

BIOMARKERS FOR DOWN SYNDROME PRENATAL DIAGNOSIS

CROSS-REFERENCE TO RELATED APPLICATIONS

[001] This application claims the benefit of priority of Singapore patent application No. 201207172-6, filed 26 September 2012, the contents of it being hereby incorporated by reference in its entirety for all purposes.

FIELD OF THE INVENTION

[002] The present invention relates to biochemistry in particular biomarkers/biomarker regions. In particular, the present invention relates to biomarker/biomarker regions associated with Down syndrome and methods of using the biomarkers to determine the likelihood that a foetus will have Down syndrome.

BACKGROUND OF THE INVENTION

[003] Current methods for screening congenital diseases in foetus include ultrasound such as foetal nuchal translucency (NT) and other tests to detect biomarkers found in maternal serum. For example, in screens for Down syndrome, biomarkers measured include the amount of alpha fetoprotein (AFP) and human chorionic gonadotropin, which are produced by the foetus and the placenta and can be detected in the maternal serum. Together with the age of the mother and results of foetal nuchal translucency scan, the measurements of alpha fetoprotein and human chorionic gonadotropin are used to calculate the risk of the baby having Down syndrome. When the resulting numerical risk is classified as high risk, to confirm the results, an invasive test using chorionic villus sampling (CVS) or amniocentesis to obtain foetal tissue is required. As chorionic villus sampling or amniocentesis involves the insertion of a fine needle into the womb, these procedures may cause miscarriage.

[004] Down syndrome, or Mongolism, is a congenital condition caused by a defect in the chromosomes. An individual born with Down syndrome has three copies of chromosome 21, instead of the usual two, thus causing the disease to be also known as trisomy 21.

[005] The cause of Down syndrome is unclear and no direct genotype-phenotype associations have been established. However, certain conditions such as advanced maternal

age and history of having another child or previous pregnancy with Down syndrome are found to increase risk of having a foetus with Down syndrome. As an individual with Down syndrome would develop complex clinical features and symptoms such as lifelong mental retardation, development delays and other problems such as seizures, thyroid disorders, cardiac defects, an increased risk of leukaemia, infertility, gastrointestinal defects and early aging, there is a need to provide for an accurate detection of a foetus with trisomy 21.

[006] Since the current screening markers offer low specificity and reliable screening methods rely on the collection of amniotic fluid via amniocentesis or chorionic villus sampling sample, there is a need to provide alternative methods or biomarkers that can be used to screen diseases in foetus.

SUMMARY OF THE INVENTION

[007] In one aspect, there is provided an isolated biomarker/biomarker region comprising a DNA region of the human genome selected from a DNA region listed in any one of tables 3 to 6 (groups 3, 4, 1' and 2').

[008] In another aspect, there is provided an isolated biomarker/biomarker region for detecting trisomy 21 or partial trisomy 21, comprising a DNA region of the human genome selected from a DNA region listed in any one of tables 3 to 6 (groups 3, 4, 1' and 2').

[009] In yet another aspect, there is provided an isolated biomarker/biomarker region comprising a DNA region of the human genome selected from a DNA region listed in any one of tables 1 to 4 and 7 to 8 (groups 1 to 4 and Mix10 Group 1 and Mix10 Group 2 respectively), wherein the level of DNA-methylation of any one of the biomarker/biomarker regions in a diseased sample is different from the level of DNA-methylation in the same biomarker/biomarker region of a non-diseased control DNA.

[0010] In yet another aspect there is provided a method determining the likelihood of a foetus to suffer from a specific disease. The method comprising the steps of: a) providing an isolated total DNA sample from a pregnant woman, comprising foetal DNA and maternal DNA. Further comprising the steps of: b) removing maternal DNA background; c) measuring a signal indicative for the level of foetal DNA based on one or more biomarkers/biomarker regions, where in the case where the maternal DNA background had a level of methylation below 10%, the signal is the level of methylated foetal DNA and in the case where the

maternal DNA background had a level of methylation above 90%, the signal is the level of unmethylated foetal DNA; d) determining a ratio of signals obtained under step c) by dividing the signals of one or more of Group 1 and/or Group 3 biomarkers/biomarker regions over the signals of one or more of Group 2 and/or Group 4 biomarkers/biomarker regions, wherein a ratio higher than the ratio determined in control foetal DNA obtained from a non-diseased foetus indicates that the foetus is likely to suffer from the specific disease. In one example, each of the groups is characterized by:

[0011] Group 1: maternal DNA background has a level of methylation below 10% and the signal of the biomarker/biomarker region is higher in foetal DNA obtained from a foetus suffering from the specific disease compared to the same biomarker/biomarker region in control foetal DNA obtained from a foetus not suffering from the disease.

[0012] Group 2: maternal DNA background has a level of methylation below 10% and the signal of the biomarker/biomarker region is lower in foetal DNA obtained from a foetus suffering from the specific disease compared to the same biomarker/biomarker region in control foetal DNA obtained from a foetus not suffering from the disease.

[0013] Group 3: maternal DNA background has a level of methylation above 90% and the signal of the biomarker/biomarker region is higher in foetal DNA obtained from a foetus suffering from the specific disease compared to the same biomarker/biomarker region in control foetal DNA obtained from a foetus not suffering from the disease.

[0014] Group 4: maternal DNA background has a level of methylation above 90% and the signal of the biomarker/biomarker region is lower in foetal DNA obtained from a foetus suffering from the specific disease compared to the same biomarker/biomarker region in control foetal DNA obtained from a foetus not suffering from the disease.

[0015] In yet another aspect, there is provided a method of determining the likelihood of a foetus to suffer from trisomy 21 or partial trisomy 21. The method comprising the steps of: a) providing an isolated total DNA sample from a pregnant woman, comprising foetal DNA and maternal DNA; b) removing maternal DNA background; c) measuring a signal indicative for the level of foetal DNA based on one or more biomarkers/biomarker regions listed in any one of Tables 1 to 8, where in the case where the maternal DNA background had a level of methylation below 10%; the signal is the level of methylated foetal DNA and in the case where the maternal DNA background had a level of methylation above 90%, the signal is the

level of unmethylated foetal DNA; d) determining a ratio of signals obtained under step c) by dividing the signals of one or more of Group 1 and/or Group 3 biomarkers/biomarker regions over the signals of one or more of Group 2 and/or Group 4 biomarkers/biomarker regions, wherein a ratio higher than the ratio determined in control foetal DNA obtained from a non-diseased foetus indicates that the foetus is likely to suffer from trisomy 21 or partial trisomy 21. In one example, each of the groups is characterized by:

[0016] Group 1: biomarker/biomarker region listed in Table 1 (Group 1), Table 5 (Group 1'), or Table 7 (Mix10 Group 1).

[0017] Group 2: biomarker/biomarker region listed in Table 2 (Group 2) or Table 6 (Group 2'), or Table 8 (Mix10 Group 2).

[0018] Group 3: biomarker/biomarker region listed in Table 3 (Group 3).

[0019] Group 4: biomarker/biomarker region listed in Table 4 (Group 4).

[0020] In yet another aspect there is provided a kit comprising primers for amplifying the one or more biomarkers/biomarker regions selected from any one of the DNA regions of the human genome listed in any one of tables 3 to 6 (groups 3, 4, 1' and 2'). The kit further comprises one or more reagents for measuring a signal indicative for the level of foetal DNA based on the one or more biomarkers/biomarker regions.

[0021] In yet another aspect there is provided a kit comprising primers for amplifying the one or more biomarkers/biomarker regions selected from any one of the DNA regions of the human genome listed in any one of tables 1 to 4 (groups 1 to 4). The kit further comprises one or more reagents for measuring a signal indicative for the level of foetal DNA based on the one or more biomarkers/biomarker regions.

[0022] In yet another aspect there is provided a kit comprising primers for amplifying the one or more biomarkers/biomarker regions selected from any one of the DNA regions of the human genome listed in any one of tables 7 to 8 (Mix10 Group 1 and Mix10 Group 2). The kit further comprises one or more reagents for measuring a signal indicative for the level of foetal DNA based on the one or more biomarkers/biomarker regions.

[0023] In yet another aspect there is provided a method of determining the methylation levels of a biomarker/biomarker region comprising the steps of: a) treating a sample comprising both foetal and maternal DNA with a reagent that differentially modifies methylated and non-methylated DNA. The method further comprises b) calculating the

percentage of unmodified cytosine residues over the total number of modified and unmodified cytosine residues in order to determine the methylation levels of a biomarker/biomarker region.

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BRIEF DESCRIPTION OF THE DRAWINGS

[0024] The invention will be better understood with reference to the detailed description when considered in conjunction with the non-limiting examples and the accompanying drawings.

[0025] **Figure 1:** Schematic illustration of the steps performed in Example 2, whereby a trisomy 21 (T21) foetus detection using methylation biomarkers is performed. The steps are explained in detail in the description. The result of performing these steps is a differentiation between a trisomy 21 foetus and a normal foetus by quantifying the foetal-specific DNA, utilizing a universal qPCR primer pair and group specific probes. If the ratio between group 1 and group 2 is high, then the foetus is deemed to be trisomy 21; if the ratio is low, then the foetus is deemed to be normal. The group specific probes are defined according to tables 7 and 8. Figure 1 illustrates one example of the application of the method of the present disclosure in determining the likelihood of a foetus having trisomy 21.

[0026] **Figure 2:** Signal difference between Group 1 and Group 2 biomarkers with probe mix10. This histogram shows data resulting from a DNA analysis using Group 1 and Group 2 biomarkers on trisomy 21 (T21) and normal samples. The difference shown here is the result of different methylations between the trisomy 21 and the normal group when using probe mix10, which contains 35 biomarkers from Group 1 and 26 biomarkers from Group 2. The details of probe sequences and their target biomarkers are listed in Mix10 Group 1 (see Table 7) and Mix10 Group 2 (see Table 8). Thus, Figure 2 demonstrates an exemplary data that may be obtained from a method of the present disclosure, showing samples obtained from trisomy 21 are markedly different from sample obtained from normal individual.

[0027] **Figure 3:** Signal difference between normal and trisomy 21 (T21) foetal DNA with probe mix10. This histogram here visualizes the difference in the signal intensity between trisomy 21 and normal tissues. $\Delta\Delta Ct$ (Group2 – Group1) values from probe mix10, whose methylation difference between normal and trisomy 21 tissues is the biggest among all combinations of probe mixtures tested, is shown. Thus, Figure 3 shows clear differences

observed in signals obtained from samples analysed using the biomarker/biomarker regions of the present disclosure.

[0028] **Figure 4:** Sensitivity assessment on probe mix10 showing (A) ΔC_t (Group2 – Group1) and (B) $\Delta\Delta C_t$ (Group2 – Group1) with different concentration of spiked foetal DNA. The sample spiked in with trisomy 21 (T21) placenta DNA (mimicking a maternal plasma sample from a woman pregnant with a trisomy 21 foetus) was clearly different from the sample spiked in with normal CVS DNA (mimicking a maternal plasma sample from a woman pregnant with a non- trisomy 21 foetus). Thus, Figure 4 shows the sensitivity of probes for the detection of biomarker/biomarker regions of the present disclosure.

[0029] **Figure 5:** Detection of methylated genomic DNA signal in maternal plasma. (A) The figure shows a one-dimensional scatter plot, representing the DNA methylation level in the examined biomarkers. (B) shows another scatter plot, this time depicting the methylation ratio of Group 1 and Group 2 from maternal plasma samples. This comparison visualizes the higher values of methylation ratio of Group 1 and Group 2 in trisomy 21 (T21) samples than in normal samples. Thus, Figure 5 demonstrates that DNA-methylation level of biomarker/biomarker regions of the present disclosure obtained from trisomy 21 sample is markedly different from DNA-methylation level of samples obtained from non-trisomy 21 sample (normal).

BRIEF DESCRIPTION OF THE TABLES

[0030] **Table A** shows the classification of different biomarker/biomarker regions as Groups 1 to 4.

[0031] **Table B** shows the DNA methylation of DNA obtained from normal chorionic villus sample versus Trisomy 21 chorionic villus sample or placenta.

[0032] **Table C** shows the DNA methylation of DNA obtained from Trisomy 21 chorionic villus sample or placenta versus normal chorionic villus sample.

[0033] **Table 1** lists biomarker/biomarker regions that fall within Group 1 as described herein.

[0034] **Table 2** lists biomarker/biomarker regions that fall within Group 2 as described herein.

[0035] **Table 3** lists biomarker/biomarker regions that fall within Group 3 as described herein.

[0036] **Table 4** lists biomarker/biomarker regions that fall within Group 4 as described herein.

[0037] **Table 5** lists biomarker/biomarker regions that fall within Group 1' as described herein.

5 [0038] **Table 6** lists biomarker/biomarker regions that fall within Group 2' as described herein.

[0039] **Table 7** lists biomarker/biomarker regions that fall within Mix10 Group 1 as described herein.

[0040] **Table 8** lists biomarker/biomarker regions that fall within Mix10 Group 2 as
10 described herein.

DETAILED DESCRIPTION OF THE PRESENT INVENTION

[0041] The inventors of the present disclosure found that depending on the
15 biomarker/biomarker region, the level of DNA-methylation in a foetal DNA and maternal DNA may be different such that the differences may be used to differentiate (1) maternal DNA from foetal DNA and (2) foetal DNA from a foetal with or without the condition or disease. As used herein, the term "disease" and "condition" are interchangeably used to refer to a condition that is not considered to be the norm, normal or healthy. In one example, the
20 disease or condition is Down syndrome or trisomy 21. As used herein, "DNA-methylation" refers to the addition of a methyl group to the cytosine or adenine nucleotides in a DNA sequence. The term "maternal DNA" refers to DNA or polynucleotide obtained from the mother of the foetus or the individual within whose womb the foetus is carried. In one example, the maternal DNA may include, but is not limited to maternal DNA obtained from
25 tissue or cell samples and maternal peripheral blood DNA. In contrast, the term "foetal DNA" refers to DNA or polynucleotide obtained from the foetus or the individual suspected to have the condition or disease.

[0042] For example, when maternal blood DNA is close to zero methylation, methylation sensitive enzymes may be used to digest maternal DNA, thus isolating the methylated foetal
30 DNA intact for further analysis. The phrase "zero methylation" means substantially none or about 1%, about 2%, about 3%, about 4%, about 5%, about 6%, about 7%, about 8%, about

9% or about 10% methylation observed. In contrast, when the region in the maternal blood DNA is highly methylated, methylation dependent enzymes can be used to digest maternal DNA to thus isolate the non-methylated foetal DNA intact for further analysis. The phrase “highly methylated” refers to fully, substantially fully or close to 100% methylation or about 100%, about 99%, about 98%, about 97%, about 96%, about 95%, about 94%, about 93%, about 92% or about 90%. Upon removal of maternal DNA by the degree of methylation observed in the maternal DNA, the level of DNA-methylation of the isolated foetal DNA is then analysed. The inventors of the present disclosure found that the isolated foetal DNA from a foetus with a condition or disease would typically be differentially methylated as compared to a foetus without the condition or disease.

[0043] Accordingly, disclosed is a method of determining the methylation levels of a biomarker/biomarker region. The method may comprise the steps of: a) treating a sample comprising both foetal and maternal DNA with a reagent that differentially modifies methylated and non-methylated DNA. The method may further comprise b) calculating the percentage of unmodified cytosine residues over the total number of modified and unmodified cytosine residues in order to determine the methylation levels of a biomarker/biomarker region. For example, the reagents may include, but are not limited to sodium bisulfite, one or more enzymes that only cleave methylated DNA, such as methylation dependent enzyme and one or more enzymes that only cleave non-methylated DNA, such as methylation sensitive enzyme. The method of determining the methylation level of biomarker/biomarker region as disclosed herein may further comprise the step of bisulfite sequencing, which may be performed before the step of calculating the percentage of unmodified cytosine residues (i.e. step (b) of the method as described herein). The step of bisulfite sequencing may be a reduced representation bisulfite sequencing (RRBS), which is used to quantify genome wide DNA-methylation profiles in placenta samples from normal individual or individual with the disease or condition. From bisulfite sequencing step, signals detected from the unmodified cytosine residues and the modified cytosine residues are compared to calculate the methylation level.

[0044] The method of determining the methylation levels of a biomarker/biomarker region paves the way to an object of the present disclosure of providing a method of screening for biomarker/biomarker regions for Down syndrome. The term “trisomy 21” may be used

interchangeably with "Down syndrome" and as used herein refers to a state where an individual or subject or foetus's karyotype is characterized by a complete or partial triplication of human chromosome 21 (HSA21). When an individual or subject or foetus's has partial triplication of human chromosome 21, the individual would be known as a partial trisomy 21. Trisomy 21 leads to complex clinical features and symptoms, for example mental retardation, Alzheimer's disease, seizures, thyroid disorders, cardiac defects, an increased risk of leukaemia, infertility, gastrointestinal defects and early aging.

[0045] When the disease or condition is Down syndrome, differentially methylated regions may be selected based on following steps. First, individual CpG sites may be selected. Methylation level of each CpG site may be calculated as: Methylation level for a CpG = Count of Cytosine / (Count of Cytosine + Count of Thymine)*100%.

[0046] Individual CpG sites may be selected using the following criteria:

- 1) present in at least two normal chorionic villus sample, three T21 chorionic villus sample/placenta, and one maternal blood samples;
- 2) with difference of average(normal) - average(T21) $\geq$ 10% or difference of average(T21) - average(normal) $\geq$ 10%;
- 3) with a Wilcoxon Rank Sum test p value of ≤ 0.05 .

[0047] Next, genomic regions with differential methylation between normal and T21 placenta samples may be selected using the following criteria:

- 1) at least 2 CpGs (preferably at least 3 CpGs) with a distance of not more than 150bp from its nearest neighbor;
- 2) the average methylation of such regions in maternal blood samples may be either $\geq 90\%$ or $\leq 10\%$;
- 3) average methylation of such regions in normal samples, named as (average(normal)(region)); and average methylation of such regions in T21 samples named as (average (T21)(region)).

[0048] The difference between average (normal)(region) and average (T21)(region) may be at least 10%, except when average maternal blood $\leq 10\%$, regions with average (normal)(region) $\geq$ average(T21)(region) may be also included.

[0049] In another example, further selection criteria may be used for more stringent final biomarker selection:

1. For methylation regions with an average(maternal blood) $\leq 10\%$, regions that may be

5 1) with average(T21) - average(normal) $\geq 25\%$; or

2) average(T21) - average(normal) between 15-25% and ratio of average(T21)/average(normal) ≥ 3 ; or

3) average(T21) - average(normal) between 10-15% and ratio of average(T21)/average(normal) ≥ 5 may be selected.

10 These regions may be listed as Group 1 biomarkers after an extension of 500-bp both up and downstream for each region.

2. For methylation regions with an average(maternal blood) $\leq 10\%$, regions that may be:

15 1) average(normal) - average(T21) $\geq 10\%$; or

2) a subset of regions with average(T21) $\leq$ average(normal) were selected. These regions may be listed as Group 2 biomarkers after an extension of 500-bp both up and downstream for each region.

3. For methylation regions with an average(maternal blood) $\geq 90\%$, regions that have:

20 1) average(normal) - average(T21) $\geq 25\%$, or

2) (average(normal) - average(T21)) between 15-25% and ratio of (1 - average(T21))/(1 - average(normal)) ≥ 2 were selected.

These regions may be listed as Group 3 biomarkers after an extension of 500-bp both up and downstream for each region.

25 4. For methylation regions with an average(maternal blood) $\geq 90\%$, regions that have:

1) average(T21) - average(normal) $\geq 25\%$ and ratio of (1 - average(normal))/(1 - average(T21)) > 2 , or

2) (average(T21) - average(normal)) between 10-25% and ratio of (1 - average(normal))/(1 - average(T21)) > 3 were selected.

30 These may be listed as Group 4 biomarkers after an extension of 500-bp both up and downstream for each region.

Table A – Classification of different biomarker/biomarker regions as Groups 1 to 4.

Group	Biomarkers falling within Group	DNA methylation level T21 vs. Normal foetus	DNA methylation level Maternal DNA
Group 1	Tables 1, 5 and 7	T21 > Normal	< 10% methylation
Group 2	Tables 2, 6 and 8	T21 < Normal	< 10% methylation
Group 3	Table 3	T21 < Normal	> 90% methylation
Group 4	Table 4	T21 > Normal	> 90% methylation

[0050] Tables B and C below list exemplary biomarker/biomarker regions where differences in DNA methylation level may be observed between DNA from maternal blood, DNA from normal sample and DNA from Trisomy 21 sample.

Table B - DNA methylation with Normal chorionic villus sample (CVS) > Trisomy 21 chorionic villus sample/placenta

Chr	Position	DNA Methylation level (%)			Regional Difference	Gene Name	Functional Group
		Maternal Blood	Normal CVS	T21 CVS/placenta			
chr7	6524601-6524671	97.5	57.7	21.2	36.5	KDELR2	Promoter
chr17	2279396-2279515	97.0	62.6	34.2	28.4	SGSM2	Intragenic
chr8	92785473-92785570	97.1	82.6	54.5	28.1		Intergenic
	177042793						Promoter
chr2	177042924	1.1	37.2	12.0	25.2	BC047481	
chr19	2621909-2621929	2.4	35.0	12.6	22.4	GNG7	Intragenic

Table C- DNA methylation with Trisomy 21 chorionic villus sample (CVS)/placenta > Normal chorionic villus sample (CVS)

Chr	Position	DNA Methylation level (%)			Regional Difference	Gene Name	Functional Group
		Maternal Blood	Normal CVS	T21 CVS/placenta			

chr15	89950295-89950453	3.3	18.7	59.3	40.6		Intergenic
chr21	38630505-38630728	1.9	47.0	82.8	35.8	DSCR3	Intragenic
chr7	49813547-49813563	2.3	5.9	40.5	34.6	VWC2	Promoter
chr4	1189243-1189282	98.2	34.6	73.4	38.8	LOC100130872	TTS
chr22	48391057-48391113	100.0	55.0	90.5	35.5		Intergenic

[0051] Tables 1 to 8 below list the various biomarker/biomarker regions of the present disclosure. All chromosome coordinates are based on hg19/GRCh37 Feb 2009 human genome build002E (which can be accessed at:

5 http://www.ncbi.nlm.nih.gov/assembly/GCF_000001405.13/).

Table 1 – the following table shows group 1 biomarker/biomarker regions

Chromosome	position±500bp
chr20	62327556-62328688
chr7	156795287-156796392
chr15	89949795-89950953
chr16	79634775-79635808
chr4	90757154-90758285
chr4	104640269-104641588
chr2	74742958-74744103
chr14	103739131-103740259
chr21	42797438-42798786
chr10	125731845-125733055
chr15	101512966-101514272
chr8	103135245-103136502
chr13	33590446-33591598
chr10	109673856-109675121
chr5	132158175-132159485
chr7	49813047-49814063
chr2	74742706-74743768
chr11	65779019-65780152
chr5	122430786-122431815
chr20	62368643-62369892
chr2	118943553-118944570
chr8	23563507-23564874
chr17	36103765-36104809
chr16	54323801-54324805
chr15	78932964-78933968

chr2	214148641-214149777
chr17	1011822-1012918
chr7	99516170-99517263
chrX	40125892-40126898
chr2	5836739-5837793
chr4	90758296-90759398
chr5	140011702-140012843
chr1	6514210-6515248
chr6	5996890-5997909
chr19	12305682-12306758
chr4	4861330-4862458
chr9	135645025-135646255
chr14	103673445-103674541
chr7	132261359-132262430
chr22	26565117-26566148
chr4	115519583-115520722
chr4	111557910-111559066
chr9	66457923-66459013
chr2	135475418-135476536
chr4	106988-108391
chr14	57274628-57275782
chr8	25901324-25902546
chr1	50890150-50891277
chr11	101453003-101454267
chr5	178421018-178422525
chr16	31548424-31549641
chr8	24812205-24813263
chr5	92915133-92916248
chr10	90342145-90343287
chr1	152083001-152084318
chr10	27547200-27548767
chr3	138663228-138664580
chr19	24154030-24155130
chr19	23653426-23654731
chr11	17741959-17743037
chr6	150335417-150336532
chr16	67207694-67209016
chr6	32632142-32633492
chr17	5000103-5001199
chr19	51829455-51830465
chr2	132181990-132183067
chr13	103052783-103053921
chr4	85418583-85419780

chr8	55366289-55367317
chr20	690183-691465
chr10	100991951-100993104
chr6	88876690-88877844
chr19	15343524-15344632
chr1	110611097-110612153
chr21	36042057-36043111
chr4	57521884-57523144
chr5	41869248-41870356
chr12	114029146-114030172
chr10	79397343-79398444
chr3	112052013-112053048
chr9	74764214-74765324
chrX	8698540-8699634
chr5	92914360-92915699
chr2	145273156-145274276
chr12	128751594-128752716
chr11	91959200-91960279
chr13	88324117-88325239
chr13	28497927-28499006
chr4	11429503-11430522
chr10	106399758-106400862
chr2	66658936-66659963
chr10	124220023-124221086
chr13	107186087-107187334
chr1	36038365-36039503
chr17	35299466-35300667
chr6	94128675-94129780
chr7	8473676-8474912
chr9	971639-972744
chr2	163199923-163200927
chr2	99553019-99554306
chr6	88876067-88877277
chr1	65991061-65992095
chr6	29855413-29856640
chr5	146613621-146614641
chr3	124930634-124932388
chr11	32354926-32355951
chr13	88323676-88324838
chr19	15342338-15343517
chr8	49782305-49783575
chr3	142839524-142840648
chr19	2250588-2251672

chr8	77592718-77593741
chr13	28493763-28495214
chr9	87284312-87285413
chrX	3262935-3264257
chr8	70981955-70983203
chr7	155249806-155251049
chr7	132261841-132262954
chr12	3599645-3600720
chr1	228224963-228226186
chr7	155249472-155250611
chr1	197880259-197881268
chr20	5296483-5297602
chr14	61114773-61116003
chr10	22764545-22765835
chr17	54990569-54991590
chr4	74734622-74735847
chr16	31711698-31712811
chr3	113251199-113252215
chr2	115918734-115919960
chr8	21646427-21647429
chr10	101088600-101089780
chr1	110626620-110627720
chr2	115919265-115920294
chr1	161228118-161229507
chr9	135465566-135466709
chr2	171673469-171674701
chr1	180203249-180204261
chr16	51185313-51186521
chr4	6223349-6224451
chr15	31775014-31776402
chr8	57231942-57233065
chr17	75954394-75955543
chr5	42951660-42952924
chr7	156796321-156797577
chr1	24228778-24229812
chr19	518649-519755
chr21	38630005-38631228
chr9	32782722-32783740
chr2	172945711-172946769
chr10	77156215-77157247
chr3	42947042-42948101
chr19	12475550-12476612
chr12	34260341-34261405

chr14	21093532-21094652
chr5	177667303-177668321
chr11	124790563-124791573
chr8	101117982-101119047
chr7	156797935-156799045
chr2	106681296-106682342
chr2	63280742-63281817
chr4	87824903-87825959
chr5	2748910-2750009
chr13	113764818-113765878
chr3	111904200-111905251
chr6	166073781-166074809
chr5	77253364-77254398
chr1	75138804-75139849
chr19	518371-519433
chr7	30721052-30722082
chr2	239071774-239072929
chr13	112714744-112715860
chr1	149672426-149673448
chr1	149615701-149616830
chr20	61048889-61049961
chr1	12227352-12228398
chr15	89948892-89949973
chr2	223161561-223162697
chr6	42694249-42695397
chr11	121970597-121971747
chr3	138678847-138679945
chr7	27141178-27142358
chr10	135341690-135342932
chr4	154604660-154606098
chr10	77166921-77167990
chr7	73416687-73417845
chr4	13524494-13525631
chr3	147114137-147115248
chr2	241496948-241498061
chr15	98418490-98419500
chr10	134600337-134601402
chr9	970745-971830
chr4	4855901-4856971
chr10	128993788-128994821
chr18	55103101-55104341
chr6	108440069-108441259
chr22	20779311-20780375

chr5	149339286-149340330
chr18	45057772-45058793
chr4	56659363-56660374
chr1	1475284-1476363
chr6	3355941-3357035
chr4	174444705-174445862
chr5	113391472-113392504
chr19	58867314-58868863
chr1	110753882-110754909
chr4	11369986-11371089
chr19	57617794-57618910
chr2	19560468-19561882
chr10	118893836-118894926
chr2	119599365-119600494
chr10	102880602-102881746
chr4	154710097-154711228
chr10	75406913-75407978
chr20	21490302-21491310
chr7	116963944-116964976
chr21	47717342-47718506
chr15	76634069-76635191
chr6	19836963-19837983
chr14	59930967-59932050
chr4	4144533-4145643
chr17	59529160-59530349
chr1	6508439-6509543
chr13	44947232-44948683
chr6	31782746-31783810
chr9	96716921-96717966
chr1	36348326-36349400
chr5	75377598-75378688
chr19	12605940-12607209
chr1	47903958-47905010
chrX	39958047-39959078
chr4	90757619-90758716
chr1	1475596-1476623
chr19	21768903-21770103
chr22	24988194-24989219
chr20	62119130-62120207
chr13	79183133-79184159
chr1	110610423-110611546
chr3	192231880-192232883
chr1	53067432-53068754

chr14	101012142-101013156
chr6	26383204-26384217
chr1	65990519-65991705
chr6	159590278-159591499
chr10	106402152-106403195
chr5	115298082-115299167
chr13	28495094-28496169
chr16	55090182-55091327
chr2	74741146-74742198
chr9	972300-973470
chr6	58147012-58148096
chr5	75378503-75379914
chr10	43697466-43698614
chr7	64029494-64030615
chr17	42733794-42734801
chr1	99469575-99470882
chr6	29894118-29895181
chr12	186616-187663
chr6	132271437-132272559
chr16	47177934-47179073
chr11	31832420-31833516
chr18	77557591-77558639
chr6	166073966-166074991
chrX	40034167-40035274
chr6	30181343-30182675
chr10	22541383-22542500
chr19	3585181-3586394
chr10	100993393-100994709
chr13	79175198-79176772
chr8	23145402-23146448
chr13	21649464-21650563
chr4	75719138-75720217
chr8	103136154-103137408
chr1	36348827-36350064
chr15	28351810-28352993
chr3	96533005-96534029
chr16	65156230-65157298
chr8	55371384-55372441
chr21	34443211-34444382
chr5	134870249-134871600
chr5	77267916-77269224
chr20	43726021-43727045
chr16	22824422-22825688

chr2	115919472-115920484
chr19	47742446-47743585
chr20	30639826-30640917
chr22	51111920-51112989
chr3	105085656-105086690
chr14	103654845-103656221
chr4	190935571-190936589
chr2	105473874-105474938
chr11	17740927-17742032
chr9	139024152-139025261
chr7	121945355-121946609
chr7	155241820-155243015
chr5	140430790-140431893
chr9	96712945-96714056
chr6	41339962-41341707
chr7	156400077-156401426
chr18	77159068-77160097
chr18	60263106-60264250
chr7	19146867-19148026
chr15	30516966-30518116
chr1	20669276-20670328
chr17	70117154-70118558
chrX	40034885-40036021
chr1	114695254-114696299
chr17	54911376-54912688
chrX	9732865-9733965
chrX	6144580-6145595
chr12	31079245-31080319
chr6	6003739-6004908
chr1	241587181-241588433
chr8	41754670-41755694
chr16	54964538-54965902
chr4	174450805-174451938
chr1	159823558-159824677
chr8	37823023-37824385
chr5	87973673-87974741
chr16	67196451-67197670
chr3	13114257-13115348
chrX	114795895-114796907
chr8	145924929-145925943
chr4	30722888-30724010
chr4	1164481-1165501
chr7	21984531-21985631

chr6	31324194-31325462
chr3	45837269-45838527
chr17	27942575-27943615
chr11	44326881-44327978
chr19	8656375-8657381
chr10	22765474-22766553
chr18	44786948-44788097
chr1	33772291-33773475
chr1	113286413-113287533
chr7	153583712-153584742
chr5	82767869-82768964
chr10	72217791-72218837
chr12	113913247-113914358
chr9	140172312-140173550
chr6	26501341-26502453
chr17	36103118-36104390
chr10	42970960-42972143
chr7	158936875-158937980
chr2	1748371-1749376
chr8	69243257-69244288
chr16	54970558-54971645
chr8	9764031-9765054
chr9	842120-843612
chr2	172952602-172953626
chr2	133427083-133428199
chr14	59930491-59931634
chr13	20692102-20693181
chr3	77088401-77089812
chr18	44337399-44338422
chr10	126135331-126137188
chr5	137577248-137578414
chr1	9712287-9713390
chr13	79176043-79177076
chr19	12605693-12606740
chr14	59931412-59932478
chr17	66097225-66098410
chr15	55581822-55582906
chr17	1012309-1013345
chr1	53098584-53099635
chr22	50241983-50243135
chr12	6664496-6665516
chr4	186048153-186049274
chr18	52988666-52989672

chr17	19482933-19483953
chr2	225306989-225308020
chr16	67449902-67450941
chr15	31775691-31776827
chrX	39955333-39956431
chr11	57194026-57195059
chr11	111169716-111170774
chr12	72665649-72666818
chr1	45249552-45250648
chr2	29338325-29339442
chr11	100997875-100998907
chr7	53286535-53287614
chr9	132312254-132313324
chr22	48971239-48972323
chr2	193059493-193060598
chr1	10057231-10058257
chr11	17742294-17743364
chr11	111169255-111170351
chr17	75954719-75955800
chr22	25816648-25817666
chr2	99438484-99439585
chr8	41624127-41625221
chr1	37499295-37500364
chr2	163200364-163201409
chr2	107502606-107503922
chr4	155664449-155665498
chrX	40013880-40014965
chrX	40013390-40014703
chr1	114694636-114695802
chrX	15872020-15873196
chr11	30606334-30607434
chr14	99740085-99741112
chrX	39963638-39964797
chr19	35454254-35455374
chr13	22248958-22249968
chr4	187025401-187026581
chr17	66192588-66193620
chr2	5836246-5837312
chr21	34443877-34445016
chr7	97360924-97362033
chr1	91183001-91184015
chr21	27944733-27945766
chr6	132271747-132272781

chr2	169312310-169313377
chr19	59073628-59074697
chr19	36821970-36823132
chr14	57274109-57275116
chr16	88449175-88450191
chr11	32460086-32461090
chr1	53098242-53099332
chrX	16729550-16730618
chr6	105583839-105584865

Table 2 – Group 2 biomarker/biomarker regions

Chromosome	position±500bp
chr2	177042293-177043424
chr17	75447059-75448078
chr4	7194255-7195262
chr5	91589-92757
chr11	120856622-120857640
chr19	59024982-59026094
chr19	940516-941795
chr4	7193938-7195038
chr4	146654036-146655094
chr11	615557-616696
chr15	79382604-79383650
chr3	196728977-196730077
chr11	16631959-16633059
chr16	68572564-68573664
chr12	124941370-124942436
chr5	58335092-58336193
chr5	91749-92889
chr7	5467057-5468077
chr5	139493102-139494173
chr14	105662974-105663985
chr8	145805846-145806961
chr8	79428036-79429146
chr6	35265258-35266346
chr16	27329710-27330719
chr15	45458347-45459411
chr9	34989359-34990445
chr7	128530459-128531546
chr19	52206950-52208110
chr17	42430714-42431746

chr16	58549538-58550654
chr7	104624049-104625214
chr8	11659326-11660461
chr9	123630409-123631417
chr1	201367906-201369041
chr3	195633644-195634695
chr18	10131461-10132610
chr17	43095855-43096977
chr1	27667983-27669068
chr2	160653729-160654977
chr19	7547731-7548834
chr19	2621909-2621929

Table 3 – Group 3 biomarker/biomarker regions

Chromosome	position±500bp
chr19	1992317-1993338
chr9	83918469-83919525
chr8	92784973-92786070
chr2	131887213-131888412
chr1	40349978-40351047
chr2	7737860-7738957
chr7	6524101-6525171
chr17	2278896-2280015
chr5	112127163-112128218
chr5	107937663-107938712
chr4	190867762-190868792
chr15	42790858-42791890
chr6	25218781-25219919
chrX	49580354-49581444
chr22	46477261-46478350
chr12	33042280-33043306
chr14	65238928-65240072
chr19	13117111-13118120
chr15	34446506-34447535
chr13	113475775-113476794
chr2	88279238-88280344
chr19	1214463-1215477
chr16	583117-584271
chr16	16165071-16166219
chr7	75794867-75795908
chr14	101528133-101529142
chr12	76192585-76193641

chrX	88311105-88312110
chr6	150947074-150948101
chr3	51509119-51510233
chr5	179003888-179005014
chr16	3706134-3707245
chr16	585552-586647
chr1	245365516-245366599
chr19	5632940-5634086
chr17	18866459-18867472
chr5	149776287-149777294
chr14	102446215-102447310

Table 4 – Group 4 biomarker/biomarker regions

Chromosome	position±500bp
chr22	48390557-48391613
chr1	1563371-1564396
chr17	64955445-64956457
chr17	25308644-25309779
chr22	20711278-20712384
chr11	65634906-65635965
chr19	36430013-36431127
chr2	120753853-120755007
chr3	88352344-88353355
chr12	118902540-118903563
chr9	108281416-108282562
chr5	140051482-140052560
chr1	160898735-160899806
chr18	76572890-76573894
chr4	1188743-1189782
chr8	63055627-63056660
chr21	46408455-46409519
chr17	26369562-26370575
chr12	34357829-34359018
chr16	3422508-3423625
chr11	4355415-4356462
chr12	96503707-96504816
chr6	113010397-113011423
chr14	70700394-70701467
chr2	127817448-127818633
chr13	21833885-21835024
chr1	8086820-8087841
chr4	1008272-1009402

chr16	4164416-4165435
chr9	133925782-133926857
chr5	71715634-71716664
chr8	143376293-143377346
chr9	136341686-136342707
chr4	125571381-125572445
chr16	31499692-31500779
chr22	41331407-41332439
chr5	1123224-1124264
chr10	134944203-134945216
chr7	157234758-157235834
chr19	1108075-1109206
chr3	153827190-153828263
chr2	25855948-25857091
chr20	30768268-30769293
chr19	612209-613468
chr1	16810272-16811297
chr10	35394872-35395895
chr6	33632081-33633160
chr7	70502101-70503116
chr19	56459039-56460056
chr4	126665714-126666723
chr21	44145263-44146282
chr22	46302758-46303769
chr18	77169944-77170972
chr17	80138618-80139679
chr11	129910118-129911305
chr18	74769508-74770618
chr22	31050196-31051220
chr18	77246168-77247325
chr17	54927087-54928213
chr17	4352117-4353135
chr20	37035846-37036880
chr20	58507122-58508294
chr6	70176629-70177636
chr2	190260853-190261931
chr1	1228438-1229528
chr7	6193070-6194100
chr8	122839743-122840792
chr4	48294388-48295416
chr4	751860-752999
chr9	34038719-34039789
chr6	131845507-131846517

chr6	149559266-149560360
chr2	241826047-241827158
chr8	144945405-144946658
chr7	150094165-150095247
chr12	66282137-66283144
chr19	4217153-4218288
chr22	50721576-50722871
chr22	45808306-45809481
chr15	100550723-100551746
chr10	131903386-131904407
chr1	245427653-245428772
chr7	150093627-150094712
chr2	182985970-182987017
chr22	50721296-50722325

Table 5 – Group 1' biomarker/biomarker regions

Chromosome	position±500bp
chr20	62327556-62328688
chr7	156795287-156796392
chr15	89949795-89950953
chr16	79634775-79635808
chr4	90757154-90758285
chr4	104640269-104641588
chr2	74742958-74744103
chr14	103739131-103740259
chr21	42797438-42798786
chr10	125731845-125733055
chr15	101512966-101514272
chr8	103135245-103136502
chr13	33590446-33591598
chr10	109673856-109675121
chr5	132158175-132159485
chr2	74742706-74743768
chr5	122430786-122431815
chr20	62368643-62369892
chr2	118943553-118944570
chr8	23563507-23564874
chr17	36103765-36104809
chr16	54323801-54324805
chr15	78932964-78933968
chr2	214148641-214149777
chr17	1011822-1012918

chr7	99516170-99517263
chrX	40125892-40126898
chr2	5836739-5837793
chr4	90758296-90759398
chr5	140011702-140012843
chr1	6514210-6515248
chr6	5996890-5997909
chr19	12305682-12306758
chr9	135645025-135646255
chr14	103673445-103674541
chr7	132261359-132262430
chr22	26565117-26566148
chr4	115519583-115520722
chr9	66457923-66459013
chr2	135475418-135476536
chr4	106988-108391
chr14	57274628-57275782
chr8	25901324-25902546
chr1	50890150-50891277
chr11	101453003-101454267
chr16	31548424-31549641
chr8	24812205-24813263
chr5	92915133-92916248
chr10	90342145-90343287
chr1	152083001-152084318
chr10	27547200-27548767
chr3	138663228-138664580
chr19	24154030-24155130
chr19	23653426-23654731
chr11	17741959-17743037
chr6	150335417-150336532
chr16	67207694-67209016
chr6	32632142-32633492
chr17	5000103-5001199
chr19	51829455-51830465
chr2	132181990-132183067
chr13	103052783-103053921
chr4	85418583-85419780
chr8	55366289-55367317
chr20	690183-691465
chr10	100991951-100993104
chr6	88876690-88877844
chr19	15343524-15344632

chr1	110611097-110612153
chr21	36042057-36043111
chr4	57521884-57523144
chr5	41869248-41870356
chr12	114029146-114030172
chr10	79397343-79398444
chr3	112052013-112053048
chr9	74764214-74765324
chrX	8698540-8699634
chr5	92914360-92915699
chr2	145273156-145274276
chr12	128751594-128752716
chr11	91959200-91960279
chr13	88324117-88325239
chr13	28497927-28499006
chr4	11429503-11430522
chr10	106399758-106400862
chr2	66658936-66659963
chr10	124220023-124221086
chr13	107186087-107187334
chr1	36038365-36039503
chr17	35299466-35300667
chr7	8473676-8474912
chr9	971639-972744
chr2	163199923-163200927
chr2	99553019-99554306
chr6	88876067-88877277
chr1	65991061-65992095
chr6	29855413-29856640
chr5	146613621-146614641
chr3	124930634-124932388
chr11	32354926-32355951
chr13	88323676-88324838
chr19	15342338-15343517
chr8	49782305-49783575
chr3	142839524-142840648
chr19	2250588-2251672
chr8	77592718-77593741
chr13	28493763-28495214
chr9	87284312-87285413
chrX	3262935-3264257
chr8	70981955-70983203
chr7	132261841-132262954

chr12	3599645-3600720
chr1	228224963-228226186
chr20	5296483-5297602
chr14	61114773-61116003
chr10	22764545-22765835
chr17	54990569-54991590
chr4	74734622-74735847
chr16	31711698-31712811
chr2	115918734-115919960
chr8	21646427-21647429
chr10	101088600-101089780
chr1	110626620-110627720
chr2	115919265-115920294
chr1	161228118-161229507
chr9	135465566-135466709
chr1	180203249-180204261
chr16	51185313-51186521
chr4	6223349-6224451
chr15	31775014-31776402
chr8	57231942-57233065
chr17	75954394-75955543
chr5	42951660-42952924
chr7	156796321-156797577

chr1	24228778-24229812
chr19	518649-519755
chr21	38630005-38631228
chr9	32782722-32783740
chr2	172945711-172946769
chr10	77156215-77157247
chr3	42947042-42948101
chr19	12475550-12476612
chr12	34260341-34261405
chr14	21093532-21094652
chr5	177667303-177668321
chr11	124790563-124791573
chr8	101117982-101119047
chr7	156797935-156799045
chr2	106681296-106682342
chr2	63280742-63281817
chr4	87824903-87825959
chr5	2748910-2750009
chr13	113764818-113765878
chr3	111904200-111905251

chr6	166073781-166074809
chr5	77253364-77254398
chr1	75138804-75139849
chr19	518371-519433
chr7	30721052-30722082
chr2	239071774-239072929
chr13	112714744-112715860
chr1	149672426-149673448
chr1	149615701-149616830
chr20	61048889-61049961
chr15	89948892-89949973
chr2	223161561-223162697
chr6	42694249-42695397
chr11	121970597-121971747
chr3	138678847-138679945
chr7	27141178-27142358
chr10	135341690-135342932
chr4	154604660-154606098
chr10	77166921-77167990
chr7	73416687-73417845
chr4	13524494-13525631
chr3	147114137-147115248
chr2	241496948-241498061
chr15	98418490-98419500
chr10	134600337-134601402
chr9	970745-971830
chr4	4855901-4856971
chr10	128993788-128994821
chr18	55103101-55104341
chr6	108440069-108441259
chr22	20779311-20780375
chr5	149339286-149340330
chr18	45057772-45058793
chr4	56659363-56660374
chr1	1475284-1476363
chr6	3355941-3357035
chr4	174444705-174445862
chr5	113391472-113392504
chr19	58867314-58868863
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chr10	102880602-102881746
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chr6	19836963-19837983
chr14	59930967-59932050
chr4	4144533-4145643
chr17	59529160-59530349
chr1	6508439-6509543
chr13	44947232-44948683
chr6	31782746-31783810
chr9	96716921-96717966
chr1	36348326-36349400
chr5	75377598-75378688
chr19	12605940-12607209
chr1	47903958-47905010
chrX	39958047-39959078
chr4	90757619-90758716
chr1	1475596-1476623
chr19	21768903-21770103
chr22	24988194-24989219
chr20	62119130-62120207
chr13	79183133-79184159
chr1	110610423-110611546
chr3	192231880-192232883
chr14	101012142-101013156
chr6	26383204-26384217
chr1	65990519-65991705
chr6	159590278-159591499
chr10	106402152-106403195
chr5	115298082-115299167
chr13	28495094-28496169
chr16	55090182-55091327
chr9	972300-973470
chr6	58147012-58148096
chr5	75378503-75379914
chr10	43697466-43698614
chr7	64029494-64030615
chr17	42733794-42734801

chr1	99469575-99470882
chr6	29894118-29895181
chr12	186616-187663
chr6	132271437-132272559
chr16	47177934-47179073
chr11	31832420-31833516
chr18	77557591-77558639
chr6	166073966-166074991
chrX	40034167-40035274
chr6	30181343-30182675
chr10	22541383-22542500
chr19	3585181-3586394
chr10	100993393-100994709
chr13	79175198-79176772
chr8	23145402-23146448
chr13	21649464-21650563
chr4	75719138-75720217
chr8	103136154-103137408
chr1	36348827-36350064
chr15	28351810-28352993
chr3	96533005-96534029
chr16	65156230-65157298
chr8	55371384-55372441
chr21	34443211-34444382
chr5	134870249-134871600
chr5	77267916-77269224
chr20	43726021-43727045
chr16	22824422-22825688
chr2	115919472-115920484
chr19	47742446-47743585
chr20	30639826-30640917
chr22	51111920-51112989
chr3	105085656-105086690
chr14	103654845-103656221
chr4	190935571-190936589
chr2	105473874-105474938
chr11	17740927-17742032
chr9	139024152-139025261
chr7	121945355-121946609
chr7	155241820-155243015
chr5	140430790-140431893
chr9	96712945-96714056
chr6	41339962-41341707

chr7	156400077-156401426
chr18	77159068-77160097
chr18	60263106-60264250
chr7	19146867-19148026
chr15	30516966-30518116
chr1	20669276-20670328
chr17	70117154-70118558
chrX	40034885-40036021
chr1	114695254-114696299
chrX	9732865-9733965
chrX	6144580-6145595
chr12	31079245-31080319
chr6	6003739-6004908
chr1	241587181-241588433
chr8	41754670-41755694
chr16	54964538-54965902
chr4	174450805-174451938
chr1	159823558-159824677
chr8	37823023-37824385
chr5	87973673-87974741
chr16	67196451-67197670
chr3	13114257-13115348
chrX	114795895-114796907
chr8	145924929-145925943
chr4	30722888-30724010
chr4	1164481-1165501
chr7	21984531-21985631
chr6	31324194-31325462
chr17	27942575-27943615
chr19	8656375-8657381
chr10	22765474-22766553
chr18	44786948-44788097
chr1	33772291-33773475
chr1	113286413-113287533
chr7	153583712-153584742
chr5	82767869-82768964
chr10	72217791-72218837
chr12	113913247-113914358
chr6	26501341-26502453
chr17	36103118-36104390
chr10	42970960-42972143
chr7	158936875-158937980
chr2	1748371-1749376

chr8	69243257-69244288
chr16	54970558-54971645
chr8	9764031-9765054
chr9	842120-843612
chr2	172952602-172953626
chr2	133427083-133428199
chr14	59930491-59931634
chr13	20692102-20693181
chr3	77088401-77089812
chr18	44337399-44338422
chr10	126135331-126137188
chr5	137577248-137578414
chr1	9712287-9713390
chr13	79176043-79177076
chr19	12605693-12606740
chr14	59931412-59932478
chr17	66097225-66098410
chr15	55581822-55582906
chr17	1012309-1013345
chr1	53098584-53099635
chr22	50241983-50243135
chr4	186048153-186049274
chr18	52988666-52989672
chr17	19482933-19483953
chr2	225306989-225308020
chr16	67449902-67450941
chr15	31775691-31776827
chrX	39955333-39956431
chr11	57194026-57195059
chr11	111169716-111170774
chr12	72665649-72666818
chr1	45249552-45250648
chr2	29338325-29339442
chr11	100997875-100998907
chr7	53286535-53287614
chr9	132312254-132313324
chr22	48971239-48972323
chr2	193059493-193060598
chr1	10057231-10058257
chr11	17742294-17743364
chr11	111169255-111170351
chr17	75954719-75955800
chr22	25816648-25817666

chr2 99438484-99439585
 chr8 41624127-41625221
 chr1 37499295-37500364
 chr2 163200364-163201409
 chr4 155664449-155665498
 chrX 40013880-40014965
 chrX 40013390-40014703
 chr1 114694636-114695802
 chrX 15872020-15873196
 chr11 30606334-30607434
 chr14 99740085-99741112
 chrX 39963638-39964797
 chr19 35454254-35455374
 chr13 22248958-22249968
 chr4 187025401-187026581
 chr17 66192588-66193620
 chr2 5836246-5837312
 chr21 34443877-34445016
 chr7 97360924-97362033
 chr1 91183001-91184015
 chr21 27944733-27945766
 chr6 132271747-132272781
 chr2 169312310-169313377
 chr19 59073628-59074697
 chr19 36821970-36823132
 chr14 57274109-57275116
 chr16 88449175-88450191
 chr11 32460086-32461090
 chr1 53098242-53099332
 chrX 16729550-16730618
 chr6 105583839-105584865

Table 6 – Group 2' biomarker/biomarker regions

Chromosome	position±500bp
chr2	177042293-177043424
chr17	75447059-75448078
chr4	7194255-7195262
chr5	91589-92757
chr11	120856622-120857640
chr19	59024982-59026094
chr19	940516-941795
chr4	7193938-7195038

chr4	146654036-146655094
chr11	615557-616696
chr15	79382604-79383650
chr3	196728977-196730077
chr11	16631959-16633059
chr16	68572564-68573664
chr12	124941370-124942436
chr5	58335092-58336193
chr5	91749-92889
chr7	5467057-5468077
chr5	139493102-139494173
chr14	105662974-105663985
chr8	145805846-145806961
chr8	79428036-79429146
chr6	35265258-35266346
chr16	27329710-27330719
chr15	45458347-45459411
chr9	34989359-34990445
chr7	128530459-128531546
chr19	52206950-52208110
chr17	42430714-42431746
chr16	58549538-58550654
chr7	104624049-104625214
chr8	11659326-11660461
chr9	123630409-123631417
chr1	201367906-201369041
chr3	195633644-195634695
chr18	10131461-10132610
chr17	43095855-43096977
chr1	27667983-27669068
chr19	7547731-7548834

Table 7 – Mix10 Group 1 biomarkers

Chromosome	position±500bp	Probe set	First Probe sequence (5'→3')	SEQ ID NO:	Second Probe sequence (5'→3')	SEQ ID NO:
chr20	62327556-62328688	1	GCATGGCTGCTGAGATCGTTCACAGTATGAATCTCTACTCCGCGTACAGCCGGC ACCGG	1	CAGCAGGGCAGGCAGCGCCAACATCGATGCGAACGTGCG	2
chr7	156795287-156796392	2	GCATGGCTGCTGAGATCGTTCACAGTATGAATCTCTCCGGACCTGCGCCGGCC CCTGCG	3	CCTCCAGCAGGCCGCTCAGTCCGCCGGATCGATGCGAACGTGCG	4
chr15	89949795-89950953	3	GCATGGCTGCTGAGATCGTTCACAGTATGAATCTCTGGGACAGAGCGCAGGAT CCTCTGCG	5	CTTGCGGACTGGGAGCGGGCGGATCGATGCGAACGTGCG	6
chr16	79634775-79635808	4	GCATGGCTGCTGAGATCGTTCACAGTATGAATCTCTCCAGCCTTCTGGGAGG CGCAT	7	GGCCGGGCGGCCAGCCCTCGATGCGAACGTGCG	8
chr4	90757154-90758285	5	GCATGGCTGCTGAGATCGTTCACAGTATGAATCTCTCGCGTTTCCCGGGGAAAA GCGGA	9	TCCCGGAGAAGCAGCCTAATCTCTCAGCCCTTCGATGCGAACGTGCG	10
chr4	104640269-104641588	6	GCATGGCTGCTGAGATCGTTCACAGTATGAATCTCTCGTGGTGAGTGGGAG GGTCCG	11	GGACTGCAGACCGGTGGCGATGGCTCGATGCGAACGTGCG	12
chr2	74742958-74744103	7	GCATGGCTGCTGAGATCGTTCACAGTATGAATCTCTGGCGTGATCTGCGCAA CGCTG	13	GCTTGGGAGCCGGCCGGTGGTGGTCTGATGCGAACGTGCG	14
chr14	103739131-103740259	8	GCATGGCTGCTGAGATCGTTCACAGTATGAATCTCTCCAGCTTCGCGTACCTG CGCG	15	CACACCGGGTCCCCCGCGGCTTCGATGCGAACGTGCG	16
chr14	103739131-103740259	9	GCATGGCTGCTGAGATCGTTCACAGTATGAATCTCTCGCCCCGGAGCGGGCGC GTCCT	17	TCTGGACCAACAGGCTTGGCGAGGTTCGATGCGAACGTGCG	18
chr21	42797438-42798786	10	GCATGGCTGCTGAGATCGTTCACAGTATGAATCTCTCCAATGCCCTTCTCGCGC TCCT	19	AGCGCGTTTACTGGGCGCTGCCCTCGATGCGAACGTGCG	20
chr21	42797438-42798786	11	GCATGGCTGCTGAGATCGTTCACAGTATGAATCTCTGCCCGCTGTGCCCCGTG CTCC	21	GGAGCGCTAGTCTCCGCCACGAACGTCGATGCGAACGTGCG	22
chr15	101512966-101514272	12	GCATGGCTGCTGAGATCGTTCACAGTATGAATCTCTCCGGTGTCTCCCCATCG TTACGCAG	23	CGCGTTCTGTCCAGGGCAGGTCTGTCTGATGCGAACGTGCG	24
chr8	103135245-103136502	13	GCATGGCTGCTGAGATCGTTCACAGTATGAATCTCTGTGGATCCCGGGCCTGC GGAGT	25	CTGCCCCGAAAGGCCACAGGAGGCTCGATGCGAACGTGCG	26
chr13	33590446-33591598	14	GCATGGCTGCTGAGATCGTTCACAGTATGAATCTCTGCAGCCGCTCCAGAGGC GCCG	27	GTAGTAGCGCAGCCCTCGCGTTGGTCTGATGCGAACGTGCG	28
chr10	109673856-109675121	15	GCATGGCTGCTGAGATCGTTCACAGTATGAATCTCTGTGAGCGGTTCTCTGGC GGCG	29	CGGCGCGCGCTCTGGGTCTCTCTGATGCGAACGTGCG	30
chr5	132158175-132159485	16	GCATGGCTGCTGAGATCGTTCACAGTATGAATCTCTGTGCGAGCACTACCGTG GAGGAGC	31	TGCGCGTCTATTGCGCCCTGCTGCTGATGCGAACGTGCG	32
chr7	49813047-49814063	17	GCATGGCTGCTGAGATCGTTCACAGTATGAATCTCTACCTGCGCGCTCCGCCG GCGC	33	GCACACCGGGCTAGGGCGTCTCTGGTCTGATGCGAACGTGCG	34
chr11	65779019-65780152	18	GCATGGCTGCTGAGATCGTTCACAGTATGAATCTCTGACCAGGGCCAGGCC AGCGC	35	CAGCGGGAGGTTGGAACGCGCCATTCTGATGCGAACGTGCG	36
chr8	23563507-23564874	19	GCATGGCTGCTGAGATCGTTCACAGTATGAATCTCTTCTGCGACTGAGCGCG AGCGG	37	AGTGCCCCCGCGCTTCGCCACATCGATGCGAACGTGCG	38
chr8	23563507-23564874	20	GCATGGCTGCTGAGATCGTTCACAGTATGAATCTCTGCTCTGCACCTTCTCCCC CAGCGCT	39	TTCTTCCCGCGCCGTCGAATCTCTGATGCGAACGTGCG	40

chr15	78932964-78933968	21	GCATGGCTGCTGAGATCGTTCACAGTATGAATCTCTACCGGCCCCGCGGCCA TGAGG	41	CGCGCGCCTTCCCTGGTCTTTCTCGATGCGAACGTGCG	42
chr2	214148641-214149777	22	GCATGGCTGCTGAGATCGTTCACAGTATGAATCTCTCCAACGGCCCCGCGGAG CTCTC	43	GCGAGATCCGGCATCTCTACCCCCGATGCGAACGTGCG	44
chr17	1011822-1012918	23	GCATGGCTGCTGAGATCGTTCACAGTATGAATCTCTATGAGTCCCGGGTCGGG AGCCC	45	CAGGCCGGGCGCGCGGGGTAGATCGATGCGAACGTGCG	46
chrX	40125892-40126898	24	GCATGGCTGCTGAGATCGTTCACAGTATGAATCTCTGCGCGGGCGGCTGCG CGTCC	47	CGGATGCGTCCGCGGCGAGAAGATGTCGATGCGAACGTGCG	48
chr4	90758296-90759398	25	GCATGGCTGCTGAGATCGTTCACAGTATGAATCTCTGAGGGCAAAGCGCTCTC GGCGG	49	TGCGGTGTGAGCCACCTCCCGCGCTCGATGCGAACGTGCG	50
chr5	140011702-140012843	26	GCATGGCTGCTGAGATCGTTCACAGTATGAATCTCTATACTGCCGCGGGTCGGC GTCCG	51	CATCGACGCGCTTTAGAAACGGCTCTAGGTTTCGATGCGAACGTGCG	52
chr1	6514210-6515248	27	GCATGGCTGCTGAGATCGTTCACAGTATGAATCTCTCGGCGCGGATCGACGGTG AAGCG	53	CGGCGCCGACAGGGCGGCCGAGATTCGATGCGAACGTGCG	54
chr9	135645025-135646255	28	GCATGGCTGCTGAGATCGTTCACAGTATGAATCTCTCGGGCCCGAGGCCAG CCCCGC	55	GCCAAGGGCCACCCCTCGGCGCTCGATGCGAACGTGCG	56
chr8	25901324-25902546	29	GCATGGCTGCTGAGATCGTTCACAGTATGAATCTCTAACCCGGCGCTGGCAGT GGCGG	57	CGCGGGAGGAAAGCCGGCTTCGTGTCGATGCGAACGTGCG	58
chr19	23653426-23654731	30	GCATGGCTGCTGAGATCGTTCACAGTATGAATCTCTGCGAGGGCGTGAGAC CCCCGC	59	CGGCGCAGAACGCGCTGGCCAGTCGATGCGAACGTGCG	60
chr20	690183-691465	31	GCATGGCTGCTGAGATCGTTCACAGTATGAATCTCTGCGCCCGAGGCCCGCC GCCGC	61	GCTGCCGTCTCGCACCCATCCGCGCTCGATGCGAACGTGCG	62
chr21	36042057-36043111	32	GCATGGCTGCTGAGATCGTTCACAGTATGAATCTCTGCGGCTCACCCGAGACCC GGCGC	63	GCCCTTCTGCCGACCCTCGGCTCGATGCGAACGTGCG	64
chr13	107186087-107187334	33	GCATGGCTGCTGAGATCGTTCACAGTATGAATCTCTGGCTGGCGGCGCGGTGC CAAGG	65	CGCCCCGGCTCCAGGGCTCTGCGTCGATGCGAACGTGCG	66
chr5	42951660-42952924	34	GCATGGCTGCTGAGATCGTTCACAGTATGAATCTCTATTCGCCGGCTTCGCCGG ACGC	67	CACAGGCAGAGTGCCGCGGGTCGATGCGAACGTGCG	68
chr7	156796321-156797577	35	GCATGGCTGCTGAGATCGTTCACAGTATGAATCTCTGCAGCAGCGCTCCACACC GCGG	69	CTGCAGCGGCTCCGGGTTAATCAGCTCGATGCGAACGTGCG	70

Table 8 – Mix10 Group 2 biomarker/biomarker regions

Chromosome	position±500bp	Probe set	First Probe sequence (5'→3')	SEQ ID NO:	Second Probe sequence (5'→3')	SEQ ID NO:
chr2	177042293-177043424	1	GCATGGCTGCTGAGATCGTTCCACACATAGAGTTCTTGACGCTGGCCGCTCGC TGAGGC	71	GCGGAGAGCGCGGAACGAGCGCGTCGATGCGAACGTGCG	72
chr17	75447059-75448078	2	GCATGGCTGCTGAGATCGTTCCACACATAGAGTTCTTGACGCGCCGAGAG CCGCG	73	GCCCCCCACCAGGTCACATGGTCGATGCGAACGTGCG	74
chr4	7194255-7195262	3	GCATGGCTGCTGAGATCGTTCCACACATAGAGTTCTTAGACCTGCGCCCGCAA GCCAC	75	TCCGCCAGCGCGCGCCCGCTTCGATGCGAACGTGCG	76
chr5	91589-92757	4	GCATGGCTGCTGAGATCGTTCCACACATAGAGTTCTTAGCTCCCGCTAGCCCC GGACGC	77	GCCCCCTGGGCGGCTCTCCCTCGATGCGAACGTGCG	78
chr5	91589-92757	5	GCATGGCTGCTGAGATCGTTCCACACATAGAGTTCTTGCCGAGGCCGCTTC GCCGC	79	GCGCGTTCGCGGGGCCCGCTTCGATGCGAACGTGCG	80
chr11	120856622-120857640	6	GCATGGCTGCTGAGATCGTTCCACACATAGAGTTCTTGACGCCCCAGCCAGA GACCGC	81	GCCCCGTGAGGAGCAGGGTCCATCGATGCGAACGTGCG	82
chr19	59024982-59026094	7	GCATGGCTGCTGAGATCGTTCCACACATAGAGTTCTTGACACCGCGTGC GCCTTC	83	AGTGCCTGCTGCGCAAGCGCTTCACTCGATGCGAACGTGCG	84
chr19	59024982-59026094	8	GCATGGCTGCTGAGATCGTTCCACACATAGAGTTCTTCGCGCTCGGGCCGG TGAGTG	85	CGCATGTGCACGTTGAGCGAGCTTTTCGATGCGAACGTGCG	86
chr19	940516-941795	9	GCATGGCTGCTGAGATCGTTCCACACATAGAGTTCTTGACCCGACGGCCAGG CGGTGGC	87	GCAGGGGCCAGATAAGGCTTTCGCGCTCGATGCGAACGTGCG	88
chr4	146654036-146655094	10	GCATGGCTGCTGAGATCGTTCCACACATAGAGTTCTTGACGCGAGCGGGCTCC TTCCCGC	89	GCCCCAGTCTGTCAGCCCCGTGTCGATGCGAACGTGCG	90
chr11	615557-616696	11	GCATGGCTGCTGAGATCGTTCCACACATAGAGTTCTTAGCGGAACCCCGCCCG GCCAGC	91	GCGGAGTAGGGAGGAGTGAGGGCGTCGATGCGAACGTGCG	92
chr15	79382604-79383650	12	GCATGGCTGCTGAGATCGTTCCACACATAGAGTTCTTCGCTGCCCTCGCCAG CGCG	93	CAGATCGCCAGTCCCTCAGTTTGCCCGCTCGATGCGAACGTGCG	94
chr3	196728977-196730077	13	GCATGGCTGCTGAGATCGTTCCACACATAGAGTTCTTCGCTCCCCACTGTG GCCCGC	95	CCAGTCCATCCCGGCCAGCCGCTCGATGCGAACGTGCG	96
chr11	16631959-16633059	14	GCATGGCTGCTGAGATCGTTCCACACATAGAGTTCTTAGCCCCACGGCGCA AGAACC	97	CCGGGCGCTGAGTCCGGTCTCGATGCGAACGTGCG	98
chr16	68572564-68573664	15	GCATGGCTGCTGAGATCGTTCCACACATAGAGTTCTTAGCCGGGCGCGCAA CTAACT	99	TCCCAGAGGCCCGCGCGCAGGTCTGATGCGAACGTGCG	100
chr5	58335092-58336193	16	GCATGGCTGCTGAGATCGTTCCACACATAGAGTTCTTGACACACGCTCGCGC CCGCGC	101	CTCTCGTCTGCACTTCAAAGCGAGTGGCGCTCGATGCGAACGTGCG	102
chr5	91749-92889	17	GCATGGCTGCTGAGATCGTTCCACACATAGAGTTCTTCGAGGCCGCTTCGCC GCGCG	103	CGTTCGCGGGGCCCGCCGATCTCGATGCGAACGTGCG	104
chr5	91749-92889	18	GCATGGCTGCTGAGATCGTTCCACACATAGAGTTCTTCCGTGCCCTCTTACC CGGGC	105	GCCTGGGCCGCCCGCCGCTTCGATGCGAACGTGCG	106
chr7	5467057-5468077	19	GCATGGCTGCTGAGATCGTTCCACACATAGAGTTCTTGCGTTGGCCGACCTAG ACTGGCGC	107	TGTATCGCGCTGGGCTGTGCGATCGATGCGAACGTGCG	108
chr8	145805846-145806961	20	GCATGGCTGCTGAGATCGTTCCACACATAGAGTTCTTCGCTCGCGGAGAGC AGCCCCG	109	GGCGGGCAGCAGCGTCAGCCGTCGATGCGAACGTGCG	110

chr16	27329710-27330719	21	GCATGGCTGCTGAGATCGTTCCACACATAGAGTTCTCCCGGGCTGGCACTCG AGATATGTG	111	AGCGCGTGTTACGTTCAACTTTGCTTTGCAGTCGATGCGAACGTGCG	112
chr9	34989359-34990445	22	GCATGGCTGCTGAGATCGTTCCACACATAGAGTTCTCCGGGACCCCGAGTCC TGGCTT	113	GGCGCCCTCACCGGTGAGGAGCTGCTCGATGCGAACGTGCG	114
chr16	58549538-58550654	23	GCATGGCTGCTGAGATCGTTCCACACATAGAGTTCTCCGGGGCTGCAGCTC GTGGAGC	115	AGCGCCAGCAGCAGTGGCACCCAGCTCGATGCGAACGTGCG	116
chr8	11659326-11660461	24	GCATGGCTGCTGAGATCGTTCCACACATAGAGTTCTTCCAGTCCCACAGCGT TCGCGC	117	TCCAGCCGGGGTAAGCGGAAGAAAATCGATGCGAACGTGCG	118
chr9	123630409-123631417	25	GCATGGCTGCTGAGATCGTTCCACACATAGAGTTCTTACCTCAAGGGGAGAAC AGAAGGCCGGC	119	TCCGCGTGGCATCTAGCACTGTGGAGCTCGATGCGAACGTGCG	120
chr3	195633644-195634695	26	GCATGGCTGCTGAGATCGTTCCACACATAGAGTTCTTCAGCTTCATCCATGCC TGTCGCGC	121	TGCCACCCAAACACTCATGCACCGGAAGTTCGATGCGAACGTGCG	122

[0052] Accordingly, also provided is an isolated biomarker/biomarker region comprising a DNA region of the human genome selected from a DNA region listed in any one of tables 3 to 6 (groups 3, 4, 1' and 2'). In one example, the isolated biomarker/biomarker region consists of the DNA region of the human genome selected from a DNA region listed in any one of tables 3 to 6 (groups 3, 4, 1' and 2'). Advantageously, unlike biomarker/biomarker regions known in the art, which are typically obtained from comparison data of DNA obtained from trisomy 21 and normal placenta, the biomarker/biomarker regions of the present disclosure may be used for DNA obtained from bodily fluids.

[0053] The term "isolated" as used herein with respect to biomarker/biomarker regions relates to nucleic acids, such as DNA or RNA. In particular, the term "isolated" refers to molecules separated from other DNAs or RNAs, respectively that are present in the natural source of the macromolecule as well as polypeptides. The term "isolated" is meant to include nucleic acid fragments which are not naturally occurring as fragments and would not be found in the natural state. For example, the term "isolated" means separated from constituents, cellular and otherwise, in which the cell, tissue, polynucleotide, peptide, polypeptide, protein, antibody or fragment(s) thereof, which are normally associated in nature. An isolated polynucleotide is separated from the 3' and 5' contiguous nucleotides with which it is normally associated in its native or natural environment, e.g., on the chromosome.

[0054] As used herein, the term "biomarker" or "biomarker region" refer to molecular indicators of a specific biological property, a biochemical feature or facet that can be used to determine the presence or absence and/or severity of a particular disease or condition. As used herein, the term "biomarker" or "biomarker regions" refers to polynucleotide or DNA region whose presence may be associated to a disease or condition. The biomarkers may be differentially present (i.e. partially, complete and/or otherwise present) in a foetus with the disease or condition, the presence of one or more of which can be used to distinguish foetus with an increased risk of the disease or condition and foetus that do not have an increased risk of the disease or condition.

[0055] The inventors of the present disclosure found that the biomarker/biomarker region as provided in the present disclosure is identified to be related to Down syndrome or trisomy 21. Thus, in another aspect, there is provided an isolated biomarker/biomarker region for detecting trisomy 21 or partial trisomy 21. The isolated biomarker/biomarker region

comprising a DNA region of the human genome selected from a DNA region listed in any one of tables 3 to 6 (groups 3, 4, 1' and 2'). In one example, the isolated biomarker/biomarker region consists of the DNA region of the human genome selected from a DNA region listed in any one of tables 3 to 6 (groups 3, 4, 1' and 2').

5 [0056] The term "partial" as used herein refers to partial triplication of chromosome 21. That is, the extra copy of chromosome 21 is not complete, incomplete or existing only in part.

[0057] One way of determining whether the isolated biomarker/biomarker region is related to a disease or condition present in the foetus is by observing the presence of abnormalities or differences as compared to a control sample. For example, the isolated
10 biomarker/biomarker region may have increased or decreased DNA-methylation or DNA mutations such as deletion, frame-shift, insertion, missense, nonsense, point, silent, splice site or translocation.

[0058] Thus, in one example, the level of DNA-methylation of any one of the biomarker/biomarker regions in a diseased sample is different from the level of DNA-
15 methylation in the same biomarker/biomarker region of a non-diseased control DNA. The level of DNA-methylation differences may be observed in any one, two, three, four, five, six, seven, eight, nine, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255,
20 260, 265, 270, 275, 280, 285, 290, 295, 300, 305, 310, 315, 320, 325, 330, 335, 340, 345, 350, 355, 360, 365, 370, 375, 380, 385, 390, 395 or all of the biomarker/biomarker regions of a non-diseased control DNA.

[0059] The "non-diseased control DNA" or "negative control" refers to DNA or sample from an individual or a group of individual who do not have the condition or disease and/or
25 who are not carrying a diseased foetus or a foetus with a condition. For example, the "non-diseased control DNA" may include DNA or sample obtained from an individual or group of individual who do not have trisomy 21 or not carrying trisomy 21- or Down syndrome-foetus.

[0060] As used herein, the term "different" refers to not the same as the level of DNA-methylation as observed in a non-diseased control DNA. For example, the level of DNA-
30 methylation isolated biomarker/biomarker region may be less or more than a non-diseased control DNA.

[0061] In one example, the isolated biomarker/biomarker region referred to in Table 5 (group 1') or 6 (group 2') may be methylated at a level less than about 10% in maternal DNA. Thus, the isolated biomarker/biomarker region referred to in Table 5 (group 1') or 6 (group 2') may be methylated at a level less than about 9%, less than about 8%, less than about 7%, less than about 6%, less than about 5%, less than about 4%, less than about 3%, less than about 2% or less than about 1% in maternal DNA. That is, the isolated biomarker/biomarker region of a diseased or individual with the condition may be methylated at a level of less than about 10% of the DNA-methylation observed in maternal DNA.

[0062] In another example, the isolated biomarker/biomarker region referred to in Table 3 (group 3) or 4 (group 4) may be methylated at a level more than about 90% in maternal DNA. Thus, the isolated biomarker/biomarker region referred to in Table 3 (group 3) or 4 (group 4) is methylated at a level more than about 91%, more than about 92%, more than about 93%, more than about 94%, more than about 95%, more than about 96%, more than about 97%, more than about 98%, more than about 99% or about 100% in maternal DNA. That is, the isolated biomarker/biomarker region of a diseased or individual with the condition may be methylated at a level of more than about 90% of the DNA-methylation observed in maternal DNA.

[0063] In one example, the level of the DNA-methylation of the biomarker referred to in Tables 5 (group 1') or 4 (group 4) in a diseased sample may be higher than the level of DNA-methylation in the same region of a non-diseased control DNA. As used herein, the term "higher" refers to the level of the DNA-methylation to be at least about 10%, at least about 11%, at least about 12%, at least about 13%, at least about 14%, at least about 15%, at least about 16%, at least about 17%, at least about 18%, at least about 19%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95% or at least about 100% higher than the level of DNA-methylation in the same region of a non-diseased control DNA.

[0064] On the other hand, the level of DNA-methylation of the biomarker referred to in Tables 6 (group 2') or 3 (group 3) in a diseased sample may be lower than the level of DNA-methylation in the same region of a non-diseased control DNA. The term "lower" refers to the level of the DNA-methylation to be at least about 1%, at least about 5%, at least about

10%, at least about 11%, at least about 12%, at least about 13%, at least about 14%, at least about 15%, at least about 16%, at least about 17%, at least about 18%, at least about 19%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95% or at least about 100% lower than the level of DNA-methylation in the same region of a non-diseased control DNA.

[0065] The present disclosure also provides for an isolated biomarker/biomarker region comprising a DNA region of the human genome selected from a DNA region listed in any one of tables 1 to 4 and 7 to 8 (groups 1 to 4 and Mix10 Group 1 and Mix10 Group 2 respectively). In one example, the isolated biomarker/biomarker region consists of the DNA region of the human genome selected from the DNA region listed in any one of tables 1 to 4 and 7 to 8 (groups 1 to 4 and Mix10 Group 1 and Mix10 Group 2 respectively). The isolated biomarker/biomarker region may be selected from any two, three, four, five or all of tables 1 to 4 and 7 to 8 (groups 1 to 4 and Mix10 Group 1 and Mix10 Group 2 respectively).

[0066] The level of DNA-methylation of any one of the biomarker/biomarker regions of tables 1 to 4 and 7 to 8 (groups 1 to 4 and Mix10 Group 1 and Mix10 Group 2 respectively) in a diseased sample may be different from the level of DNA-methylation in the same biomarker/biomarker region of a non-diseased control DNA.

[0067] In one example, the isolated biomarker/biomarker region referred to in Tables 1 to 2 and 7 to 8 (Group 1, Group 2, Mix 10 Group 1 and Mix 10 Group 2 respectively) may be methylated at a level less than about 10% in maternal DNA. Thus, the isolated biomarker/biomarker region referred to in Tables 1 to 2 and 7 to 8 (Group 1, Group 2, Mix 10 Group 1 and Mix 10 Group 2 respectively) may be methylated at a level less than about 9%, less than about 8%, less than about 7%, less than about 6%, less than about 5%, less than about 4%, less than about 3%, less than about 2% or less than about 1% in maternal DNA. That is, the isolated biomarker/biomarker region of a diseased or individual with the condition may be methylated at a level of less than about 10% of the DNA-methylation observed in maternal DNA.

[0068] In another example, the isolated biomarker/biomarker region referred to in Table 3 (group 3) or 4 (group 4) may be methylated at a level more than about 90% in maternal DNA. Thus, the isolated biomarker/biomarker region referred to in Table 3 (group 3) or 4 (group 4)

is methylated at a level more than about 91%, more than about 92%, more than about 93%, more than about 94%, more than about 95%, more than about 96%, more than about 97%, more than about 98%, more than about 99% or about 100% in maternal DNA. That is, the isolated biomarker/biomarker region of a diseased or individual with the condition may be methylated at a level of more than about 90% of the DNA-methylation observed in maternal DNA.

[0069] In one example, the level of the DNA-methylation of the biomarker referred to in Table 1 (group 1) or Table 4 (group 4) or Table 7 (Mix10 Group 1) in a diseased sample may be higher than the level of DNA-methylation in the same region of a non-diseased control DNA. As used herein, the term "higher" refers to the level of the DNA-methylation to be at least about 10%, at least about 11%, at least about 12%, at least about 13%, at least about 14%, at least about 15%, at least about 16%, at least about 17%, at least about 18%, at least about 19%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95% or at least about 100% higher than the level of DNA-methylation in the same region of a non-diseased control DNA.

[0070] On the other hand, the level of DNA-methylation of the biomarker referred to in Table 2 (group 2) or Table 3 (group 3) or Table 8 (Mix 10 Group 2) in a diseased sample may be lower than the level of DNA-methylation in the same region of a non-diseased control DNA. The term "lower" refers to the level of the DNA-methylation to be at least about 1%, at least about 5%, at least about 10%, at least about 11%, at least about 12%, at least about 13%, at least about 14%, at least about 15%, at least about 16%, at least about 17%, at least about 18%, at least about 19%, or at least about 20%, at least about 25%, at least about 30%, at least about 35% or at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95% or at least about 100% lower than the level of DNA-methylation in the same region of a non-diseased control DNA.

[0071] As the isolated biomarker/biomarker region as described herein are related to a specific disease or condition, it was found that they may be used in the screening of a specific disease or condition in a foetus. Thus, in yet another aspect of the present disclosure there is provided a method determining the likelihood of a foetus to suffer from a specific disease.

The method comprising the steps of: a) providing an isolated total DNA sample from a pregnant woman, comprising foetal DNA and maternal DNA. Further comprising the steps of: b) removing maternal DNA background; c) measuring a signal indicative for the level of foetal DNA based on one or more biomarkers/biomarker regions, where in the case where the maternal DNA background had a level of methylation below 10%, the signal is the level of methylated foetal DNA and in the case where the maternal DNA background had a level of methylation above 90%, the signal is the level of unmethylated foetal DNA; d) determining a ratio of signals obtained under step c) by dividing the signals of one or more of Group 1 and/or Group 3 biomarkers/biomarker regions over the signals of one or more of Group 2 and/or Group 4 biomarkers/biomarker regions, wherein a ratio higher than the ratio determined in control foetal DNA obtained from a non-diseased foetus indicates that the foetus is likely to suffer from the specific disease.

[0072] In one example, each of the groups is characterized by:

[0073] Group 1: maternal DNA background has a level of methylation below 10% and the signal of the biomarker/biomarker region is higher in foetal DNA obtained from a foetus suffering from the specific disease compared to the same biomarker/biomarker region in control foetal DNA obtained from a foetus not suffering from the disease.

[0074] Group 2: maternal DNA background has a level of methylation below 10% and the signal of the biomarker/biomarker region is lower in foetal DNA obtained from a foetus suffering from the specific disease compared to the same biomarker/biomarker region in control foetal DNA obtained from a foetus not suffering from the disease.

[0075] Group 3: maternal DNA background has a level of methylation above 90% and the signal of the biomarker/biomarker region is higher in foetal DNA obtained from a foetus suffering from the specific disease compared to the same biomarker/biomarker region in control foetal DNA obtained from a foetus not suffering from the disease.

[0076] Group 4: maternal DNA background has a level of methylation above 90% and the signal of the biomarker/biomarker region is lower in foetal DNA obtained from a foetus suffering from the specific disease compared to the same biomarker/biomarker region in control foetal DNA obtained from a foetus not suffering from the disease.

[0077] Specific diseases that may be screened using the method as described herein include Trisomy 18, 13, X and Y and other diseases associated with placenta such as preterm labour, pre-eclampsia and/or eclampsia, intrauterine growth restriction (IUGR), congenital

heart diseases. It would be appreciated by the person skilled in the art that for each of these specific diseases, the biomarker/biomarker regions would be those known to be related to the individual specific disease.

[0078] In particular, the present disclosure found a method of screening for Down syndrome. Thus, in yet another aspect there is provided a method of determining the likelihood of a foetus to suffer from trisomy 21 or partial trisomy 21. The method comprising the steps of: a) providing an isolated total DNA sample from a pregnant woman, comprising foetal DNA and maternal DNA; b) removing maternal DNA background; c) measuring a signal indicative for the level of foetal DNA based on one or more biomarkers/biomarker regions listed in any one of Tables 1 to 8, where in the case where the maternal DNA background had a level of methylation below 10%, the signal is the level of methylated foetal DNA and in the case where the maternal DNA background had a level of methylation above 90%, the signal is the level of unmethylated foetal DNA; d) determining a ratio of signals obtained under step c) by dividing the signals of one or more of Group 1 and/or Group 3 biomarkers/biomarker regions over the signals of one or more of Group 2 and/or Group 4 biomarkers/biomarker regions, wherein a ratio higher than the ratio determined in control foetal DNA obtained from a non-diseased foetus indicates that the foetus is likely to suffer from trisomy 21 or partial trisomy 21.

[0079] In one example, each of the groups is characterized by:

[0080] Group 1: biomarker/biomarker region listed in Table 1 (Group 1), Table 5 (Group 1'), or Table 7 (Mix10 Group 1).

[0081] Group 2: biomarker/biomarker region listed in Table 2 (Group 2) or Table 6 (Group 2'), or Table 8 (Mix10 Group 2).

[0082] Group 3: biomarker/biomarker region listed in Table 3 (Group 3).

[0083] Group 4: biomarker/biomarker region listed in Table 4 (Group 4).

[0084] One example of the method of determining the likelihood of a foetus to suffer from a specific disease such as Down syndrome is illustrated in Figure 1. The method may comprise the steps as described herein. In brief, total genomic DNA obtained from a sample, which may comprise both maternal and foetal DNA, may be processed to remove maternal DNA. The removal of maternal DNA, which may be performed by restriction enzyme digestion based on the methylation status of the maternal DNA, ensure only foetal-specific DNA is analysed in the method as described herein. Once foetal-specific DNA is substantially

free of maternal DNA, at least one of the specific region (such as the biomarker/biomarker region as disclosed herein) of the foetal-specific DNA may then be analysed for its signal by using methods known in the art, such as by using detectable label or quantitatively using quantitative polymerase chain reaction (qPCR). When qPCR is used, probes required may include, but are not limited to probes to specific region of a particular group (e.g. region for Group 1, Group 2, Group 3 or Group 4 as described herein), a first universal probe for detection of foetal-specific DNA region and a second universal probe for detection of foetal-specific DNA region. To facilitate with the analysis of foetal-specific DNA after the isolation from maternal DNA, the enzyme digested DNA may be treated with an enzyme that can catalyses the removal of nucleotides from single-stranded DNA in 3' to 5' direction and/or to facilitate the removal of the 3' overhang of the enzyme digested DNA, for example Exonuclease I. The method may also further comprise steps required prior to qPCR, such as target-specific probe hybridization, ligation and beads purification. The ratio of signals obtained from two or more different groups may be calculated and if the ratio is high, the foetus is considered to have trisomy 21. If the ratio is low, the foetus is considered to not have trisomy 21 (i.e. normal).

[0085] In the present disclosure, the isolated total DNA may be obtained from biological sample such as, but not limited to biological fluid, cell or tissue sample obtained from an individual suspected of having the disease or condition or the pregnant woman, which can be assayed for biomarkers. For example, the isolated total DNA from step a) in the methods as described herein may be obtained from bodily fluid, tissue sample obtained from the pregnant woman and the like. The "bodily fluid" as used herein refers to any biological fluid, which can comprise cells or be substantially cell free, which can be assayed for biomarkers, including, but is not limited to whole blood, tears, sweat, vaginal secretion, saliva, urine and amniotic fluid. As used herein, whole blood may include, but is not limited to blood cells, plasma and serum. That is, the total DNA as used in the methods of the present disclosure may be obtained from plasma or serum, or the like.

[0086] In another example, the isolated total DNA may be obtained from tissue sample obtained from the pregnant woman. In which case, the tissues may include, but are not limited to placental tissue and amniotic sac tissue.

[0087] As used herein, when the biological sample is obtained from a pregnant individual, the sample may be obtained in the first, second or third trimester of pregnancy. The term

“first trimester” as used herein refers to the period of time within the first third of a pregnant individual's gestation. For example, the “first trimester” can be the period of time within the first three months, the first 12 weeks or about the first 90 days of gestation, for example human gestation. The term “second trimester” as used herein refers to the period of time
5 within the second third of a pregnant individual's gestation. For example, the “second trimester” comprises the period of time within the fourth through sixth months, 13th through 27th weeks, or about days 91 to 180 of gestation, for example human gestation. The term “third trimester” as used herein refers to the period of time within the third or last third of a pregnant individual's gestation. For example, the “third trimester” comprises the period of
10 time within the seventh months through ninth months, 28th weeks through 41st weeks, or about days 181 to 270 of gestation, for example human gestation. Accordingly, as used in the methods as disclosed herein, the maternal DNA may include, but is not limited to maternal DNA obtained from tissue or cell samples and maternal peripheral blood DNA.

[0088] To ensure accurate execution of the method of the present disclosure, it is
15 important to remove the maternal DNA from the isolated total DNA. As used herein, the phrase “removing maternal DNA background” refers to partial or full removal of DNA that is not from the foetus or individual suspected to have the condition or disease. The removal of maternal DNA background may lead to substantially no maternal DNA present. As mentioned above, the inventors of the present disclosure discovers that the level of DNA-methylation on
20 the DNA of a foetus and the maternal DNA may be different at different sites. Thus, when the maternal DNA background has a level of methylation below 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2% or 1%, the signal as measured in the methods of the present disclosure may be the level of methylated foetal DNA. On the other hand, if the maternal DNA background has a level of methylation above 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%, the
25 signal as measured in the methods of the present disclosure may be the level of unmethylated foetal DNA.

[0089] The difference in DNA-methylation level in maternal DNA and foetal DNA may be utilised in the step of removing maternal DNA background of the method of the present disclosure. For example, the step of removing maternal DNA background may be performed
30 by treating the total isolated DNA with a reagent that differentially modifies methylated or non-methylated DNA, such as by treating total isolated DNA with an antibody or a protein that can specifically binds to methylated cytosine. For example, the reagents may include, but

are not limited to sodium bisulfite, one or more enzymes that only cleave methylated DNA, such as methylation dependent enzyme and one or more enzymes that only cleave non-methylated DNA, such as methylation sensitive enzyme. The enzymes may include, but are not limited to MspJI, LpnPI, FspEI, DpnI, DpnII, McrBC, MspI, HapII, AatII, AciI, AclI, AfeI, AgeI, AscI, AsiSI, Aval, BceAI, BmgBI, BsaAI, BsaHI, BsiEI, BsiWI, BsmBI, BspDI, BsrFI, BssHII, BstBI, BstUI, ClaI, EagI, Faul, FseI, FspI, HaeII, HgaI, HhaI, HinPII, HpaII, Hpy99I, HpyCH4IV, KsaI, MluI, NaeI, NarI, NgoMIV, NotI, NruI, Nt.BsmAI, NtCviPII, PaeR7I, PmlI, PvuI, RsrII, SacII, SalI, SfoI, SgrAI, SmaI, TspMI, ZraI and the like.

[0090] As known in the art, prior to measuring the signal indicative for the level of foetal DNA based on one or more biomarkers/biomarker regions, the total DNA may be treated with an enzyme which catalyses the removal of nucleotides from single-stranded DNA in the 3' to 5' direction, for example enzymes such as, but are not limited to exonucleases such as Exonuclease I. This step ensures the removal of the 3' overhang of a digested DNA. For avoidance of doubt, the 3' end of a single strand DNA refers to the terminating or tail end of DNA strand which is characterised by the hydroxyl group of the third carbon in the sugar-ring; the 5' end of a single-strand DNA refers to the end of the DNA that has the fifth carbon in the sugar-ring of the deoxyribose or ribose at its terminus.

[0091] To facilitate the detection of biomarker/biomarker regions, the method of the present disclosure may require the addition of one or more probe sets. Thus, in one example, the total DNA of the method of the present disclosure may be incubated with one or more probe sets. In one example, the total DNA may be incubated with one or two or three or four or five or six or seven or eight or nine or ten or 50 or 100 or 200 or 300 or 400 or 500 or 600 or 700 or 800 or 900 or 1000 or 2000 or in order of thousands or more probe sets.

[0092] The first probe may include, but is not limited to a sequence for binding a forward primer, a sequence for binding a third probe and a sequence for binding to the one or more biomarker/biomarker regions. The first probe, which binds to a third probe may include, but is not limited to TaqMan® probe or the like. The sequences of the first probe in a probe set may be selected from any one of the probe sets listed in Tables 7 or 8. That is, the first probe in a probe set may include any one, two, three, four, five, six, seven, eight, nine, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34 or all of the probes listed in Table 7 and/or may include one, two, three, four, five, six, seven, eight,

nine, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or all of the probes listed in Table 8.

[0093] The second probe may include, but is not limited to a sequence for binding a reverse primer and sequence for binding to one or more biomarker/biomarker regions. The second probe may be phosphorylated at the 5' end. The second probe may include further modification, which allows the probe to be isolated by affinity purification. Such modification may include, but not limited to a 3' Biotin-TEG modification, which allows the probe to be isolated by bead purification. The sequences of the second probe in a probe set may be selected from any one of the probe sets listed in Tables 7 or 8. That is, the second probe in a probe set may include any one, two, three, four, five, six, seven, eight, nine, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34 or all of the probes listed in Table 7 and/or may include one, two, three, four, five, six, seven, eight, nine, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or all of the probes listed in Table 8.

[0094] In view of the above, the sequences of the first probe and second probe in each probe set may be selected from any one of the probe sets listed in Tables 7 and/or 8. When one probe is selected from the probe sets listed in Tables 7 and/or 8, the first probe and second probe from each probe set may be ligated together. That is, the two probes from each probe set may be ligated together. If two or more probes are ligated together, any excess probes which have not been ligated may be removed. Thus, the method of the present disclosure further comprises the step of removing the excess probes which have not been ligated together. The step of removing the excess probes may be performed using bead purification, such as but is not limited to streptavidin beads.

[0095] The third probe may include binding sequences that is different for each of biomarker/biomarker region groups 1 to 4. That is, the binding sequence for third probe for the Group 1 biomarker/biomarker region may comprise or consists of the sequence 5'-CCACAGTATGAATCTCT-3' (SEQ ID NO: 123). For Group 2 biomarker/biomarker region, the binding sequence for third probe may comprise or consists of the sequence 5'-CCACACATAGAGTTCTT-3' (SEQ ID NO: 124). In one example, the third probe may comprise or consists of the sequence 5'-FAM-CCACAGTATGAATCTCT-MGB-3' (SEQ ID NO: 125), which is suitable for Mix10 Group 1. In another example, the third probe may

comprise or consists of the sequence 5'-VIC-CCACACATAGAGTTCTT-MGB-3' (SEQ ID NO: 126), which is suitable for Mix 10 Group 2.

[0096] In any of the methods of the present disclosure, the signal indicative of the level of foetal DNA may be measured using a detectable label, such as a fluorophore, radioactive moiety, enzyme, biotin/avidin label, chromophore, chemiluminescent label, or the like. Thus, in one example, the signal which is indicative of the level of foetal DNA in step (c) of the methods as described herein may be a fluorescent signal. Different fluorescent signals may be provided and measured for each of biomarker/biomarker region groups 1 to 4. When fluorescent signals are used to detect the level of foetal DNA, the signal would originate from one or more probes having fluorophores thereon.

[0097] In an alternative example, the signal indicative of the level of foetal DNA may be measured quantitatively. For example, the signal which is indicative of the level of foetal DNA in step (c) of the methods as described herein may be measured by quantitative polymerase chain reaction.

[0098] To facilitate detection, probes of the present disclosure may further comprise forward primer and reverse primers. In one example, the forward primer may comprise or consists of the sequence 5'-GCATGGCTGCTGAGATCGT-3' (SEQ ID NO: 127). The reverse primer may comprise or consists of the sequence 5'-CGCACGTTTCGCATCGA-3' (SEQ ID NO: 128).

[0099] In one example, the probe set may comprise 5'-FAM-CGGCTGCCACCCG-MGB-3' (SEQ ID NO: 129), which is a specific probe suitable for Group 1, 5'-VIC-CGCGCCTTCCAGTG-MGB-3' (SEQ ID NO: 130), which is a specific probe suitable for Group 2, 5'-ACCCACAGCGGAGCTC-3' (SEQ ID NO: 131), which is a forward primer suitable for Group 1 and 5'-AACACATGGTCACGCACACC-3' (SEQ ID NO: 132), which is a forward primer suitable for Group 2, 5'-AGAAAAGGACCAGGGAAGGC-3' (SEQ ID NO: 133), which is a reverse primer suitable for group 1 and 5'-CGCTTGGCGCAGACG-3' (SEQ ID NO: 134), which is a reverse primer suitable for group 2.

[00100] The present disclosure also contemplates a variety of kits for use in the disclosed methods. For example, there is provided a kit comprising primers for amplifying the one or more biomarkers/biomarker regions selected from any one of the DNA regions of the human genome listed in any one of tables 3 to 6 (groups 3, 4, 1' and 2'). The kit may further

comprise one or more reagents for measuring a signal indicative for the level of foetal DNA based on the one or more biomarkers/biomarker regions.

[00101] In yet another example there is provided a kit comprising primers for amplifying the one or more biomarkers/biomarker regions selected from any one of the DNA regions of the human genome listed in any one of tables 1 to 4 (groups 1 to 4). The kit may further
5 comprise one or more reagents for measuring a signal indicative for the level of foetal DNA based on the one or more biomarkers/biomarker regions.

[00102] In yet another example there is provided a kit comprising primers for amplifying the one or more biomarkers/biomarker regions selected from any one of the DNA regions of
10 the human genome listed in any one of tables 7 to 8 (Mix10 Group 1 and Mix10 Group 2). The kit may further comprise one or more reagents for measuring a signal indicative for the level of foetal DNA based on the one or more biomarkers/biomarker regions.

[00103] The reagents that are suitable for measuring a signal may include reagents that may incorporate a detectable label, such as a fluorophore, radioactive moiety, enzyme,
15 biotin/avidin label, chromophore, chemiluminescent label, or the like, or the kits may include reagents for labeling the nucleic acid primers, the nucleic acid probes or the nucleic acid primers and nucleic acid probes for detecting the presence or absence of the biomarker/biomarker region as described herein. The primers and/or probes, calibrators and/or controls can be provided in separate containers or pre-dispensed into an appropriate
20 assay format, for example, into microtiter plates. The kit may further comprises reagents including, but are not limited to reagents for isolating DNA from samples, reagents for differentially modifying methylated or non-methylated DNA, reagents for polymerase chain reaction and reagents for quantitative polymerase chain reaction. For example, the kits may include reagents used in the Experimental sections below, in particular Example 2 and
25 Example 3.

[00104] The kit may further comprise instructions that may be provided in paper form or in computer-readable form, such as a disc, CD, DVD or the like. The kits may optionally include quality control reagents, such as sensitivity panels, calibrators, and positive controls.

[00105] The kits can optionally include other reagents required to conduct a diagnostic
30 assay or facilitate quality control evaluations, such as buffers, salts, enzymes, enzyme co-factors, substrates, detection reagents, and the like. Other components, such as buffers and solutions for the isolation and/or treatment of a test sample (e.g., pretreatment reagents), may

also be included in the kit. The kit may additionally include one or more other controls. One or more of the components of the kit may be lyophilized and the kit may further comprise reagents suitable for the reconstitution of the lyophilized components.

[00106] The various components of the kit optionally are provided in suitable containers.

- 5 As indicated above, one or more of the containers may be a microtiter plate. The kit further can include containers for holding or storing a sample (e.g., a container or cartridge for a blood or urine sample). Where appropriate, the kit may also optionally contain reaction vessels, mixing vessels and other components that facilitate the preparation of reagents or the test sample. The kit may also include one or more instruments for assisting with obtaining a
10 test sample, such as a syringe, pipette, forceps, measured spoon, or the like.

[00107] As used herein, the term "about", in the context of level of DNA-methylation, typically means $\pm 5\%$ of the stated value, more typically $\pm 4\%$ of the stated value, more typically $\pm 3\%$ of the stated value, more typically, $\pm 2\%$ of the stated value, even more typically $\pm 1\%$ of the stated value, and even more typically $\pm 0.5\%$ of the stated value.

- 15 [00108] As used herein, the term "one or more" refers to one, two, three, four, five, six, seven, eight, nine, ten or more possible probes or any other feature that is recited as "one or more".

- [00109] The invention illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed
20 herein. Thus, for example, the terms "comprising", "including", "containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible
25 within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the inventions embodied therein herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

- 30 [00110] The invention has been described broadly and generically herein. Each of the narrower species and sub-generic groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or

negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

5

EXPERIMENTAL SECTION

Materials and Methods

[00111] Clinical samples: Women with euploidy and Down syndrome (DS) (also known as
10 Trisomy 21, or T21) pregnancies, who attended KK Women's and Children's Hospital, Singapore, were recruited. Informed consent was obtained under the ethics approval from the SingHealth CRIB Committee.

[00112] Ten mL of peripheral blood from each subject was collected into EDTA tubes. The blood samples were centrifuged at 1,790g for 10 min at 4 °C. After removing the supernatant
15 plasma, the blood cells were transferred to a new microcentrifuge tube and centrifuged at 2,300g for 5 min at room temperature to remove the residual plasma. The blood cells containing buffy coat were then collected and stored at -80 °C. DNA was extracted from 200 µL of blood cells from pregnancies using QIAamp DNA Blood Mini Kit (QIAGEN GmbH, Germany), according to manufacturer's instructions. DNA samples eluted with 50 µL of
20 DNase and RNase-free water (Sigma) were stored at -80 °C.

[00113] Chorionic villus samples from subjects carrying a normal or DS foetus at the first or second trimesters of pregnancy were collected by chorionic villus sampling (CVS). Placenta villi samples (foetal side) from DS foetuses were collected from termination of pregnancy (TOP). All tissue samples were washed with diethylpyrocarbonate (Sigma-
25 Aldrich, USA) treated water. Tissues were stored at -80 °C for DNA analysis. Genomic DNA extraction from tissues was performed with QIAamp DNA Mini Kit (QIAGEN GmbH, Germany), according to manufacturer's instructions.

Example 1 - discovery of DNA methylation biomarkers

30 [00114] The maternal plasma DNA from peripheral blood of a pregnant woman contains both maternal DNA (derived primarily from leukocytes) and foetal DNA (derived from placental cells). Foetal DNA constitutes about 10% of all cell-free DNA in maternal plasma.

One can distinguish foetal and maternal DNA based on DNA methylation differences of specific genomic regions between foetal and maternal DNA. DNA methylation differences are also present between normal and disease fetuses in placenta DNA. In some genomic regions DNA methylation levels are higher in disease samples while in other regions DNA methylation levels are lower in disease samples compared with normal samples.

[00115] Reduced representation bisulfite sequencing (RRBS) was used for quantifying genome-wide DNA methylation profiles in normal and Trisomy 21 placenta samples. Two restriction enzymes (Taq α I and MspI, both from New England Biolabs) were used to digest the genomic DNA samples. DNA fragments were purified with the QIAquick PCR Purification Kit (QIAGEN GmbH), and were end-repaired, 3'-end-adenylated, and adapter-ligated using ChIP-Seq Sample Preparation Kit (Illumina, USA). Illumina's RRBS for Methylation Analysis protocol was followed, except that 10 μ L the methylation adapter oligonucleotides were used and the ligation was performed for 15 min at 20 °C in the adapter-ligation step. Two different sizes of fragments (150-197 bp and 207-230 bp) were selected by gel electrophoresis with a 3% agarose gel. The purified fragments were then bisulfite treated using the EZ DNA Methylation-Gold Kit (Zymo Research, USA). The converted DNA was amplified using HotStarTaq DNA Polymerase Kit (QIAGEN GmbH), with 1x reaction buffer, 1.5 mM of additional MgCl₂, 300 μ M of dNTP mix, 500 nM each of PCR primer PE 1.0 and 2.0, and 2.5 U of HotStarTaq DNA polymerase. The thermocycling condition was 15 min at 94 °C for heat activation, and 8-12 cycles of 20 sec at 94 °C, 30 sec at 65 °C and 30 sec at 72 °C, followed by a 5 min final extension at 72 °C. The amplified fragments were purified by gel electrophoresis and further quantified by the Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Each DNA library was analyzed by two lanes of paired-end sequencing (2 $\times$ 36 bp) read on an Illumina Genome Analyzer IIx.

[00116] Sequencing data was analyzed. The human genome was converted into two reference genomes for sequencing alignment. The C2T converted reference genome was derived by converting all cytosines to thymines. The G2A converted reference genome was derived by converting all guanines to adenosines. After initial quality control based on their Phred scores (Ewing *et al.* Genome Res 1998; 8 (3): 186-94) and fragment ending with expected tri-nucleotides after enzymatic reaction, the sequencing reads were aligned to two reference genomes separately using Bowtie aligner (Langmead *et al.* Genome Biol 2009; 10 (3): R25). The newly added cytosines in the "end-repair" step were excluded from

methylation analysis and CpGs overlapping with potential polymorphisms were also excluded. Methylation level of each CpG site was calculated as: Methylation level for a CpG = Count of Cytosine / (Count of Cytosine + Count of Thymine)*100%.

5

Example 2 – T21 foetus detection using methylation biomarkers

[00117] Figure 1 shows a schematic describing the following steps. Step 1: Removal of unmethylated DNA for selected biomarkers by methylation-sensitive restriction enzymes. In the case of a foetal/maternal DNA mixture experiment, this step removed maternal DNA background since the biomarkers regions were mostly unmethylated. 25 ng of genomic DNA was subjected to methylation-sensitive restriction enzyme digestion in a 15 µL system, containing 1 x buffer 4, 1 x BSA, 9 units of BstUI, 10 units of HpaII and 10 units of HhaI (New England Biolabs, USA). Mock digestion without restriction enzymes was set up as control. The samples were incubated at 37 °C for 2 hr and then 60 °C for 2 hr.

15 [00118] Step 2: Exonuclease I treatment was used to remove the 3' overhang for the digested DNA. 10 units of Exonuclease I (New England Biolabs, USA) was added to the enzyme digested sample, and incubated at 37 °C for 1 hr, followed by heat inactivation at 80 °C for 20 min.

[00119] Step 3: Denaturation of the genomic DNA and probe hybridization. A mixture of probe sets containing 1000 amole (atto mole) of each probe set was added to samples from Step 2. Each probe set contains 2 probes. The first probe contained three sequences: a sequence for the qPCR forward primer (in bold), a sequence for the TaqMan probe (underlined) (for Group 1 biomarkers: 5'-GCATGGCTGCTGAGATCGTTCCACAGTATGAATCTCT-3'(SEQ ID NO: 127 and SEQ ID NO: 123); for Group 2 biomarkers: 5'-GCATGGCTGCTGAGATCGTTCCACACATAGAGTTCTT-3'(SEQ ID NO: 127 and SEQ ID NO: 124)), and a biomarker-specific sequence. The second probe contained two sequences, a sequence for the qPCR reverse primer (5'-TCGATGCGAACGTGCG-3'(SEQ ID NO: 135)) and a biomarker-specific sequence. The second probe is phosphorylated at the 5' end and with an optional 3' Biotin-TEG modification (Integrated DNA technologies, USA). The sample was then incubated at 95 °C for 10 min to denature the genomic DNA, followed by incubation at 60 °C for 16-18 hr for probe hybridization.

[00120] Step 4: Ligation of annealed probes. When the two probes from each probe set were hybridized to their target sequences, they were ligated in a 20 μ L system, containing 18.5 mM Tris, 41.9 mM potassium acetate, 9.3 mM magnesium acetate, 10 mM DTT, 1 mM NAD, 0.02% Triton X-100, and 20 units of Taq DNA ligase (New England Biolabs, USA), at 60 °C for 2 hr.

[00121] Step 5: Beads purification to remove excess of probes. After ligation, the excess of probes were removed either by Agencourt AMPure XP beads (Beckman Coulter, USA) or by Dynabeads MyOne Streptavidin C1 beads (Life Technologies, USA), according to manufacturer's instructions.

[00122] Step 6: Detection of methylated foetal DNA by quantitative real-time PCR (qPCR). Beads purified DNA from Step 5 was then subjected to qPCR to detect methylated foetal DNA. Each reaction contains 1 x TaqMan Universal PCR Master Mix (Life Technologies, USA), 300 nM each of forward primer (5'-GCATGGCTGCTGAGATCGT-3'; SEQ ID NO: 127) and reverse primer (5'-CGCACGTTTCGCATCGA-3'; SEQ ID NO: 128), 100 nM each of TaqMan probes (Group 1 biomarkers: 5'-FAM-CCACAGTATGAATCTCT-MGB-3' (SEQ ID NO: 125); Group 2 biomarkers: 5'-VIC-CCACACATAGAGTTCTT-MGB-3' (SEQ ID NO: 126)) (Life Technologies, USA), and DNA from Step 5. The qPCR assays were performed in the ABI 7500 Real-Time PCR System (Life Technologies, USA). The thermo profile is 50 °C for 2 min, and 95 °C heat activation for 10 min, followed by 50 cycles of 95 °C for 15 sec and 60 °C for 1 min. Result was analyzed by 7500 Software v2.0.1.

Example 3 - T21 fetus detection using methylation biomarkers.

[00123] Ten mL of peripheral blood from each subject was collected into EDTA tubes. The blood samples were centrifuged at 1,790g for 10 min at 4 °C. The supernatant was transferred to a new microcentrifuge tube and centrifuged at 16,100g for 10 min at 4 °C. The supernatant cell-free plasma was then collected and stored at -80 °C. DNA was extracted from 1.6 mL of plasma from pregnancies using QIAamp DNA Blood Mini Kit (QIAGEN GmbH, Germany), according to manufacturer's instructions. DNA samples eluted with 75 μ L of DNase and RNase-free water (Sigma) were stored at -80 °C.

[00124] Step 1: Removal of unmethylated DNA for selected biomarkers by methylation-sensitive restriction enzymes. In the case of a foetal/maternal DNA mixture experiment, this step removed maternal DNA background since the biomarker regions were mostly

unmethylated. Half of genomic DNA extracted from maternal plasma was subjected to methylation-sensitive restriction enzyme digestion in a 45 μ L system, containing 1 x buffer 4, 1 x BSA, 20 units of *Bst*UI, 20 units of *Hpa*II and 20 units of *Hha*I (New England Biolabs, USA). Mock digestion without restriction enzymes was set up as control. The samples were

5 incubated at 37 °C for 2 hr and then 60 °C for 2 hr.

[00125] Step 2: Detection of methylated foetal DNA by quantitative real-time PCR (qPCR). Restriction enzyme digested DNA from Step 1 was then subjected to qPCR to detect methylated foetal DNA. Two biomarkers were assayed, assay 1 (chr15:78,933,445-78,933,521) from Group 1 and assay 2 (chr19:59,025,557-59,025,614) from Group 2. Assay

10 1 reaction contains 1 x TaqMan Universal PCR Master Mix (Life Technologies, USA), 300 nM each of forward primer (5'-ACCCACAGCGGAGCTC-3'; SEQ ID NO: 131) and reverse primer (5'-AGAAAAGGACCAGGGAAGGC-3'; SEQ ID NO: 133), 200 nM of TaqMan probe (5'-FAM-CGGCTGCCACCCG-MGB-3'; SEQ ID NO: 129) (Life Technologies, USA), and 10 μ L of DNA in a 50 μ L system. Assay 2 reaction contains 1 x

15 TaqMan Universal PCR Master Mix (Life Technologies, USA), 300 nM each of forward primer (5'-AACACATGGTCACGCACACC-3'; SEQ ID NO: 132) and reverse primer (5'-CGCTTGGCGCAGACG-3'; SEQ ID NO: 134), 150 nM of TaqMan probe (5'-VIC-CGCGCCTTCCAGTG-MGB-3'; SEQ ID NO: 130) (Life Technologies, USA), and 10 μ L of DNA in a 50 μ L system. The qPCR assays were performed in the ABI 7500 Real-Time PCR

20 System (Life Technologies, USA). The thermo profile is 50 °C for 2 min, and 95 °C heat activation for 10 min, followed by 50 cycles of 95 °C for 15 sec and 60 °C for 1 min. Result was analyzed by 7500 Software v2.0.1.

Results

[00126] Different combinations of probe mixtures were examined with genomic DNA from

25 one normal and one T21 cases. Cycle Threshold (Ct) values for Group 1 and Group 2 biomarkers were determined in qPCR. The signal ratio for Group 1 and Group 2 was determined by calculating the Ct difference (Δ Ct). Δ Ct = Ct(Group 2) - Ct(Group 1) where a higher Δ Ct value is expected in T21 samples as compared to normal samples. Figure 2 illustrates the methylation difference between Group 1 and Group 2 biomarkers in normal

30 and T21 samples using probe mix 10, which contains 35 biomarkers from Group 1 and 26 biomarkers from Group 2. The details of probe sequences and their target biomarkers are listed in Mix10 Group 1 (see Table 7) and Mix10 Group 2 (see Table 8).

[00127] Alternatively, a mock digestion was performed for each sample. A mock digestion was exactly the same as the real digestion (specified in Steps 1-6 in Example 2), except no restriction enzyme was added in Step 1 and no Exonuclease I was added in Step 2. Ct difference between enzyme-digested sample and its mock-digested control was calculated where for each group (Group 1 and Group 2) $\Delta Ct = Ct(\text{"enzyme-digested"}) - Ct(\text{"mock-digested-control"})$. This ΔCt value represents DNA methylation level for all measured biomarkers in each Group. The difference of the ΔCt ("enzyme-digested" – "mock-digested-control") values between Group 1 and Group 2 biomarkers were then compared to obtain $\Delta\Delta Ct$ (Group2 – Group1). The calculated $\Delta\Delta Ct$ (Group2 – Group1) value represents the ratio of targeted methylated DNA in Group 1 and Group 2. Figure 3 shows the $\Delta\Delta Ct$ (Group2 – Group1) values from probe mix10, whose methylation difference between normal and T21 tissues is the biggest among all combinations of probe mixtures tested.

[00128] DNA samples obtained from maternal plasma in first trimester contain roughly 10% of foetal DNA and 90% of maternal DNA. To mimic maternal plasma samples, we generated two types of DNA mixture samples, with foetal DNA at 10% and 5% of total DNA, respectively. We used purified placental DNA (foetal origin) or CVS DNA and purified peripheral blood DNA from pregnant women, as these two tissues are the main contributors of foetal and maternal DNA in maternal plasma during pregnancy. As shown in Figure 4, the same mix10 probe sets was used. The sample spiked in with T21 placenta DNA (mimicking a maternal plasma sample from a woman pregnant with a T21 foetus) was clearly different from the sample spiked in with normal CVS DNA (mimicking a maternal plasma sample from a woman pregnant with a non-T21 foetus).

[00129] Two biomarkers were examined with genomic DNA extracted from maternal plasma samples from pregnancies carrying normal (N = 31) or T21 (N = 2) foetus. Assay 1 represents biomarkers from Group 1 where T21 cases yield higher signal than normal cases, and assay 2 represents biomarkers from Group 2 where normal cases yield higher signal than T21 cases. Methylation-sensitive restriction enzyme digestion was performed to remove unmethylated foetus signal as well as maternal background. A mock digestion was performed for each sample as control. Ct difference between enzyme-digested sample and its mock-digested control was calculated where for each group (Group 1 and Group 2) $\Delta Ct = Ct(\text{"enzyme-digested"}) - Ct(\text{"mock-digested-control"})$. DNA methylation level was than

calculated as following: DNA methylation (%) = $2^{-[Ct(\text{"enzyme-digested"}) - Ct(\text{"mock-digested-control"})]}$ x 100%.

[00130] The methylation difference between biomarkers from Group 1 and Group 2 was obtained by calculating the methylation ratio of Group 1 and Group 2. Figure 5 shows the DNA methylation level in the examined biomarkers and the methylation ratio of Group 1 and Group 2 from maternal plasma samples, demonstrating higher values of methylation ratio of Group 1 and Group 2 in T21 samples than in normal samples.

Claims

1. An isolated biomarker/biomarker region comprising a DNA region of the human genome selected from a DNA region listed in any one of tables 3 to 6 (groups 3, 4, 1' and 2').
2. An isolated biomarker/biomarker region for detecting trisomy 21 or partial trisomy 21, comprising a DNA region of the human genome selected from a DNA region listed in any one of tables 3 to 6 (groups 3, 4, 1' and 2').
3. The isolated biomarker/biomarker region according to any one of claims 1 to 2, wherein the level of DNA-methylation of any one of the biomarker/biomarker regions in a diseased sample is different from the level of DNA-methylation in the same biomarker/biomarker region of a non-diseased control DNA.
4. The isolated biomarker/biomarker region according to claim 3, wherein the isolated biomarker/biomarker region referred to in Table 5 (group 1') or 6 (group 2') is methylated at a level less than 10% in maternal DNA.
5. The isolated biomarker/biomarker region according to claim 3, wherein the isolated biomarker/biomarker region referred to in Table 3 (group 3) or 4 (group 4) is methylated at a level more than 90% in maternal DNA.
6. The isolated biomarker/biomarker region according to any one of claims 3 to 5, wherein the level of the DNA-methylation of the biomarker referred to in Tables 5 (group 1') or 4 (group 4) in a diseased sample is higher than the level of DNA-methylation in the same region of a non-diseased control DNA.
7. The isolated biomarker/biomarker region according to any one of claims 3 to 6, wherein the level of the DNA-methylation of the biomarker referred to in Tables 6 (group 2') or 3 (group 3) in a diseased sample is lower than the level of DNA-methylation in the same region of a non-diseased control DNA.

8. An isolated biomarker/biomarker region comprising a DNA region of the human genome selected from a DNA region listed in any one of tables 1 to 4 and 7 to 8 (groups 1 to 4 and Mix10 Group 1 and Mix10 Group 2 respectively), wherein the level of DNA-methylation of any one of the biomarker/biomarker regions in a diseased sample is different from the level of DNA-methylation in the same biomarker/biomarker region of a non-diseased control DNA.
9. The isolated biomarker/biomarker region according to claim 8, wherein the isolated biomarker/biomarker region referred to in any one of Tables 1 to 2 and 7 to 8 (Group 1, Group 2, Mix 10 Group 1 and Mix 10 Group 2 respectively) is methylated at a level less than 10% in maternal DNA.
10. The isolated biomarker/biomarker region according to claim 8, wherein the isolated biomarker/biomarker region referred to in Table 3 (group 3) or Table 4 (group 4) is methylated at a level more than 90% in maternal DNA.
11. The isolated biomarker/biomarker region according to any one of claims 8 to 10, wherein the level of the DNA-methylation of the biomarker referred to in Table 1 (group 1) or Table 4 (group 4) or Table 7 (Mix10 Group 1), or in a diseased sample is higher than the level of DNA-methylation in the same region of a non-diseased control DNA.
12. The isolated biomarker/biomarker region according to any one of claims 8 to 11, wherein the level of the DNA-methylation of the biomarker referred to in Table 2 (group 2) or Table 3 (group 3) or Table 8 (Mix 10Group 2) in a diseased sample is lower than the level of DNA-methylation in the same region of a non-diseased control DNA.
13. The isolated biomarker/biomarker region according to any one of claims 4 to 12, wherein the maternal DNA is maternal peripheral blood DNA.

14. A method of determining the likelihood of a foetus to suffer from trisomy 21 or partial trisomy 21, comprising the steps of:

a) providing an isolated total DNA sample from a pregnant woman, comprising foetal DNA and maternal DNA;

b) removing maternal DNA background;

c) measuring a signal indicative for the level of foetal DNA based on one or more biomarkers/biomarker regions listed in any one of Tables 1 to 8, where in the case where the maternal DNA background had a level of methylation below 10%, the signal is the level of methylated foetal DNA and in the case where the maternal DNA background had a level of methylation above 90%, the signal is the level of unmethylated foetal DNA;

d) determining a ratio of signals obtained under step c) by dividing the signals of one or more of Group 1 and/or Group 3 biomarkers/biomarker regions over the signals of one or more of Group 2 and/or Group 4 biomarkers/biomarker regions, wherein a ratio higher than the ratio determined in control foetal DNA obtained from a non-diseased foetus indicates that the foetus is likely to suffer from trisomy 21 or partial trisomy 21;

wherein each of the groups is characterized by:

Group 1: biomarker/biomarker region listed in Table 1 (Group 1), Table 5 (Group 1'), or Table 7 (Mix10 Group 1);

Group 2: biomarker/biomarker region listed in Table 2 (Group 2) or Table 6 (Group 2'), or Table 8 (Mix10 Group 2);

Group 3: biomarker/biomarker region listed in Table 3 (Group 3); and

Group 4: biomarker/biomarker region listed in Table 4 (Group 4).

15. The method according to claim 14, wherein the isolated total DNA from step (a) is obtained from the group consisting of a bodily fluid or a tissue sample obtained from the pregnant woman.

16. The method according to claim 15, wherein the bodily fluid is selected from the group consisting of whole blood, saliva, urine and amniotic fluid.

17. The method according to claim 16, wherein the bodily fluid is whole blood comprising blood cells, plasma and serum.

18. The method according to any one of claims 15 to 17, wherein the total DNA is obtained from plasma or serum.

19. The method according to claim 15, wherein the tissue is selected from the group consisting of placental tissue and amniotic sac tissue.

20. The method according to any one of claims 14 to 19, wherein the maternal DNA is maternal peripheral blood DNA.

21. The method according to any one of claims 14 to 20, wherein step (b) is performed by treating the total isolated DNA with a reagent that differentially modifies methylated or non-methylated DNA.

22. The method according to claim 21, wherein the reagent is selected from the group consisting of sodium bisulfite, one or more enzymes that only cleaves methylated DNA and one or more enzymes that only cleaves non-methylated DNA.

23. The method according to claim 22, wherein the enzyme is selected from the group consisting of MspJI, LpnPI, FspEI, DpnI, DpnII, McrBC, MspI, HapII, AatII, AclI, AclI, AfeI, AgeI, AscI, AsiSI, AvaI, BceAI, BmgBI, BsaAI, BsaHI, BsiEI,

BsiWI, BsmBI, BspDI, BsrFI, BssHII, BstBI, BstUI, ClaI, EagI, FauI, FseI, FspI, HaeII, HgaI, HhaI, HinP1I, HpaII, Hpy99I, HpyCH4IV, KsaI, MluI, NaeI, NarI, NgoMIV, NotI, NruI, Nt.BsmAI, NtCviPII, PaeR7I, PmlI, PvuI, RsrII, SacII, Sall, SfoI, SgrAI, SmaI, TspMI and ZraI.

- 5
24. The method according to any one of claims 14 to 23, wherein prior to step (c), the total DNA is treated with an enzyme which catalyses the removal of nucleotides from single-stranded DNA in the 3' to 5' direction.
- 10
25. The method according to any one of claims 14 to 24, wherein the total DNA is incubated with one or more probe sets.
26. The method according to claim 25, wherein each probe set comprises:
- 15
- (a) a first probe, comprising a sequence for binding a forward primer, a sequence for binding a third probe and a sequence for binding to the one or more biomarker/biomarker regions; and
- (b) a second probe, comprising a sequence for binding a reverse primer and a sequence for binding to the one or more biomarker/biomarker regions.
- 20
27. The method according to claim 26 wherein the second probe is phosphorylated at the 5' end.
28. The method according to any one of claims 26 to 27, wherein the binding sequence for the third probe is different for each of biomarker/biomarker region groups 1 to 4.
- 25
29. The method according to claim 28, wherein the binding sequence for the third probe for the Group 1 biomarker/biomarker region comprises the sequence 5'-CCACAGTATGAATCTCT-3' (SEQ ID NO: 123).
- 30
30. The method according to any one of claims 28 to 29, wherein the binding sequence for the third probe for the Group 2 biomarker/biomarker region comprises the sequence

5'-CCACACATAGAGTTCTT-3' (SEQ ID NO: 124).

31. The method according to any one of claims 26 to 30, wherein the sequences of the first probe and second probe in each probe set is selected from any one of the probe sets listed in Tables 7 or 8.

32. The method according to any one of claims 26 to 31, wherein the two probes from each probe set are ligated together.

33. The method according to claim 32, further comprising the step of removing the excess probes which have not been ligated together.

34. The method according to claim 32, wherein the step of removing the excess probes is performed using bead purification.

35. The method according to any one of claims 14 to 34, wherein the signal which is indicative of the level of foetal DNA in step (c) is a fluorescent signal.

36. The method according to claim 35, wherein a different fluorescent signal is measured for each of biomarker/biomarker region groups 1 to 4.

37. The method according to claim 35, wherein the fluorescent signals originate from one or more probes having fluorophores thereon.

38. The method according to any one of claims 26 to 37, wherein the forward primer comprises the sequence selected from the group consisting of 5'-GCATGGCTGCTGAGATCGT-3' (SEQ ID NO: 127).

39. The method according to any one of claims 26 to 38, wherein the reverse primer comprises the sequence selected from the group of 5'-CGCACGTTTCGCATCGA-3' (SEQ ID NO: 128).

40. The method according to any one of claims 26 to 39, wherein the third probe comprises the sequence selected from the group consisting of 5'-FAM-CCACAGTATGAATCTCT-MGB-3' (SEQ ID NO: 125).

5 41. The method according to any one of claims 26 to 40, wherein the third probe comprises the sequence selected from the group consisting of 5'-VIC-CCACACATAGAGTTCTT-MGB-3' (SEQ ID NO: 126).

10 42. The method according to any one of claims 14 to 38, wherein the signal indicative of the level of foetal DNA in step (c) is measured by quantitative polymerase chain reaction.

15 43. A kit comprising primers for amplifying the one or more biomarkers/biomarker regions selected from any one of the DNA regions of the human genome listed in any one of tables 3 to 6 (groups 3, 4, 1' and 2') and one or more reagents for measuring a signal indicative for the level of foetal DNA based on the one or more biomarkers/biomarker regions.

20 44. A kit comprising primers for amplifying the one or more biomarkers/biomarker regions selected from any one of the DNA regions of the human genome listed in any one of tables 1 to 4 (groups 1 to 4) and one or more reagents for measuring a signal indicative for the level of foetal DNA based on the one or more biomarkers/biomarker regions.

25 45. A kit comprising primers for amplifying the one or more biomarkers/biomarker regions selected from any one of the DNA regions of the human genome listed in any one of tables 7 to 8 (Mix10 Group 1 and Mix10 Group 2) and one or more reagents for measuring a signal indicative for the level of foetal DNA based on the one or more biomarkers/biomarker regions.

30 46. The kit of any one of claims 43 to 45, wherein the reagents are selected from the group consisting of reagents for isolating DNA from samples, reagents for

differentially modifying methylated or non-methylated DNA, reagents for polymerase chain reaction and reagents for quantitative polymerase chain reaction.

5 47. A method of determining the methylation levels of a biomarker/biomarker region comprising the steps of:

(a) treating a sample comprising both foetal and maternal DNA with a reagent that differentially modifies methylated and non-methylated DNA;

10 (b) calculating the percentage of unmodified cytosine residues over the total number of modified and unmodified cytosine residues in order to determine the methylation levels of a biomarker/biomarker region.

15 48. The method according to claim 47, wherein the reagent in step (a) is selected from the group consisting of sodium bisulfite, one or more enzymes that preferentially cleaves methylated DNA and one or more enzymes that preferentially cleaves non-methylated DNA.

49. The method according to any one of claims 47 to 48, further comprising bisulfite sequencing prior to step (b).

20 50. The method according to claim 49, where signals detected from the unmodified cytosine residues and the modified cytosine residues are compared to calculate the methylation level.