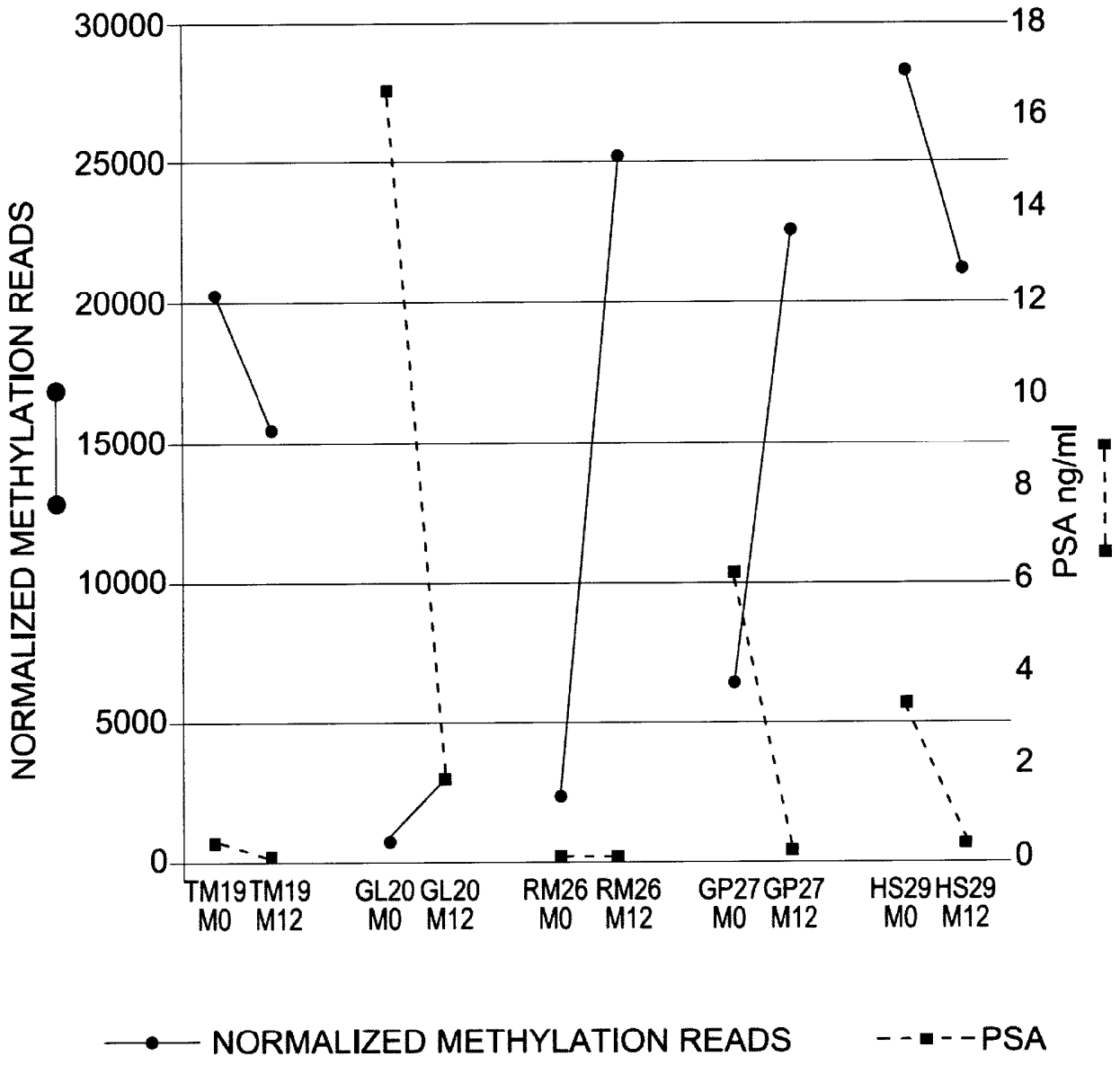

30000
25000
20000
15000
10000
5000
0
NORMALIZED METHYLATION READS
18
16
14
12
10
8
6
4
2
0
PSA ng/ml
TM19 M0
TM19 M12
GL20 M0
GL20 M12
RM26 M0
RM26 M12
GP27 M0
GP27 M12
HS29 M0
HS29 M12
NORMALIZED METHYLATION READS
PSA

Fig. 24

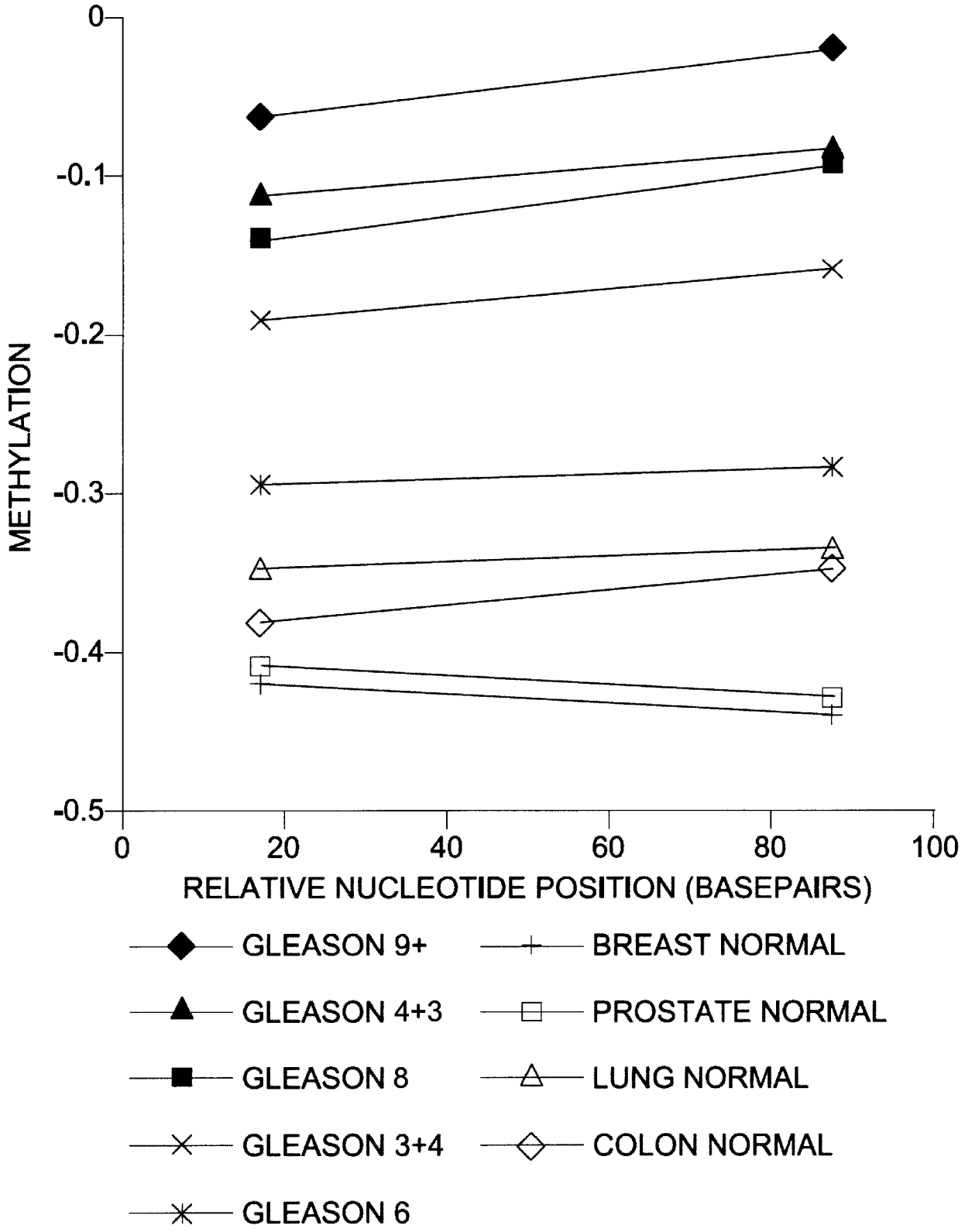

0
-0.1
-0.2
-0.3
-0.4
-0.5
METHYLATION
0
20
40
60
80
100
RELATIVE NUCLEOTIDE POSITION (BASEPAIRS)
GLEASON 9+
GLEASON 4+3
GLEASON 8
GLEASON 3+4
GLEASON 6
BREAST NORMAL
PROSTATE NORMAL
LUNG NORMAL
COLON NORMAL

Fig. 25

Name	Chromosome	Nucleotide	Basal	Her2	LumA	LumB	Normal-Lik	Tissue	
			82	31	280	127	17	89	# Tumours
CTSA,NEURL2	chr20	43952209	0.30	0.71	0.89	0.91	0.24	0.01	
CHR5:43054200	chr5	43054200	0.15	0.77	0.87	0.90	0.35	0.06	
ADCY4	chr14	23873713	0.26	0.94	0.83	0.90	0.41	0.06	
DDAH2	chr6	31806197	0.18	0.61	0.81	0.84	0.18	0.00	
USP44	chr12	94467038	0.43	0.90	0.80	0.95	0.35	0.01	
L1TD1	chr1	62433212	0.95	0.90	0.78	0.94	0.35	0.03	
PRDM15	chr21	42110158	0.37	0.74	0.78	0.79	0.53	0.02	
HOXD11	chr2	176680809	0.20	0.65	0.76	0.89	0.35	0.03	
GNG4	chr1	233880632	0.35	0.87	0.75	0.91	0.24	0.02	
COL16A1	chr1	31942288	0.24	0.84	0.75	0.92	0.18	0.00	
TAL1	chr1	47470535	0.77	0.87	0.74	0.90	0.35	0.02	
CDH23	chr10	72826383	0.11	0.81	0.73	0.83	0.24	0.01	
SHF	chr15	43267047	0.21	1.00	0.72	0.91	0.53	0.03	
CHST11	chr12	103376569	0.12	0.52	0.72	0.77	0.18	0.01	
PRKCB	chr16	23755057	0.32	0.68	0.71	0.83	0.12	0.00	
AFF3	chr2	99542136	0.00	0.32	0.71	0.57	0.18	0.04	
ATP5G2	chr12	52357357	0.40	0.74	0.70	0.89	0.12	0.03	
CHR19:51071769	chr19	51071769	0.34	0.97	0.70	0.83	0.24	0.02	
C1orf114	chr1	167663259	0.61	0.90	0.69	0.88	0.24	0.00	
CHR1:90967747	chr1	90967747	0.52	0.74	0.68	0.80	0.12	0.01	
NT5E	chr6	86215916	0.45	0.61	0.68	0.79	0.12	0.01	
PLTP	chr20	43973373	0.20	0.84	0.68	0.82	0.18	0.00	
MAST1	chr19	12839611	0.59	0.87	0.67	0.88	0.29	0.00	
ITPRIPL1	chr2	96354641	0.61	0.90	0.67	0.84	0.18	0.00	
KIAA1751	chr1	1925149	0.18	0.71	0.66	0.70	0.18	0.01	
ACVRL1	chr12	50587178	0.38	0.81	0.66	0.89	0.18	0.02	
GABRA4	chr4	46690498	0.33	0.52	0.66	0.85	0.06	0.00	
TOB2P1	chr6	28283425	0.77	0.65	0.65	0.80	0.18	0.00	
FLI1	chr11	128068895	0.12	0.61	0.65	0.77	0.06	0.00	
CD38	chr4	15389336	0.21	0.77	0.65	0.79	0.18	0.00	
HOTAIR	chr12	52646064	0.84	1.00	0.64	0.77	0.53	0.04	
PRDM13	chr6	100167832	0.74	0.81	0.64	0.82	0.24	0.00	
HNF1B	chr17	33177343	0.30	0.65	0.64	0.75	0.06	0.00	
RGS17	chr6	153494417	0.10	0.77	0.64	0.79	0.24	0.02	
COL11A2	chr6	33269254	0.07	0.55	0.64	0.70	0.18	0.01	
CHR11:68379305	chr11	68379315	0.16	1.00	0.63	0.87	0.18	0.02	
RUNX1T1	chr8	93183326	0.35	0.42	0.63	0.80	0.18	0.01	
VWC2	chr7	49783648	0.11	0.65	0.63	0.76	0.18	0.00	
RTN4RL1	chr17	1827755	0.48	0.52	0.62	0.76	0.12	0.03	
CHR17:69460208	chr17	69460208	0.35	0.90	0.62	0.79	0.18	0.01	
TMEM132C	chr12	127317993	0.78	0.71	0.62	0.73	0.18	0.00	
C19orf41	chr19	55358350	0.35	0.45	0.61	0.77	0.12	0.00	
SHISA3	chr4	42094600	0.04	0.26	0.61	0.75	0.18	0.00	
SOX2OT	chr3	182919839	0.56	0.77	0.60	0.80	0.18	0.01	
ALX4	chr11	44289229	0.28	0.68	0.60	0.79	0.41	0.03	
HLA-F	chr6	29799979	0.26	0.42	0.60	0.62	0.29	0.02	
KCNJ2	chr17	65676379	0.10	0.65	0.60	0.76	0.06	0.00	
ERNA4	chr1	153310037	0.59	0.90	0.60	0.78	0.18	0.01	
HIF3A	chr19	51491904	0.09	0.55	0.59	0.71	0.12	0.01	
CHR8:95315690	chr8	95315690	0.57	0.84	0.59	0.80	0.29	0.02	
GIPC2	chr1	78284188	0.60	0.77	0.59	0.76	0.24	0.04	
CRYM	chr16	21202756	0.39	0.58	0.59	0.80	0.29	0.01	
HIVEP3	chr1	41901042	0.00	0.35	0.59	0.80	0.24	0.01	
SLC2A2	chr3	172228901	0.40	0.68	0.58	0.80	0.12	0.00	
CYTL1	chr4	5072014	0.13	0.58	0.58	0.76	0.06	0.00	
PON3	chr7	94863672	0.33	0.55	0.58	0.64	0.12	0.00	

Fig. 26A

Name	Chromoso	Nucleotide	Basal	Her2	LumA	LumB	Normal-LikTissue		
;			82	31	280	127	17	89	# Tumours
TRIM71	chr3	32834411	0.27	0.52	0.58	0.74	0.18	0.00	
CHR12:61	chr12	61311757	0.18	0.55	0.58	0.69	0.24	0.00	
C5orf39	chr5	43076337	0.41	0.48	0.58	0.78	0.24	0.01	
HOXA10	chr7	27180568	0.10	0.29	0.58	0.75	0.06	0.00	
ESPN	chr1	6430566	0.21	0.77	0.57	0.73	0.24	0.03	
POU4F1	chr13	78075701	0.12	0.58	0.57	0.74	0.18	0.00	
TXNRD1	chr12	103133606	0.38	0.77	0.56	0.78	0.24	0.00	
EGFLAM	chr5	38293298	0.10	0.48	0.56	0.68	0.00	0.00	
NR5A2	chr1	198278409	0.66	0.52	0.56	0.59	0.12	0.00	
LHX1	chr17	32368589	0.10	0.35	0.56	0.67	0.06	0.00	
TNFRSF10I	chr8	23077458	0.28	0.35	0.56	0.76	0.06	0.00	
STK33	chr11	8572251	0.05	0.65	0.56	0.65	0.18	0.00	
CHR2:174	chr2	174899291	0.26	0.65	0.55	0.75	0.06	0.00	
ID4	chr6	19944994	0.35	0.68	0.55	0.67	0.18	0.00	
ZFPM2	chr8	106401171	0.38	0.52	0.55	0.69	0.29	0.00	
TTBK1	chr6	43319186	0.34	0.71	0.55	0.72	0.18	0.00	
CHR5:429	chr5	42987870	0.24	0.52	0.55	0.67	0.00	0.00	
NKX2-1	chr14	36058194	0.15	0.52	0.55	0.69	0.24	0.00	
INA	chr10	105026691	0.22	0.52	0.54	0.72	0.12	0.00	
CHST2	chr3	144322268	0.04	0.58	0.54	0.72	0.12	0.00	
HOXD8	chr2	176702611	0.27	0.48	0.53	0.56	0.12	0.00	
PTPRN2	chr7	157176096	0.67	0.58	0.53	0.67	0.12	0.00	
CHR1:238	chr1	238227872	0.29	0.58	0.52	0.75	0.24	0.00	
ALX1	chr12	84198427	0.27	0.77	0.52	0.66	0.18	0.00	
TSPAN33	chr7	128596336	0.54	0.77	0.51	0.70	0.18	0.01	
FOXL2,C3c	chr3	140148674	0.38	0.74	0.51	0.72	0.18	0.00	
CDK5R2	chr2	219532295	0.26	0.61	0.51	0.50	0.18	0.00	
RECK	chr9	36027340	0.02	0.52	0.51	0.69	0.24	0.00	
SHOX2	chr3	159304101	0.46	0.68	0.51	0.78	0.06	0.00	
SFRP5	chr10	99521787	0.12	0.52	0.51	0.60	0.18	0.00	
CHR8:680	chr8	68037587	0.11	0.52	0.51	0.70	0.29	0.01	
NRN1	chr6	5952522	0.09	0.77	0.51	0.68	0.00	0.00	
MIR129-2	chr11	43559433	0.24	0.52	0.51	0.72	0.06	0.00	
LRRC4,SNI	chr7	127459694	0.48	0.61	0.50	0.69	0.41	0.07	
SP9	chr2	174907892	0.33	0.81	0.50	0.71	0.12	0.00	
TBX15	chr1	119332123	0.30	0.61	0.49	0.68	0.18	0.00	
POU3F3	chr2	104836990	0.15	0.52	0.49	0.66	0.00	0.00	
EVX1	chr7	27248876	0.39	0.77	0.49	0.74	0.18	0.00	
FABP5	chr8	82355156	0.09	0.74	0.48	0.61	0.12	0.01	
PDE4B	chr1	66030623	0.10	0.61	0.48	0.67	0.06	0.00	
T	chr6	166501919	0.13	0.65	0.48	0.69	0.12	0.00	
CHR1:119	chr1	119344859	0.45	0.61	0.47	0.71	0.06	0.00	
CHR12:52	chr12	52897710	0.33	0.74	0.47	0.63	0.29	0.01	
LOC25516	chr5	6636380	0.33	0.84	0.47	0.62	0.35	0.00	
NUDT16P	chr3	132563617	0.77	0.81	0.47	0.56	0.47	0.04	
IRF4	chr6	336743	0.57	0.74	0.47	0.72	0.18	0.00	
MSC	chr8	72918612	0.22	0.61	0.47	0.69	0.12	0.00	
UDB	chr6	29629547	0.62	0.45	0.46	0.68	0.29	0.00	
C1orf230	chr1	149960747	0.24	0.55	0.46	0.69	0.12	0.00	
HOXA9	chr7	27171749	0.50	0.42	0.46	0.68	0.06	0.01	
CARD11	chr7	3049859	0.65	0.71	0.45	0.69	0.18	0.00	
SPAG6	chr10	22674438	0.78	0.61	0.45	0.68	0.12	0.00	
IRF8	chr16	84490167	0.43	0.45	0.45	0.60	0.12	0.00	
CDO1	chr5	115180312	0.38	0.45	0.45	0.67	0.12	0.00	
CHR3:148	chr3	14827760	0.29	0.74	0.44	0.70	0.06	0.00	
GALR3	chr22	36550955	0.70	0.81	0.43	0.70	0.24	0.02	

Fig. 26B

Name	Chromoso	Nucleotide	Basal	Her2	LumA	LumB	Normal-Li	Tissue
s			82	31	280	127	17	89 # Tumours
CHR7:646:	chr7	64675139	0.28	0.61	0.43	0.69	0.18	
ALOX5	chr10	45234531	0.13	0.65	0.43	0.66	0.18	0.00
PDK1	chr13	27389540	0.62	0.61	0.43	0.50	0.12	0.00
CHR19:48	chr19	48895423	0.35	0.58	0.43	0.68	0.06	0.00
MIR155HG	chr21	25856447	0.09	0.68	0.43	0.53	0.12	0.04
AKR1B1	chr7	133794363	0.15	0.77	0.43	0.60	0.06	0.00
TMEM90B	chr20	24398353	0.61	0.58	0.42	0.52	0.18	0.00
KCNK17	chr6	39389863	0.23	0.68	0.42	0.59	0.24	0.01
DMBX1	chr1	46723905	0.76	0.65	0.41	0.61	0.18	0.00
DMRTA2	chr1	50659537	0.66	0.68	0.41	0.68	0.18	0.00
chr5:7263	chr5	72634721	0.74	0.65	0.41	0.48	0.12	0.01
CPXM1	chr20	2729316	0.38	0.94	0.40	0.62	0.18	0.01
CHR2:236	chr2	236737696	0.59	0.68	0.39	0.57	0.12	0.00
FLJ41350,I	chr10	102980016	0.24	0.71	0.39	0.57	0.18	0.01
NKX6-2	chr10	134449831	0.22	0.58	0.39	0.69	0.06	0.00
SNX32	chr11	65357904	0.12	0.81	0.38	0.66	0.24	0.00
SFRP2	chr4	154929278	0.16	0.65	0.38	0.54	0.12	0.00
CHR10:43	chr10	43138371	0.48	0.77	0.38	0.63	0.12	0.00
GALR1	chr18	73090725	0.43	0.48	0.37	0.62	0.18	0.00
NOTUM	chr17	77512868	0.15	0.77	0.37	0.64	0.12	0.01
CD8A	chr2	86871615	0.10	0.68	0.37	0.54	0.24	0.00
NPHS2	chr1	177811696	0.30	0.23	0.37	0.51	0.06	0.00
BOLL	chr2	198359232	0.62	0.74	0.36	0.64	0.29	0.00
FZD2	chr17	39990836	0.37	0.58	0.36	0.61	0.18	0.01
CDKL2	chr4	76774796	0.72	0.61	0.35	0.43	0.18	0.01
BARHL2	chr1	90965039	0.68	0.23	0.35	0.50	0.00	0.00
SYNGR3	chr16	1980982	0.30	0.58	0.34	0.60	0.06	0.00
EPSTI1	chr13	42464421	0.49	0.48	0.34	0.61	0.06	0.00
OSTM1/NF	chr6	108546973	0.61	0.77	0.33	0.51	0.12	0.00
AP3B1	chr5	77304490	0.30	0.19	0.32	0.57	0.00	0.00
LASS1,GDF	chr19	18868311	0.04	0.65	0.32	0.52	0.18	0.00
DRD4	chr11	627035	0.56	0.61	0.32	0.54	0.12	0.00
PAX6	chr11	31797204	0.84	0.71	0.30	0.47	0.18	0.00
HSPA12B	chr20	3661341	0.21	0.65	0.30	0.58	0.12	0.01
TLX1NB	chr10	102871266	0.40	0.61	0.30	0.54	0.12	0.00
C6orf186	chr6	110785613	0.11	0.68	0.30	0.54	0.00	0.00
CCDC8	chr19	51608360	0.48	0.52	0.29	0.55	0.00	0.00
SALL3	chr18	74841079	0.37	0.42	0.29	0.59	0.06	0.00
KCNK4	chr11	63816492	0.11	0.68	0.29	0.52	0.06	0.00
C17orf64	chr17	55853653	0.32	0.52	0.28	0.54	0.06	0.00
NRC	chr19	3386252	0.63	0.52	0.27	0.36	0.24	0.01
DPP10	chr2	115635476	0.38	0.42	0.27	0.44	0.18	0.00
CCL28	chr5	43432924	0.88	0.23	0.24	0.28	0.06	0.04
MIR548G,F	chr3	101077583	0.79	0.19	0.24	0.39	0.06	0.03
NR2E1	chr6	108596488	0.55	0.71	0.23	0.42	0.18	0.00
C20orf56	chr20	22507676	0.44	0.16	0.23	0.34	0.00	0.00
SLC7A4	chr22	19716885	0.65	0.77	0.23	0.54	0.12	0.00
TCTEX1D1	chr1	66990668	0.57	0.39	0.19	0.38	0.00	0.00
PHOX2B	chr4	41447005	0.57	0.42	0.19	0.22	0.06	0.02
CA9	chr9	35666104	0.55	0.52	0.15	0.37	0.12	0.00
LEF1,LOC6	chr4	109307487	0.63	0.32	0.10	0.13	0.06	0.01
PRSS27	chr16	2705621	0.68	0.23	0.09	0.15	0.12	0.02
SIM1	chr6	101021823	0.59	0.26	0.07	0.13	0.00	0.00
PPFIA3	chr19	54337905	0.82	0.58	0.05	0.16	0.29	0.00
CHR10:12	chr10	125024561	0.59	0.03	0.00	0.00	0.00	0.00

Fig. 26C

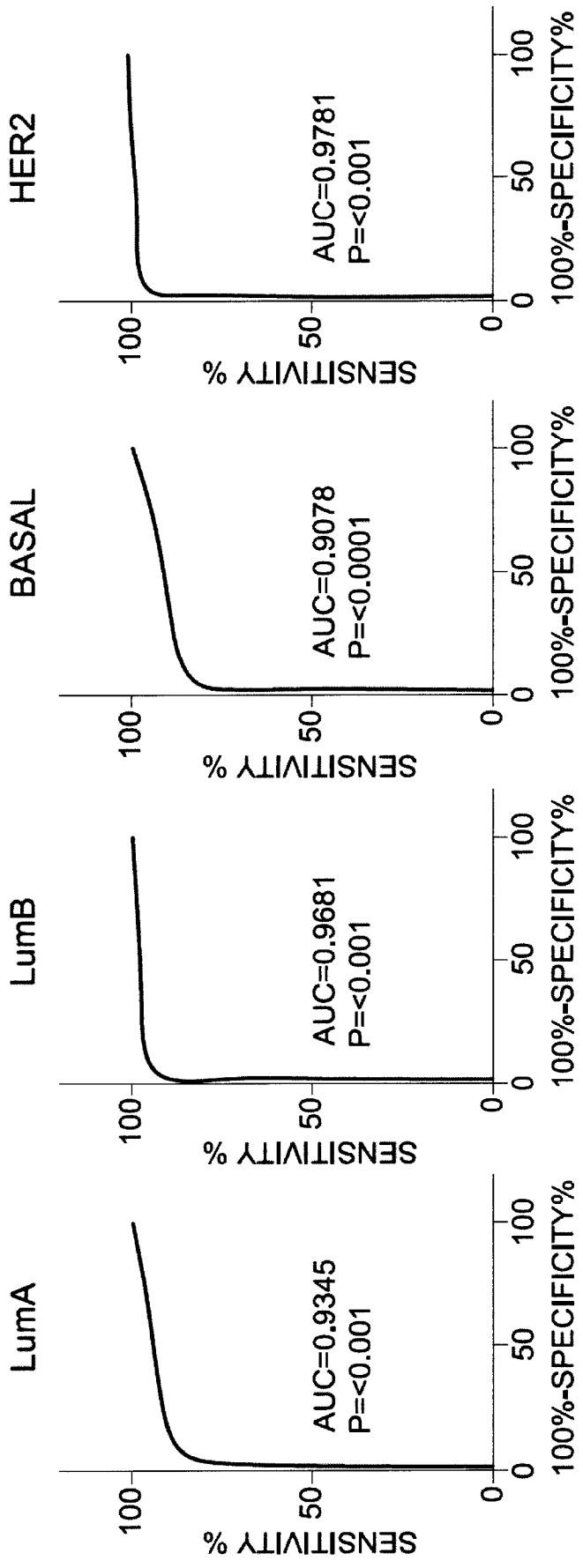

LumA
AUC=0.9345
P=<0.001
LumB
AUC=0.9681
P=<0.001
BASAL
AUC=0.9078
P=<0.0001
HER2
AUC=0.9781
P=<0.001
SENSITIVITY %
100%-SPECIFICITY%
0
50
100

Fig. 27

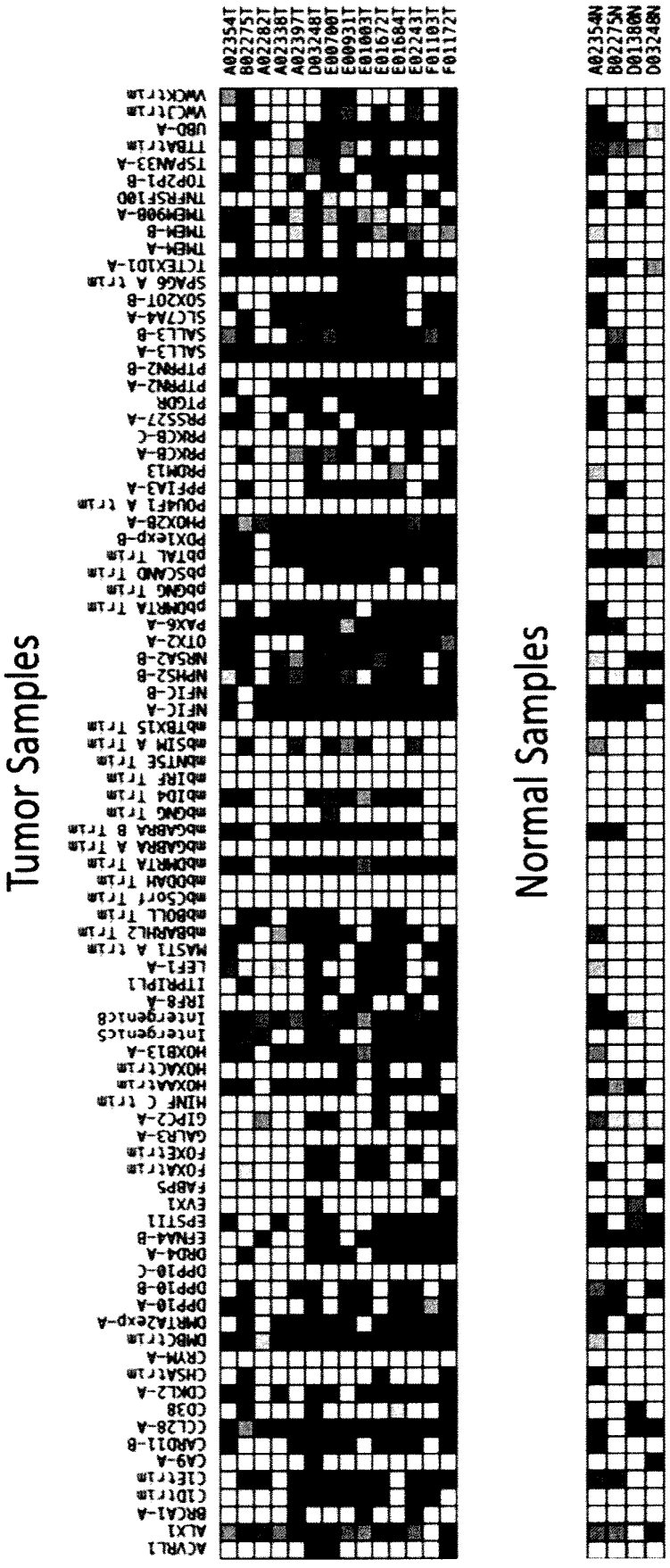
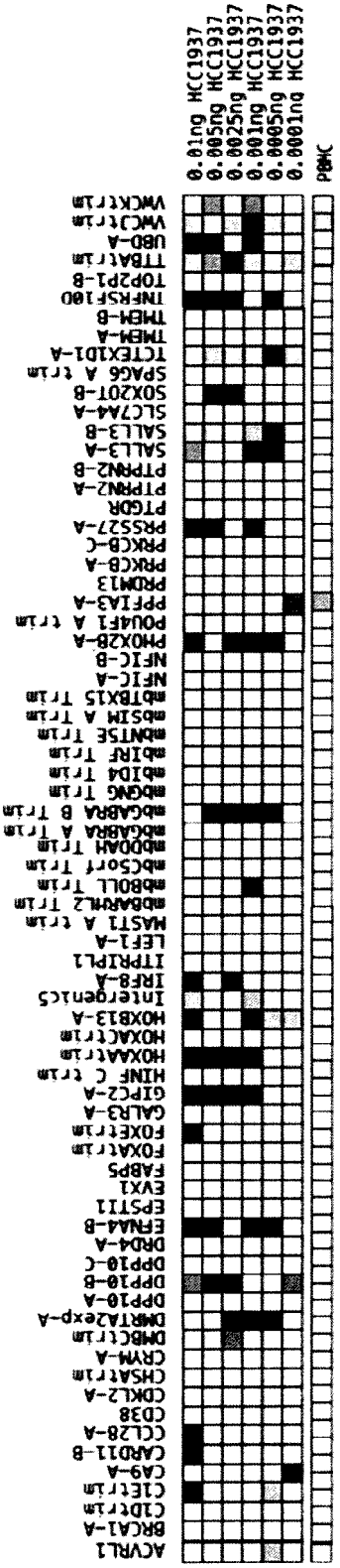

Tumor Samples
Normal Samples
A02354T
B02275T
A02282T
A02338T
A02397T
D03248T
E00700T
E00931T
E01003T
E01672T
E01684T
E02243T
F01103T
F01172T
A02354N
B02275N
D01380N
D03248N

Fig. 28


0.01ng HCC1937
0.005ng HCC1937
0.0025ng HCC1937
0.001ng HCC1937
0.0005ng HCC1937
0.0001ng HCC1937
PBMC
ACVRL1
BRCA1-A
C1Dtrim
C1Etrim
CA9-A
CARD11-B
CCL28-A
CD38
CDKL2-A
CHSAtrim
CRYM-A
DMBCtrim
DNRTA2exp-A
DPP10-A
DPP10-B
DPP10-C
DRD4-A
EFNA4-B
EPS111
EVX1
FABP5
FOXAtrim
FOXEtrim
GALR3-A
GIPC2-A
HINF C trim
HOXAAtrim
HOXACtrim
HOXB13-A
Intergenic5
IRF8-A
ITPRIPL1
LEF1-A
MAST1 A trim
mbBARHL2 Trim
mbBOLL Trim
mbCSorf Trim
mbDDAH Trim
mbGABRA A Trim
mbGABRA B Trim
mbGNG Trim
mbIDA Trim
mbIRF Trim
mbNTSE Trim
mbSIM A Trim
mbTBX15 Trim
NFIC-A
NFIC-B
PHOXZB-A
POU4F1 A trim
PPFIA3-A
PRDM13
PRKCB-A
PRKCB-C
PRS527-A
PTGDR
PTPRN2-A
PTPRN2-B
SALL3-A
SALL3-B
SLC7A4-A
SOX20T-B
SPAG6 A trim
TCTEX1D1-A
TMEM-A
TMEM-B
TNFRSF10D
TOP2P1-B
TTBAtrim
UBD-A
VWCJtrim
VWCKtrim

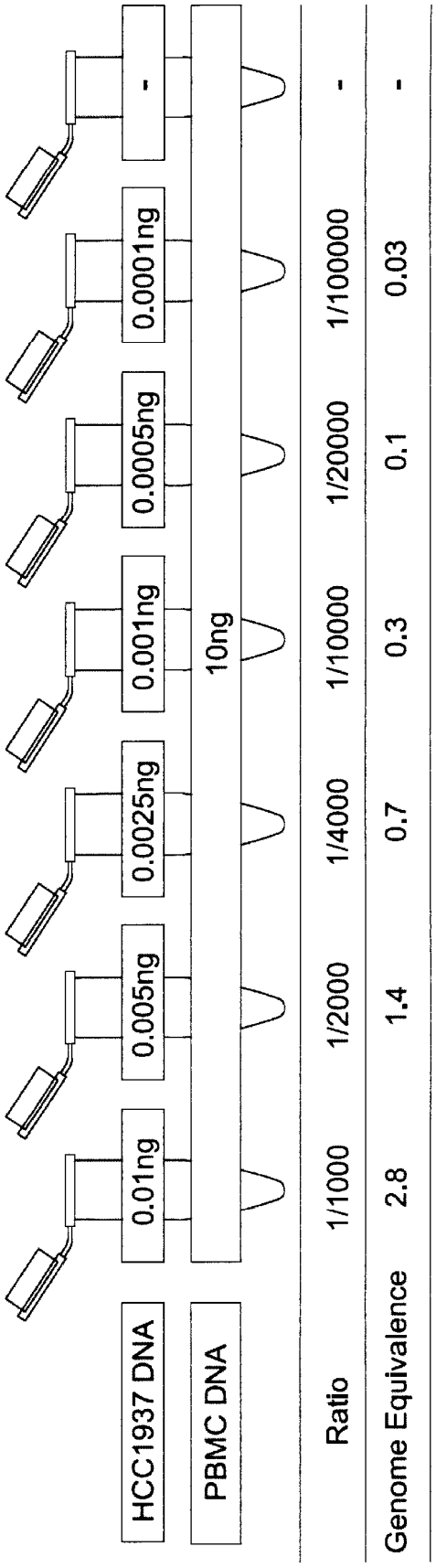

HCC1937 DNA
PBMC DNA
0.01ng
0.005ng
0.0025ng
0.001ng
0.0005ng
0.0001ng
-
10ng
Ratio 1/1000 1/2000 1/4000 1/10000 1/20000 1/100000 -
Genome Equivalence 2.8 1.4 0.7 0.3 0.1 0.03 -

Fig. 29

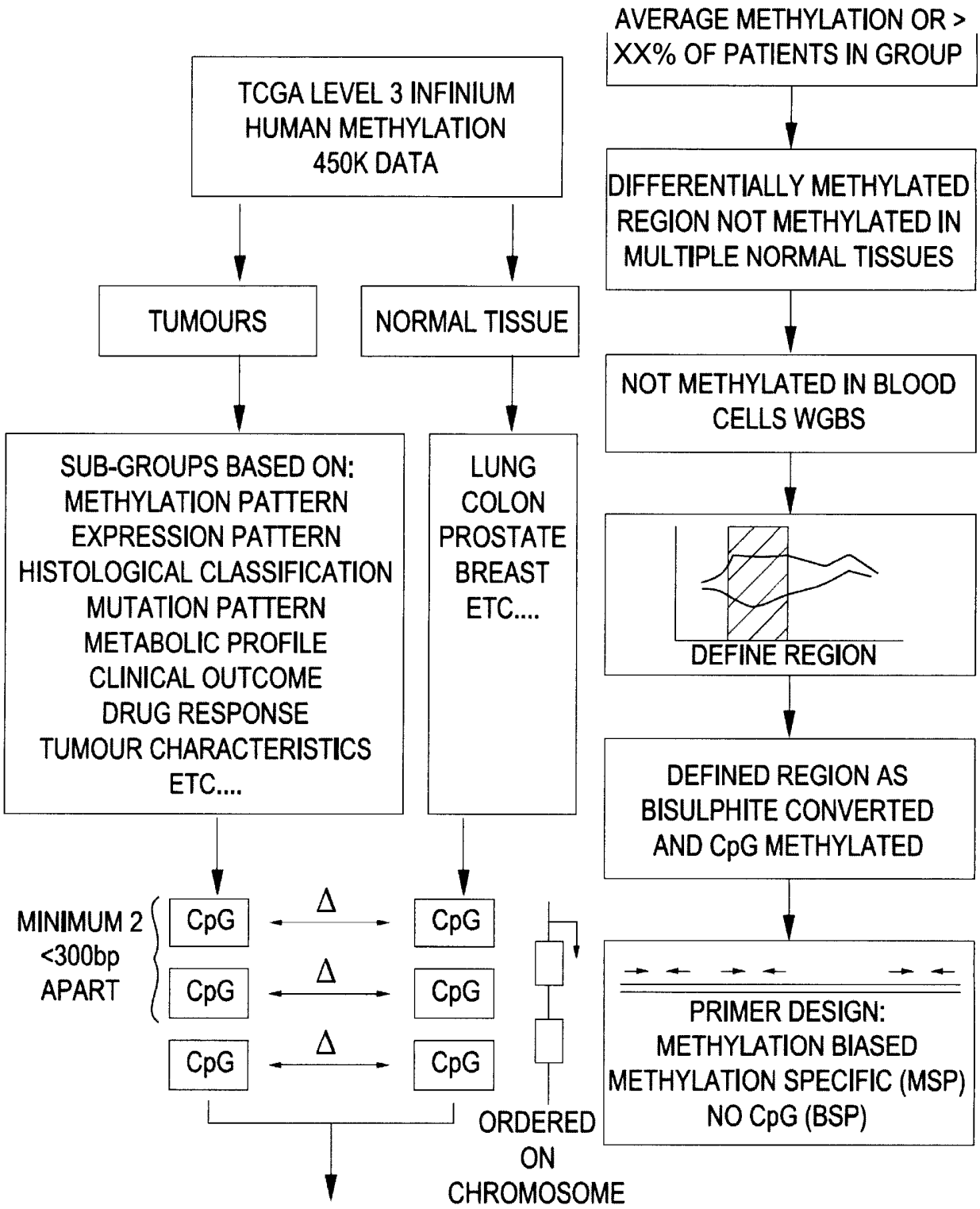

TCGA LEVEL 3 INFINIUM HUMAN METHYLATION 450K DATA
TUMOURS
NORMAL TISSUE
SUB-GROUPS BASED ON:
METHYLATION PATTERN
EXPRESSION PATTERN
HISTOLOGICAL CLASSIFICATION
MUTATION PATTERN
METABOLIC PROFILE
CLINICAL OUTCOME
DRUG RESPONSE
TUMOUR CHARACTERISTICS
ETC....
LUNG
COLON
PROSTATE
BREAST
ETC....
MINIMUM 2
<300bp
APART
CpG
Δ
ORDERED
ON
CHROMOSOME
AVERAGE METHYLATION OR >
XX% OF PATIENTS IN GROUP
DIFFERENTIALLY METHYLATED REGION NOT METHYLATED IN MULTIPLE NORMAL TISSUES
NOT METHYLATED IN BLOOD CELLS WGBS
DEFINE REGION
DEFINED REGION AS BISULPHITE CONVERTED AND CpG METHYLATED
PRIMER DESIGN:
METHYLATION BIASED
METHYLATION SPECIFIC (MSP)
NO CpG (BSP)

Fig. 30

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- US 2009305256 A1 **[0006]**
- WO 2014043763 A1 **[0006]**
- WO 2015159292 A1 **[0006]**

Non-patent literature cited in the description

- Identification of tissue-specific cell death using methylation patterns of circulating DNA. **LEHMANN-WERMAN** ; **RONI et al.** Proceedings of the National Academy of Sciences. scientific publication, 2016, E1826-E1834 **[0006]**
- **SONG Q et al.** A reference methylome database and analysis pipeline to facilitate integrative and comparative epigenomics. *PLOS ONE*, 2013, vol. 8 (12), e81148, http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0081148 **[0115]**
- **GERTZ J et al.** Analysis of DNA methylation in a three-generation family reveals widespread genetic influence on epigenetic regulation. *PLoS Genet.*, 2011, vol. 7 (8), e1002228, http://journals.plos.org/plosgenetics/article?id=10.1371/journal.pgen.1002228 **[0116]**
- **HEYN H et al.** Distinct DNA methylomes of newborns and centenarians. *Proc. Natl. Acad. Sci. U.S.A.*, 2012, vol. 109 (26), 10522-7, http://www.pnas.org/content/109/26/10522 **[0116]**
- **HON GC et al.** Global DNA hypomethylation coupled to repressive chromatin domain formation and gene silencing in breast cancer. *Genome Res.*, 2012, vol. 22 (2), 246-58, http://genome.cshlp.org/content/22/2/246 **[0116]**
- **HEYN H et al.** Whole-genome bisulfite DNA sequencing of a DNMT3B mutant patient. *Epigenetics*, 2012, vol. 7 (6), 542-50, http://www.tandfonline.com/doi/abs/10.4161/epi.20523#.VsS_gdIUVIw **[0116]**
- **HOW KA** ; **NIELSEN HM** ; **TOST J.** DNA methylation based biomarkers: practical considerations and applications. *Biochimie*, 2012, vol. 94, 2314-37 **[0216]**
- **MIKESKA T** ; **CRAIG JM.** DNA methylation biomarkers: cancer and beyond. *Genes (Basel)*, 2014, vol. 5, 821-64 **[0216]**
- **NOEHAMMER C** ; **PULVERER W** ; **HASSLER MR** ; **HOFNER M** ; **WIELSCHER M** ; **VIERLINGER K et al.** Strategies for validation and testing of DNA methylation biomarkers. *Epigenomics*, 2014, vol. 6, 603-22 **[0216]**
- **WARTON K** ; **SAMIMI G.** Methylation of cell-free circulating DNA in the diagnosis of cancer. *Front Mol.Biosci.*, 2015, vol. 2, 13 **[0216]**
- **WITTENBERGER T** ; **SLEIGH S** ; **REISEL D** ; **ZIKAN M** ; **WAHL B** ; **ALUNNI-FABBRONI M et al.** DNA methylation markers for early detection of women's cancer: promise and challenges. *Epigenomics*, 2014, vol. 6, 311-27 **[0216]**
- **USADEL H** ; **BRABENDER J** ; **DANENBERG KD** ; **JERONIMO C** ; **HARDEN S** ; **ENGLES J et al.** Quantitative adenomatous polyposis coli promoter methylation analysis in tumour tissue, serum, and plasma DNA of patients with lung cancer. *Cancer Res.*, 2002, vol. 62, 371-5 **[0216]**
- **ESTELLER M** ; **SANCHEZ-CESPEDES M** ; **ROSELL R** ; **SIDRANSKY D** ; **BAYLIN SB** ; **HERMAN JG**. Detection of aberrant promoter hypermethylation of tumour suppressor genes in serum DNA from non-small cell lung cancer patients. *Cancer Res.*, 1999, vol. 59, 67-70 **[0216]**
- **MAZUREK A** ; **PIERZYNA M** ; **GIGLOK M** ; **DWORZECKA U** ; **SUWINSKI R** ; **MA UE**. Quantification of concentration and assessment of EGFR mutation in circulating DNA. *Cancer Biomark*, 2015, vol. 15, 515-24 **[0216]**
- **OSTROW KL** ; **HOQUE MO** ; **LOYO M** ; **BRAIT M** ; **GREENBERG A** ; **SIEGFRIED JM et al.** Molecular analysis of plasma DNA for the early detection of lung cancer by quantitative methylation-specific PCR. *Clin.Cancer Res.*, 2010, vol. 16, 3463-72 **[0216]**
- **POWROZEK T** ; **KRAWCZYK P** ; **KUCHARCZYK T** ; **MILANOWSKI J.** Septin 9 promoter region methylation in free circulating DNA-potential role in non-invasive diagnosis of lung cancer: preliminary report. *Med.Oncol.*, 2014, vol. 31, 917 **[0216]**
- **LIN PC** ; **LIN JK** ; **LIN CH** ; **LIN HH** ; **YANG SH** ; **JIANG JK et al.** Clinical Relevance of Plasma DNA Methylation in Colorectal Cancer Patients Identified by Using a Genome-Wide High-Resolution Array. *Ann.Surg.Oncol.*, 2014 **[0216]**

- **PHILIPP AB** ; **NAGEL D** ; **STIEBER P** ; **LAMERZ R** ; **THALHAMMER I** ; **HERBST A et al.** Circulating cell-free methylated DNA and lactate dehydrogenase release in colorectal cancer. *BMC.Cancer*, 2014, vol. 14, 245 **[0216]**
- **CHIMONIDOU M** ; **STRATI A** ; **MALAMOS N** ; **GEORGOULIAS V** ; **LIANIDOU ES**. SOX17 promoter methylation in circulating tumour cells and matched cell-free DNA isolated from plasma of patients with breast cancer. *Clin.Chem.*, 2013, vol. 59, 270-9 **[0216]**
- **CHIMONIDOU M** ; **TZITZIRA A** ; **STRATI A** ; **SOTIROPOULOU G** ; **SFIKAS C** ; **MALAMOS N et al.** CST6 promoter methylation in circulating cell-free DNA of breast cancer patients. *Clin.Biochem.*, 2013, vol. 46, 235-40 **[0216]**
- **MARTINEZ-GALAN J** ; **TORRES-TORRES B** ; **NUNEZ MI** ; **LOPEZ-PENALVER J** ; **DEL MR** ; **RUIZ DE ALMODOVAR JM et al.** ESR1 gene promoter region methylation in free circulating DNA and its correlation with estrogen receptor protein expression in tumour tissue in breast cancer patients. *BMC.Cancer*, 2014, vol. 14, 59 **[0216]**
- **MATUSCHEK C** ; **BOLKE E** ; **LAMMERING G** ; **GERBER PA** ; **PEIPER M** ; **BUDACH W et al.** Methylated APC and GSTP1 genes in serum DNA correlate with the presence of circulating blood tumour cells and are associated with a more aggressive and advanced breast cancer disease. *Eur.J.Med.Res*, 2010, vol. 15, 277-86 **[0216]**
- **FACKLER MJ** ; **LOPEZ BZ** ; **UMBRICHT C** ; **TEO WW** ; **CHO S** ; **ZHANG Z et al.** Novel methylated biomarkers and a robust assay to detect circulating tumour DNA in metastatic breast cancer. *Cancer Res.*, 2014, vol. 74, 2160-70 **[0216]**
- **AVRAHAM A** ; **UHLMANN R** ; **SHPERBER A** ; **BIRNBAUM M** ; **SANDBANK J** ; **SELLA A et al.** Serum DNA methylation for monitoring response to neoadjuvant chemotherapy in breast cancer patients. *Int.J.Cancer*, 2012, vol. 131, E1166-E1172 **[0216]**
- **SHARMA G** ; **MIRZA S** ; **PARSHAD R** ; **GUPTA SD** ; **RALHAN R.** DNA methylation of circulating DNA: a marker for monitoring efficacy of neoadjuvant chemotherapy in breast cancer patients. *Tumour.Biol.*, 2012, vol. 33, 1837-43 **[0216]**
- **LEGENDRE C** ; **GOODEN GC** ; **JOHNSON K** ; **MARTINEZ RA** ; **LIANG WS** ; **SALHIA B.** Whole-genome bisulfite sequencing of cell-free DNA identifies signature associated with metastatic breast cancer. *Clin.Epigenetics.*, 2015, vol. 7, 100 **[0216]**
- **JONES PA.** Functions of DNA methylation: islands, start sites, gene bodies and beyond.. *Nat.Rev.Genet.*, 2012, vol. 13, 484-92 **[0216]**
- **COPE LM** ; **FACKLER MJ** ; **LOPEZ-BUJANDA Z** ; **WOLFF AC** ; **VISVANATHAN K** ; **GRAY JW et al.** Do breast cancer cell lines provide a relevant model of the patient tumour methylome?. *PLoS.One*, 2014 (9), e105545 **[0216]**
- **BECKER D** ; **LUTSIK P** ; **EBERT P** ; **BOCK C** ; **LENGAUER T** ; **WALTER J.** BiQ Analyzer HiMod: an interactive software tool for high-throughput locus-specific analysis of 5-methylcytosine and its oxidized derivatives. *Nucleic Acids Res.*, 2014, vol. 42, W501-W507 **[0216]**
- **LUTSIK P** ; **FEUERBACH L** ; **ARAND J** ; **LENGAUER T** ; **WALTER J** ; **BOCK C.** BiQ Analyzer HT: locus-specific analysis of DNA methylation by high-throughput bisulfite sequencing. *Nucleic Acids Res.*, 2011, vol. 39, W551-W556 **[0216]**
- **SOREIDE K.** Receiver-operating characteristic curve analysis in diagnostic, prognostic and predictive biomarker research. *J.Clin.Pathol.*, 2009, vol. 62, 1-5 **[0216]**
- **MADIC, J. et al.** Pyrophosphorolysis-activated polymerization detects circulating tumor DNA in metastatic uveal melanoma. *Clinical Cancer Research: an official journal of the American Association for Cancer Research*, 2012, vol. 18, 3934-3941 **[0216]**
- **BIDARD, F. C. et al.** Detection rate and prognostic value of circulating tumor cells and circulating tumor DNA in metastatic uveal melanoma. *International Journal of Cancer*, 2014, vol. 134, 1207-1213 **[0216]**
- The Molecular Taxonomy of Primary Prostate Cancer. *Cell*, 2015, vol. 163 (4), 1011-25 **[0216]**