DETECTION OF CHROMOSOME INTERACTION RELEVANT TO BREAST CANCER

Field of the Invention

The invention relates to detecting chromosome interactions.

Background of the Invention

Cancer is caused by the loss of regulation in cell growth and division. This occurs when mutations in the DNA of a cell occur, without the cell being able fix the mutation, the mutations can either be inherited (germline) or developed (acquired). There are two types of cancer: benign and malignant, benign cancers occur when a loss of regulation in cell division occurs but the tumour does not spread to other parts of the body. Malignant (or metastatic) cancers are more severe and occur when the cancerous cells migrate to other parts of the body via the bloodstream or lymph system. Breast cancer is the name for cancers that start in the breast and is the second most common cancer worldwide. An estimated 14.1 million new cancer cases occurred in 2012. Presently cancer screening using a mammogram is the gold standard to check for any breast abnormalities and if a lump is detected then a biopsy is carried out. Histological grading of invasive mammary carcinoma is used to separate patients with invasive breast cancer into three groups with distinct prognosis: good, intermediate, and poor.

Summary of the Invention

Specific Chromosome Conformation Signatures (CCSs) at loci either exist or are absent due to the regulatory epigenetic control settings associated with pathology or treatment. CCSs have mild off-rates and when representing a particular phenotype or pathology, they will only change with a physiologically signalled transition to a new phenotype, or as a result of external intervention. In addition, the measurement of these events is binary, and so this read-out is in stark contrast to the continuum readout of varying levels of DNA methylation, histone modifications and most of the non-coding RNAs. The continuum read-out used for most molecular biomarkers to date offers a challenge to data analysis, in that the magnitude of change for particular biomarkers varies greatly from patient to patient, which causes problems for classification statistics when they are used to stratify cohorts of patients. These classification statistics are better-suited to using biomarkers that are absent of magnitude and offer just a “yes or no” binary score of phenotypic differences - signifying that *EpiSwitch*™ biomarkers are an excellent resource for potential diagnostic, prognostic and predictive biomarkers.

The inventors have identified regions of the genome where chromosomal interactions are relevant to breast cancer using an approach which allows identification of subgroups in a population. Accordingly, the invention provides a process for detecting a chromosome state which represents a subgroup in a population comprising determining whether a chromosome interaction is present or absent within a defined disease-associated region of the genome, wherein said disease is breast cancer. The chromosome

interaction may optionally have been identified, or be identifiable (or derivable), by a method of determining which chromosomal interactions are relevant to a chromosome state corresponding to a breast cancer subgroup of the population, comprising contacting a first set of nucleic acids from subgroups with different states of the chromosome with a second set of index nucleic acids, and allowing complementary sequences to hybridise, wherein the nucleic acids in the first and second sets of nucleic acids represent a ligated product comprising sequences from both the chromosome regions that have come together in chromosomal interactions, and wherein the pattern of hybridisation between the first and second set of nucleic acids allows a determination of which chromosomal interactions are specific to a breast cancer subgroup.

Brief Description of the Drawings

Figure 1. Comparison of significant probes from BCa1 and BCa2 arrays. Probes adjusted p-value <0.05.

Figure 2. This Venn diagram shows the patient set broken into maker reduction set (118, set 1) and model validation set (50, set2). The other patient set refers to the controls excluded from site 2 shipment 122.

Figure 3. Cross validation plot of the GLMNET model is used to choose lambda (penalized value for logistic model) and coefficients (at min error). Y-axis is mean-squared error. X-axis is log (lambda).

Figure 4. Outlier quality control for shipment 122. Factor analysis (principle component analysis) plot of the control samples from site 2 using the 8 markers from the final BrCa model. The patients shown as triangles (site2_b2) are from shipment 122 (30 patients) and these were excluded from the total analysis, the patients shown as circles (site_2) are site 2 control from shipment 113 (25 patients), which were used. This plot shows that within these controls from the same location, there is a large variation component which separates patients that are meant to be biologically similar in context of comparison to BrCa. This is of a concern as this variation will compete for the difference between BrCa and control in the final model; hence site 2 batch 2 shipment 122 samples were removed. Y-axis is Dim 2 16.79%. X-axis is Dim 1 18.11%.

Figure 5 shows a factor analysis (principle component analysis) plot for all control samples used in the analysis (69 controls) using the 8 markers from the final BrCa model. There is slight geographical variation in the patients but this is smaller than the variation with the outlier group of the site 2 controls (shipment 122). Circles are site 1. Triangles are site 2. Dark squares are site 3. Crosses are site 4. Crossed boxes are site 5. Y-axis is Dim2 11.57%. Y-axis is Dim 1 15.47%.

Figure 6 shows a factor analysis (principle component analysis) plot for all control samples used in the analysis (69 controls), plus the 30 excluded outlier group of site 2 (shipment 122 – shown as crossed circles) using the 8 markers from the final BrCa model. The spread in the data is predominantly due to

the issues in the site 2 shipment 122. Dark diamonds are site 1. Dark circles are site 2. Dark squares are site 3. Dark triangles are site 4. Crosses are site 5. Light triangles are site 6. Crossed circles are site 2_b2. Y-axis is Dim 2 11.14%. X-axis is Dim 1 12.36%.

Figure 7 shows results for marker ATM_11_108118137_108126372_108155279_108156687_RF. The first figure shows amplification with ATM primers 54 and 56, 472bp analysis two. The second figure shows the standard curve. FAM is used. The efficiency is found to be 91.7%, R^2 is 0.996, slope is -3.539, y-int is 39.706.

Figure 8 shows amplification lines for ATM_11_108118137_108126372_108155279_108156687_RF. The first Figure shows amplification with ATM primers 54 and 56, 472bp analysis two, row C. The second figure shows amplification with ATM primers 54 and 56, 472bp analysis two, row D.

Figure 9 shows results for marker CDC6_17_38421089_38423079_38451196_38457050_FF. The first figure shows amplification with PCR 2 CDC6 FF. The second figure shows the standard curve. FAM is used. The efficiency is found to be 90.7%, R^2 is 0.990, slope is -3.568, y-int is 40.652.

Figure 10 shows results for marker FOXC1_6_1577253_1581989_1604206_1605973_FR. The first figure shows amplification with ATM 208bp FOXC1. The second figure shows the standard curve. FAM is used. The efficiency is found to be 101.6%, R^2 is 0.992, slope is -3.284, y-int is 37.746.

Figure 11 shows amplification lines for marker FOXC1_6_1577253_1581989_1604206_1605973_FR. The first Figure shows amplification with ATM 208bp, row C. The second figure shows amplification with ATM 208bp, row D.

Figure 12 shows results for marker MAP3K1_5_56102259_56110500_56140227_56144076_FF. The first figure shows amplification PCR 9 MAP3K1 cells C1-C6 (RFU versus cycles). The second figure shows melt peak for well C1 to C6. The y-axis shows –d(RFU)/dt. The x-axis shows temperature in Celsius.

Figure 13 shows results for marker MAP3K1_5_56102259_56110500_56140227_56144076_FF. The first figure shows amplification with MAP3K1 495bp. The second figure shows the standard curve. FAM is used. The efficiency is found to be 91.9%, R^2 is 0.999, slope is -3.533, y-int is 40.940.

Figure 14 shows results for marker ME3_11_86300063_86304401_86420537_86426200_FR. The first figure shows amplification ME3 PCR 12, A7-A12 (RFU versus cycles). The second figure shows melt peak. The y-axis shows –d(RFU)/dt. The x-axis shows temperature in Celsius.

Figure 15 shows results for marker ME3_11_86300063_86304401_86420537_86426200_FR. The first figure shows amplification with ME3 291bp. The second figure shows the standard curve. FAM is used. The efficiency is found to be 96.8%, R^2 is 0.998, slope is -3.400, y-int is 39.596.

Figure 16 shows results for marker MELK_9_36577630_36579243_36637050_36643005_RF. The first figure shows amplification with MELK 207bp. The second figure shows the standard curve. FAM is used. The efficiency is found to be 91.3%, R^2 is 0.995, slope is -3.550, y-int is 42.000.

Figure 17 shows results for marker MSH3_5_80021913_80025030_80153948_80159012_RF. The first figure shows amplification with MSH3 207bp. The second figure shows the standard curve. FAM is used. The efficiency is found to be 97.1%, R^2 is 0.990, slope is -3.394, y-int is 41.876.

Figure 18 shows results for marker NF1_17_29477103_29483764_29651799_29657368_FF. The first figure shows amplification with NF1 401bp. The second figure shows the standard curve. FAM is used. The efficiency is found to be 99.0%, R^2 is 0.987, slope is -3.347, y-int is 40.192.

Figure 19 shows results for marker SRD5A1_5_6634973_6639025_6667775_6669711_RF. Only standard curves are shown with no patient data. The first figure shows amplification with SRDA51. The second figure shows the standard curve. FAM is used. The efficiency is found to be 95.5%, R^2 is 0.997, slope is -3.434, y-int is 39.761.

Figure 20 shows results for marker TSPYL5_8_98276431_98282736_98316421_98318720_FF. Only standard curves are shown with no patient data. The first figure shows amplification with TSPYL5. The second figure shows the standard curve. FAM is used. The efficiency is found to be 94.2%, R^2 is 0.998, slope is -3.469, y-int is 41.344.

Figure 21 shows data corresponding to marker set 2 of Example 2. The loop detection data shows whether the marker is associated with a cancer disease sample or a control sample.

Figure 22 shows data corresponding to marker set 3 of Example 3. The loop detection data shows whether the marker is associated with a malignant disease sample or a control sample. The statistics shown are for the nested PCR work.

For all figures showing amplification curves the Y-axis is RFU, the X-axis is cycles; and for wells in row C the amplification lines for the patient samples are marked with an X, in row D the patient amplification curves are designated with triangles (Δ).

For all figures showing standard curves the Y-axis is Cq, the X-axis is log starting quantity; and the circles are standard and the crosses are unknown.

Detailed Description of the Invention

The Process of the Invention

The process of the invention comprises a typing system for detecting chromosome interactions relevant to breast cancer. This typing may be performed using the *EpiSwitch*™ system mentioned herein which is

based on cross-linking regions of chromosome which have come together in the chromosome interaction, subjecting the chromosomal DNA to cleavage and then ligating the nucleic acids present in the cross-linked entity to derive a ligated nucleic acid with sequence from both the regions which formed the chromosomal interaction. Detection of this ligated nucleic acid allows determination of the presence or absence of a particular chromosome interaction.

The chromosomal interactions may be identified using the above described method in which populations of first and second nucleic acids are used. These nucleic acids can also be generated using *EpiSwitch*™ technology.

The Epigenetic Interactions Relevant to the Invention

As used herein, the term 'epigenetic' and 'chromosome' interactions typically refers to interactions between distal regions of a chromosome, said interactions being dynamic and altering, forming or breaking depending upon the status of the region of the chromosome.

In particular processes of the invention chromosome interactions are detected by first generating a ligated nucleic acid that comprises sequence from both regions of the chromosomes that are part of the interactions. In such processes the regions can be cross-linked by any suitable means. In a preferred embodiment, the interactions are cross-linked using formaldehyde, but may also be cross-linked by any aldehyde, or D-Biotinoyl-e- aminocaproic acid-N-hydroxysuccinimide ester or Digoxigenin-3-O-methylcarbonyl-e-aminocaproic acid-N-hydroxysuccinimide ester. Para-formaldehyde can cross link DNA chains which are 4 Angstroms apart.

The chromosome interaction may reflect the status of the region of the chromosome, for example, if it is being transcribed or repressed in response to change of the physiological conditions. Chromosome interactions which are specific to subgroups as defined herein have been found to be stable, thus providing a reliable means of measuring the differences between the two subgroups.

In addition, chromosome interactions specific to a characteristic (such as a disease condition) will normally occur early in a biological process, for example compared to other epigenetic markers such as methylation or changes to binding of histone proteins. Thus the process of the invention is able to detect early stages of a biological process. This allows early intervention (for example treatment) which may as a consequence be more effective. Furthermore there is little variation in the relevant chromosome interactions between individuals within the same subgroup. Detecting chromosome interactions is highly informative with up to 50 different possible interactions per gene, and so processes of the invention can interrogate 500,000 different interactions.

Preferred Marker Sets

Specific markers are disclosed herein, any of which may used in the invention. Further sets of markers may be used, for example in the combinations or numbers disclosed herein. Marker sets 1, 2 and 3 are preferred. These may be typed by any suitable method, for example the PCR or probe based methods disclosed herein, including a qPCR method. The markers are defined herein by location or by probe and/or primer sequences.

Location and Causes of Epigenetic Interactions

Epigenetic chromosomal interactions may overlap and include the regions of chromosomes shown to encode relevant or undescribed genes, but equally may be in intergenic regions. It should further be noted that the inventors have discovered that epigenetic interactions in all regions are equally important in determining the status of the chromosomal locus. These interactions are not necessarily in the coding region of a particular gene located at the locus and may be in intergenic regions.

The chromosome interactions which are detected in the invention could be caused by changes to the underlying DNA sequence, by environmental factors, DNA methylation, non-coding antisense RNA transcripts, non-mutagenic carcinogens, histone modifications, chromatin remodelling and specific local DNA interactions. The changes which lead to the chromosome interactions may be caused by changes to the underlying nucleic acid sequence, which themselves do not directly affect a gene product or the mode of gene expression. Such changes may be for example, SNPs within and/or outside of the genes, gene fusions and/or deletions of intergenic DNA, microRNA, and non-coding RNA. For example, it is known that roughly 20% of SNPs are in non-coding regions, and therefore the process as described is also informative in non-coding situation. In one embodiment the regions of the chromosome which come together to form the interaction are less than 5 kb, 3 kb, 1 kb, 500 base pairs or 200 base pairs apart on the same chromosome.

The chromosome interaction which is detected is preferably within any of the genes mentioned in Table 9. The chromosome interaction which is detected may be within any of the genes mentioned for marker set 1, 2 or 3. However it may also be upstream or downstream of the gene, for example up to 50,000, up to 30,000, up to 20,000, up to 10,000 or up to 5000 bases upstream or downstream from the gene or from the coding sequence.

Subgroups, Diagnosis and Personalised Treatment

The aim of the present invention is to permit detection of chromosome interactions relevant to a breast cancer subgroup. Therefore the process may or may not be used for diagnosis of breast cancer. The process of the invention may be used for diagnosis of malignant breast cancer, and preferably markers from marker set 3 are used in such an embodiment.

As used herein, a “subgroup” preferably refers to a population subgroup (a subgroup in a population), more preferably a subgroup in the population of a particular animal such as a particular eukaryote, or mammal (e.g. human, non-human, non-human primate, or rodent e.g. mouse or rat). Most preferably, a “subgroup” refers to a subgroup in the human population.

The invention includes detecting and treating particular subgroups in a population. The inventors have discovered that chromosome interactions differ between subsets (for example two or at least two subsets) in a given population. Identifying these differences will allow physicians to categorize their patients as a part of one subset of the population as described in the process. The invention therefore provides physicians with a process of personalizing medicine for the patient based on their epigenetic chromosome interactions.

Generating Ligated Nucleic Acids

Certain embodiments of the invention utilise ligated nucleic acids, in particular ligated DNA. These comprise sequences from both of the regions that come together in a chromosome interaction and therefore provide information about the interaction. The EpiSwitch™ method described herein uses generation of such ligated nucleic acids to detect chromosome interactions.

Thus a process of the invention may comprise a step of generating ligated nucleic acids (e.g. DNA) by the following steps (including a method comprising these steps):

(i) cross-linking of epigenetic chromosomal interactions present at the chromosomal locus, preferably *in vitro*;

(ii) optionally isolating the cross-linked DNA from said chromosomal locus;

(iii) subjecting said cross-linked DNA to cutting, for example by restriction digestion with an enzyme that cuts it at least once (in particular an enzyme that cuts at least once within said chromosomal locus);

(iv) ligating said cross-linked cleaved DNA ends (in particular to form DNA loops); and

(v) optionally identifying the presence of said ligated DNA and/or said DNA loops, in particular using techniques such as PCR (polymerase chain reaction), to identify the presence of a specific chromosomal interaction.

These steps may be carried out to detect the chromosome interactions for any embodiment mentioned herein, such as for determining whether the individual is part of a breast cancer subgroup. The steps may also be carried out to generate the first and/or second set of nucleic acids mentioned herein.

PCR (polymerase chain reaction) may be used to detect or identify the ligated nucleic acid, for example the size of the PCR product produced may be indicative of the specific chromosome interaction which is

present, and may therefore be used to identify the status of the locus. In preferred embodiments at least 1, 2, 3, 4, 5, 6, 7 or 8 primers or primer pairs as shown in Table 10 are used in the PCR reaction. In other preferred embodiments at least 1, 2, 3, 4, 5, 6, 7 or 8 primers or primer pairs relevant to or as shown for marker set 2 or 3 are used in the PCR reaction. The skilled person will be aware of numerous restriction enzymes which can be used to cut the DNA within the chromosomal locus of interest. It will be apparent that the particular enzyme used will depend upon the locus studied and the sequence of the DNA located therein. A non-limiting example of a restriction enzyme which can be used to cut the DNA as described in the present invention is TaqI.

Embodiments such as EpiSwitch™ Technology

The EpiSwitch™ Technology also relates to the use of microarray EpiSwitch™ marker data in the detection of epigenetic chromosome conformation signatures specific for phenotypes. Embodiments such as EpiSwitch™ which utilise ligated nucleic acids in the manner described herein have several advantages. They have a low level of stochastic noise, for example because the nucleic acid sequences from the first set of nucleic acids of the present invention either hybridise or fail to hybridise with the second set of nucleic acids. This provides a binary result permitting a relatively simple way to measure a complex mechanism at the epigenetic level. EpiSwitch™ technology also has fast processing time and low cost. In one embodiment the processing time is 3 hours to 6 hours.

Samples and Sample Treatment

The process of the invention will normally be carried out on a sample. The sample will normally contain DNA from the individual. It will normally contain cells. In one embodiment a sample is obtained by minimally invasive means, and may for example be a blood sample. DNA may be extracted and cut up with a standard restriction enzyme. This can pre-determine which chromosome conformations are retained and will be detected with the EpiSwitch™ platforms. Due to the synchronisation of chromosome interactions between tissues and blood, including horizontal transfer, a blood sample can be used to detect the chromosome interactions in tissues, such as tissues relevant to disease. For certain conditions, such as cancer, genetic noise due to mutations can affect the chromosome interaction 'signal' in the relevant tissues and therefore using blood is advantageous.

Properties of Nucleic Acids of the Invention

The invention relates to certain nucleic acids, such as the ligated nucleic acids which are described herein as being used or generated in the process of the invention. These may be the same as, or have any of the properties of, the first and second nucleic acids mentioned herein. The nucleic acids of the invention typically comprise two portions each comprising sequence from one of the two regions of the chromosome which come together in the chromosome interaction. Typically each portion is at least 8, 10, 15, 20, 30 or 40 nucleotides in length, for example 10 to 40 nucleotides in length. Preferred nucleic acids

comprise sequence from any of the genes mentioned in any of the tables. Typically preferred nucleic acids comprise the specific probe sequences mentioned in Table 9; or fragments and/or homologues of such sequences. Typically preferred nucleic acids also comprise the specific probe sequences relevant to and/or mentioned for marker set 2 or 3; or fragments and/or homologues of such sequences. Preferably the nucleic acids are DNA. It is understood that where a specific sequence is provided the invention may use the complementary sequence as required in the particular embodiment.

The primers shown in Table 10 may also be used in the invention as mentioned herein. In one embodiment primers are used which comprise any of: the sequences shown in Table 10; or fragments and/or homologues of any sequence shown in Table 10. The primers relevant for and/or shown for marker set 2 or 3 may also be used in the invention as mentioned herein. In one embodiment primers are used which comprise any of: the sequences shown for marker set 2 or 3; or fragments and/or homologues of any sequence shown for marker set 2 or 3.

The Second Set of Nucleic Acids – the 'Index' Sequences

The second set of nucleic acid sequences has the function of being a set of index sequences, and is essentially a set of nucleic acid sequences which are suitable for identifying subgroup specific sequence. They can represents the 'background' chromosomal interactions and might be selected in some way or be unselected. They are in general a subset of all possible chromosomal interactions.

The second set of nucleic acids may be derived by any suitable process. They can be derived computationally or they may be based on chromosome interaction in individuals. They typically represent a larger population group than the first set of nucleic acids. In one particular embodiment, the second set of nucleic acids represents all possible epigenetic chromosomal interactions in a specific set of genes. In another particular embodiment, the second set of nucleic acids represents a large proportion of all possible epigenetic chromosomal interactions present in a population described herein. In one particular embodiment, the second set of nucleic acids represents at least 50% or at least 80% of epigenetic chromosomal interactions in at least 20, 50, 100 or 500 genes, for example in 20 to 100 or 50 to 500 genes.

The second set of nucleic acids typically represents at least 100 possible epigenetic chromosome interactions which modify, regulate or in any way mediate a disease state / phenotype in population. The second set of nucleic acids may represent chromosome interactions that affect a disease state in a species, for example chromosome interactions in genes that encode cytokines, kinases, or regulators associated with any disease state, predisposition to a disease or a disease phenotype. The second set of nucleic acids typically comprises sequences representing epigenetic interactions relevant and not relevant to a breast cancer subgroup.

In one particular embodiment the second set of nucleic acids derive at least partially from naturally occurring sequences in a population, and are typically obtained by *in silico* processes. Said nucleic acids may further comprise single or multiple mutations in comparison to a corresponding portion of nucleic acids present in the naturally occurring nucleic acids. Mutations include deletions, substitutions and/or additions of one or more nucleotide base pairs. In one particular embodiment, the second set of nucleic acids may comprise sequence representing a homologue and/or orthologue with at least 70% sequence identity to the corresponding portion of nucleic acids present in the naturally occurring species. In another particular embodiment, at least 80% sequence identity or at least 90% sequence identity to the corresponding portion of nucleic acids present in the naturally occurring species is provided.

Properties of the Second Set of Nucleic Acids

In one particular embodiment, there are at least 100 different nucleic acid sequences in the second set of nucleic acids, preferably at least 1000, 2000 or 5000 different nucleic acids sequences, with up to 100,000, 1,000,000 or 10,000,000 different nucleic acid sequences. A typical number would be 100 to 1,000,000, such as 1,000 to 100,000 different nucleic acids sequences. All or at least 90% or at least 50% or these would correspond to different chromosomal interactions.

In one particular embodiment, the second set of nucleic acids represent chromosome interactions in at least 20 different loci or genes, preferably at least 40 different loci or genes, and more preferably at least 100, at least 500, at least 1000 or at least 5000 different loci or genes, such as 100 to 10,000 different loci or genes. The lengths of the second set of nucleic acids are suitable for them to specifically hybridise according to Watson Crick base pairing to the first set of nucleic acids to allow identification of chromosome interactions specific to subgroups. Typically the second set of nucleic acids will comprise two portions corresponding in sequence to the two chromosome regions which come together in the chromosome interaction. The second set of nucleic acids typically comprise nucleic acid sequences which are at least 10, preferably 20, and preferably still 30 bases (nucleotides) in length. In another embodiment, the nucleic acid sequences may be at the most 500, preferably at most 100, and preferably still at most 50 base pairs in length. In a preferred embodiment, the second set of nucleic acids comprises nucleic acid sequences of between 17 and 25 base pairs. In one embodiment at least 100, 80% or 50% of the second set of nucleic acid sequences have lengths as described above. Preferably the different nucleic acids do not have any overlapping sequences, for example at least 100%, 90%, 80% or 50% of the nucleic acids do not have the same sequence over at least 5 contiguous nucleotides.

Given that the second set of nucleic acids acts as an 'index' then the same set of second nucleic acids may be used with different sets of first nucleic acids which represent subgroups for different characteristics,

i.e. the second set of nucleic acids may represent a 'universal' collection of nucleic acids which can be used to identify chromosome interactions relevant to different characteristics.

The First Set of Nucleic Acids

The first set of nucleic acids are normally from individuals with breast cancer. The first nucleic acids may have any of the characteristics and properties of the second set of nucleic acids mentioned herein. The first set of nucleic acids is normally derived from a sample from the individuals which has undergone treatment and processing as described herein, particularly the EpiSwitch™ cross-linking and cleaving steps. Typically the first set of nucleic acids represents all or at least 80% or 50% of the chromosome interactions present in the samples taken from the individuals.

Typically, the first set of nucleic acids represents a smaller population of chromosome interactions across the loci or genes represented by the second set of nucleic acids in comparison to the chromosome interactions represented by second set of nucleic acids, i.e. the second set of nucleic acids is representing a background or index set of interactions in a defined set of loci or genes.

Library of Nucleic Acids

Any of the types of nucleic acid populations mentioned herein may be present in the form of a library comprising at least 200, at least 500, at least 1000, at least 5000 or at least 10000 different nucleic acids of that type, such as 'first' or 'second' nucleic acids. Such a library may be in the form of being bound to an array.

Hybridisation

The invention requires a means for allowing wholly or partially complementary nucleic acid sequences from the first set of nucleic acids and the second set of nucleic acids to hybridise. In one embodiment all of the first set of nucleic acids is contacted with all of the second set of nucleic acids in a single assay, i.e. in a single hybridisation step. However any suitable assay can be used.

Labelled Nucleic Acids and Pattern of Hybridisation

The nucleic acids mentioned herein may be labelled, preferably using an independent label such as a fluorophore (fluorescent molecule) or radioactive label which assists detection of successful hybridisation. Certain labels can be detected under UV light. The pattern of hybridisation, for example on an array described herein, represents differences in epigenetic chromosome interactions between the two subgroups, and thus provides a process of comparing epigenetic chromosome interactions and

determination of which epigenetic chromosome interactions are specific to a subgroup in the population of the present invention.

The term 'pattern of hybridisation' broadly covers the presence and absence of hybridisation between the first and second set of nucleic acids, i.e. which specific nucleic acids from the first set hybridise to which specific nucleic acids from the second set, and so it not limited to any particular assay or technique, or the need to have a surface or array on which a 'pattern' can be detected.

Selecting a Subgroup with Particular Characteristics

The invention provides a process which comprises detecting the presence or absence of chromosome interactions, typically 5 to 20 or 5 to 500 such interactions, preferably 20 to 300 or 50 to 100 interactions, in order to determine the presence or absence of a characteristic relating to breast cancer in an individual. Preferably the chromosome interactions are those in any of the genes mentioned herein. In one embodiment the chromosome interactions which are typed are those represented by the nucleic acids in Table 9. The column titled 'Loop Detected' in Table 9 shows which subgroup is detected (breast cancer or control) by each probe. As can be seen the process of the invention can detect either a breast cancer subgroup and/or a control subgroup (non-breast cancer) as part of the testing.

The Individual that is Tested

Examples of the species that the individual who is tested is from are mentioned herein. In addition the individual that is tested in the process of the invention may have been selected in some way. The individual may be female, for example.

Preferred Gene Regions, Loci, Genes and Chromosome Interactions

For all aspects of the invention preferred gene regions, loci, genes and chromosome interactions are mentioned in the Table 9. Typically in the processes of the invention chromosome interactions are detected from at least 1, 2, 3, 4, 5, 6, 7 or 8 of the relevant genes listed in Table 9. Preferably the presence or absence of at least 1, 2, 3, 4, 5, 6, 7 or 8 of the relevant specific chromosome interactions represented by the probe sequences in Table 9 are detected. The disease-associated region may be upstream or downstream of any of the genes mentioned herein, for example 50 kb upstream or 20 kb downstream, for example from the coding sequence.

For all aspects of the invention preferred gene regions, loci, genes and chromosome interactions are mentioned in other tables. Typically in the processes of the invention chromosome interactions are detected from at least 1, 2, 3, 4, 5, 6, 7 or 8 of the relevant genes listed in tables, for example for marker set 2 or 3. Preferably the presence or absence of at least 1, 2, 3, 4, 5, 6, 7 or 8 of the relevant specific

chromosome interactions represented by the probe sequences in tables are detected. The disease-associated region may be upstream or downstream of any of the genes mentioned herein, for example 50 kb upstream or 20 kb downstream, for example from the coding sequence.

In one embodiment the locus (including the gene and/or place where the chromosome interaction is detected) may comprise a CTCF binding site. This is any sequence capable of binding transcription repressor CTCF. That sequence may consist of or comprise the sequence CCCTC which may be present in 1, 2 or 3 copies at the locus. The CTCF binding site sequence may comprise the sequence CCGCGNGGNGGCAG (in IUPAC notation). The CTCF binding site may be within at least 100, 500, 1000 or 4000 bases of the chromosome interaction or within any of the chromosome regions shown Table 9. The CTCF binding site may be within at least 100, 500, 1000 or 4000 bases of the chromosome interaction or within any of the chromosome regions shown in any table, for example for marker set 2 or 3.

In one embodiment the chromosome interactions which are detected are present at any of the gene regions shown Table 9. In the case where a ligated nucleic acid is detected in the process then sequence shown in any of the probe sequences in Table 9 may be detected. In another embodiment the chromosome interactions which are detected are present at any of the gene regions shown in other tables, for example for marker set 2 or 3. In the case where a ligated nucleic acid is detected in the process then sequence shown in any of the probe sequences in a table may be detected, for example for marker set 2 or 3.

Thus typically sequence from both regions of the probe (i.e. from both sites of the chromosome interaction) could be detected. In preferred embodiments probes are used in the process which comprise or consist of the same or complementary sequence to a probe shown in any table. In some embodiments probes are used which comprise sequence which is homologous to any of the probe sequences shown in the tables.

Tables Provided Herein

Table 9 shows probe (Episwitch™ marker) data and gene data representing chromosome interactions relevant to breast cancer. Other probe and gene data is shown in other tables, for example for marker set 2 or 3. The probe sequences show sequence which can be used to detect a ligated product generated from both sites of gene regions that have come together in chromosome interactions, i.e. the probe will comprise sequence which is complementary to sequence in the ligated product. The first two sets of Start-End positions show probe positions, and the second two sets of Start-End positions show the relevant 4kb region. The following information is provided in the probe data table:

- HyperG_Stats: p-value for the probability of finding that number of significant EpiSwitch™

markers in the locus based on the parameters of hypergeometric enrichment

- Probe Count Total: Total number of EpiSwitch™ Conformations tested at the locus
- Probe Count Sig: Number of EpiSwitch™ Conformations found to be statistical significant at the locus
- FDR HyperG: Multi-test (False Discovery Rate) corrected hypergeometric p-value
- Percent Sig: Percentage of significant EpiSwitch™ markers relative the number of markers tested at the locus
- logFC: logarithm base 2 of Epigenetic Ratio (FC)
- AveExpr: average log2-expression for the probe over all arrays and channels
- T: moderated t-statistic
- p-value: raw p-value
- adj. p-value: adjusted p-value or q-value
- B - B-statistic (lods or B) is the log-odds that that gene is differentially expressed.
- FC - non-log Fold Change
- FC_1 - non-log Fold Change centred around zero
- LS – Binary value this relates to FC_1 values. FC_1 value below -1.1 it is set to -1 and if the FC_1 value is above 1.1 it is set to 1. Between those values the value is 0

Table 9 shows genes where a relevant chromosome interaction has been found to occur. Other tables show similar data. The p-value in the loci table is the same as the HyperG_Stats (p-value for the probability of finding that number of significant EpiSwitch™ markers in the locus based on the parameters of hypergeometric enrichment).

The probes are designed to be 30bp away from the Taq1 site. In case of PCR, PCR primers are also designed to detect ligated product but their locations from the Taq1 site vary.

Probe locations:

Start 1 - 30 bases upstream of TaqI site on fragment 1

End 1 - TaqI restriction site on fragment 1

Start 2 - TaqI restriction site on fragment 2

End 2 - 30 bases downstream of TaqI site on fragment 2

4kb Sequence Location:

Start 1 - 4000 bases upstream of TaqI site on fragment 1

End 1 - TaqI restriction site on fragment 1

Start 2 - TaqI restriction site on fragment 2

End 2 - 4000 bases downstream of TaqI site on fragment 2

Table 10 and other tables show for each of the top PCR markers: GLMNET - procedures for fitting the entire lasso or elastic-net regularization. Lambda set to 0.5 (elastic-net)

Preferred Embodiments for Sample Preparation and Chromosome Interaction Detection

Methods of preparing samples and detecting chromosome conformations are described herein. Optimised (non-conventional) versions of these methods can be used, for example as described in this section.

Typically the sample will contain at least 2 $x10^5$ cells. The sample may contain up to 5 $x10^5$ cells. In one embodiment, the sample will contain 2 $x10^5$ to 5.5 $x10^5$ cells

Crosslinking of epigenetic chromosomal interactions present at the chromosomal locus is described herein. This may be performed before cell lysis takes place. Cell lysis may be performed for 3 to 7 minutes, such as 4 to 6 or about 5 minutes. In some embodiments, cell lysis is performed for at least 5 minutes and for less than 10 minutes.

Digesting DNA with a restriction enzyme is described herein. Typically, DNA restriction is performed at about 55°C to about 70°C, such as for about 65°C, for a period of about 10 to 30 minutes, such as about 20 minutes.

Preferably a frequent cutter restriction enzyme is used which results in fragments of ligated DNA with an average fragment size up to 4000 base pair. Optionally the restriction enzyme results in fragments of ligated DNA have an average fragment size of about 200 to 300 base pairs, such as about 256 base pairs. In one embodiment, the typical fragment size is from 200 base pairs to 4,000 base pairs, such as 400 to 2,000 or 500 to 1,000 base pairs.

In one embodiment of the EpiSwitch method a DNA precipitation step is not performed between the DNA restriction digest step and the DNA ligation step.

DNA ligation is described herein. Typically the DNA ligation is performed for 5 to 30 minutes, such as about 10 minutes.

The protein in the sample may be digested enzymatically, for example using a proteinase, optionally Proteinase K. The protein may be enzymatically digested for a period of about 30 minutes to 1 hour, for example for about 45 minutes. In one embodiment after digestion of the protein, for example Proteinase K digestion, there is no cross-link reversal or phenol DNA extraction step.

In one embodiment PCR detection is capable of detecting a single copy of the ligated nucleic acid, preferably with a binary read-out for presence/absence of the ligated nucleic acid.

Processes and Uses of the Invention

The process of the invention can be described in different ways. It can be described as a method of making a ligated nucleic acid comprising (i) *in vitro* cross-linking of chromosome regions which have come together in a chromosome interaction; (ii) subjecting said cross-linked DNA to cutting or restriction digestion cleavage; and (iii) ligating said cross-linked cleaved DNA ends to form a ligated nucleic acid, wherein detection of the ligated nucleic acid may be used to determine the chromosome state at a locus, and wherein preferably:

- the locus may be any of the loci, regions or genes mentioned in Table 9,

- and/or wherein the chromosomal interaction may be any of the chromosome interactions mentioned herein or corresponding to any of the probes disclosed in Table 9, and/or

- wherein the ligated product may have or comprise (i) sequence which is the same as or homologous to any of the probe sequences disclosed in Table 9; or (ii) sequence which is complementary to (ii).

The process of the invention can be described as a process for detecting chromosome states which represent different subgroups in a population comprising determining whether a chromosome interaction is present or absent within a defined epigenetically active (disease associated) region of the genome, wherein preferably:

- the subgroup is defined by presence or absence of breast cancer, and/or
- the chromosome state may be at any locus, region or gene mentioned in Table 9; and/or
- the chromosome interaction may be any of those mentioned in Table 9 or corresponding to any of the probes disclosed in that table.

The invention includes detecting chromosome interactions at any locus, gene or regions mentioned Table 9. The invention includes use of the nucleic acids and probes mentioned herein to detect chromosome interactions, for example use of at least 1, 2, 4, 6 or 8 such nucleic acids or probes to detect chromosome interactions in at least 1, 2, 4, 6 or 8 different loci or genes. The invention includes detection of chromosome interactions using any of the primers or primer pairs listed in Table 10 or using variants of these primers as described herein (sequences comprising the primer sequences or comprising fragments and/or homologues of the primer sequences).

In particular embodiments:

- the locus may be any of the loci, regions or genes mentioned in any table, for example for marker set 2 or 3,

- and/or wherein the chromosomal interaction may be any of the chromosome interactions mentioned herein or corresponding to any of the probes disclosed in any table, for example for marker set 2 or 3, and/or

- wherein the ligated product may have or comprise (i) sequence which is the same as or homologous to any of the probe sequences disclosed in any table, for example for marker set 2 or 3; or (ii) sequence which is complementary to (ii).

The process of the invention can be described as a process for detecting chromosome states which represent different subgroups in a population comprising determining whether a chromosome interaction is present or absent within a defined epigenetically active (disease associated) region of the genome, wherein preferably:

- the subgroup is defined by presence or absence of breast cancer, and/or
- the chromosome state may be at any locus, region or gene mentioned in any table, for example for marker set 2 or 3; and/or
- the chromosome interaction may be any of those mentioned in any table, for example for marker set 2 or 3; or corresponding to any of the probes disclosed in that table.

The invention includes detecting chromosome interactions at any locus, gene or regions mentioned in any table, for example for marker set 2 or 3. The invention includes use of the nucleic acids and probes mentioned herein to detect chromosome interactions, for example use of at least 1, 2, 4, 6 or 8 such nucleic acids or probes to detect chromosome interactions in at least 1, 2, 4, 6 or 8 different loci or genes. The invention includes detection of chromosome interactions using any of the primers or primer pairs listed in any table, for example for marker set 2 or 3, or using variants of these primers as described herein (sequences comprising the primer sequences or comprising fragments and/or homologues of the primer sequences).

Use of the Method of the Invention to Identify New Treatments

Knowledge of chromosome interactions can be used to identify new treatments for conditions. The invention provides methods and uses of chromosomes interactions defined here to identify or design new therapeutic agents for breast cancer.

Homologues

Homologues of polynucleotide / nucleic acid (e.g. DNA) sequences are referred to herein. Such homologues typically have at least 70% homology, preferably at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98% or at least 99% homology, for example over a region of at least 10, 15, 20, 30, 100 or more contiguous nucleotides, or across the portion of the nucleic acid which is from

the region of the chromosome involved in the chromosome interaction. The homology may be calculated on the basis of nucleotide identity (sometimes referred to as "hard homology").

Therefore, in a particular embodiment, homologues of polynucleotide / nucleic acid (e.g. DNA) sequences are referred to herein by reference to percentage sequence identity. Typically such homologues have at least 70% sequence identity, preferably at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98% or at least 99% sequence identity, for example over a region of at least 10, 15, 20, 30, 100 or more contiguous nucleotides, or across the portion of the nucleic acid which is from the region of the chromosome involved in the chromosome interaction.

For example the UWGCG Package provides the BESTFIT program which can be used to calculate homology and/or % sequence identity (for example used on its default settings) (Devereux *et al* (1984) Nucleic Acids Research 12, p387-395). The PILEUP and BLAST algorithms can be used to calculate homology and/or % sequence identity and/or line up sequences (such as identifying equivalent or corresponding sequences (typically on their default settings)), for example as described in Altschul S. F. (1993) J MoI Evol 36:290-300; Altschul, S, F *et al* (1990) J MoI Biol 215:403-10.

Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. This algorithm involves first identifying high scoring sequence pair (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighbourhood word score threshold (Altschul *et al*, supra). These initial neighbourhood word hits act as seeds for initiating searches to find HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extensions for the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W5 T and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a word length (W) of 11 , the BLOSUM62 scoring matrix (see Henikoff and Henikoff (1992) Proc. Natl. Acad. Sci. USA 89: 10915-10919) alignments (B) of 50, expectation (E) of 10, M=5, N=4, and a comparison of both strands.

The BLAST algorithm performs a statistical analysis of the similarity between two sequences; see e.g., Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90: 5873-5787. One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two polynucleotide sequences would occur by chance. For example, a sequence is considered similar to another sequence if the smallest sum probability in comparison of the first sequence to the second sequence is less than about 1, preferably less than about

0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

The homologous sequence typically differs by 1, 2, 3, 4 or more bases, such as less than 10, 15 or 20 bases (which may be substitutions, deletions or insertions of nucleotides). These changes may be measured across any of the regions mentioned above in relation to calculating homology and/or % sequence identity.

Arrays

The second set of nucleic acids may be bound to an array, and in one embodiment there are at least 15,000, 45,000, 100,000 or 250,000 different second nucleic acids bound to the array, which preferably represent at least 300, 900, 2000 or 5000 loci. In one embodiment one, or more, or all of the different populations of second nucleic acids are bound to more than one distinct region of the array, in effect repeated on the array allowing for error detection. The array may be based on an Agilent SurePrint G3 Custom CGH microarray platform. Detection of binding of first nucleic acids to the array may be performed by a dual colour system.

Therapeutic Agents

Therapeutic agents are mentioned herein. The invention provides such agents for use in preventing or treating the breast cancer in certain individuals, for example those identified by a process of the invention. This may comprise administering to an individual in need a therapeutically effective amount of the agent. The invention provides use of the agent in the manufacture of a medicament to prevent or treat breast cancer in certain individuals.

Preferred therapeutic agents are cytotoxic drugs which are used to disrupt the growth of cancer cells. There are a number of different chemotherapy drugs that are commonly used to treat breast cancer. These include Cyclophosphamide, Epirubicin, Fluorouracil (5FU), Methotrexate, Mitomycin, Mitozantrone, Doxorubicin, Docetaxel (Taxotere) and Gemcitabine (Gemzar). Usually patients have a combination of about three chemotherapy drugs together. The therapeutic agent may reduce the level of hormones that trigger the growth of cancerous cells. The various drugs used for hormonotherapy include Anastrozole (Arimidex), Exemestane (Aromasin), Letrozole (Femara) and Tamoxifen. The therapeutic agent may be a biological therapy, such as drugs that interrupt interactions between cancerous cells, and thereby stop cell division and growth. Commonly used drugs for biological therapy include Herceptin (Trastuzumab), Lapatinib (Tyverb), Pertuzumab (Perjeta) and Everolimus (Afinitor).

The formulation of the agent will depend upon the nature of the agent. The agent will be provided in the form of a pharmaceutical composition containing the agent and a pharmaceutically acceptable carrier or diluent. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-

buffered saline. Typical oral dosage compositions include tablets, capsules, liquid solutions and liquid suspensions. The agent may be formulated for parenteral, intravenous, intramuscular, subcutaneous, transdermal or oral administration.

The dose of an agent may be determined according to various parameters, especially according to the substance used; the age, weight and condition of the individual to be treated; the route of administration; and the required regimen. A physician will be able to determine the required route of administration and dosage for any particular agent. A suitable dose may however be from 0.1 to 100 mg/kg body weight such as 1 to 40 mg/kg body weight, for example, to be taken from 1 to 3 times daily.

Forms of the Substance Mentioned Herein

Any of the substances, such as nucleic acids or therapeutic agents, mentioned herein may be in purified or isolated form. They may be in a form which is different from that found in nature, for example they may be present in combination with other substance with which they do not occur in nature. The nucleic acids (including portions of sequences defined herein) may have sequences which are different to those found in nature, for example having at least 1, 2, 3, 4 or more nucleotide changes in the sequence as described in the section on homology. The nucleic acids may have heterologous sequence at the 5' or 3' end. The nucleic acids may be chemically different from those found in nature, for example they may be modified in some way, but preferably are still capable of Watson-Crick base pairing. Where appropriate the nucleic acids will be provided in double stranded or single stranded form. The invention provides all of the specific nucleic acid sequences mentioned herein in single or double stranded form, and thus includes the complementary strand to any sequence which is disclosed.

The invention also provides a kit for carrying out any process of the invention, including detection of a chromosomal interaction associated with breast cancer or diagnosis of breast cancer. Such a kit can include a specific binding agent capable of detecting the relevant chromosomal interaction, such as agents capable of detecting a ligated nucleic acid generated by processes of the invention. Preferred agents present in the kit include probes capable of hybridising to the ligated nucleic acid or primer pairs, for example as described herein, capable of amplifying the ligated nucleic acid in a PCR reaction.

The invention also provides a device that is capable of detecting the relevant chromosome interactions. The device preferably comprises any specific binding agents, probe or primer pair capable of detecting the chromosome interaction, such as any such agent, probe or primer pair described herein.

Detection Methods

In one embodiment quantitative detection of the ligated sequence which is relevant to a chromosome interaction is carried out using a probe which is detectable upon activation during a PCR reaction, wherein said ligated sequence comprises sequences from two chromosome regions that come together

in an epigenetic chromosome interaction, wherein said method comprises contacting the ligated sequence with the probe during a PCR reaction, and detecting the extent of activation of the probe, and wherein said probe binds the ligation site. The method typically allows particular interactions to be detected in a MIQE compliant manner using a dual labelled fluorescent hydrolysis probe.

The probe is generally labelled with a detectable label which has an inactive and active state, so that it is only detected when activated. The extent of activation will be related to the extent of template (ligation product) present in the PCR reaction. Detection may be carried out during all or some of the PCR, for example for at least 50% or 80% of the cycles of the PCR.

The probe can comprise a fluorophore covalently attached to one end of the oligonucleotide, and a quencher attached to the other end of the nucleotide, so that the fluorescence of the fluorophore is quenched by the quencher. In one embodiment the fluorophore is attached to the 5'end of the oligonucleotide, and the quencher is covalently attached to the 3' end of the oligonucleotide. Fluorophores that can be used in the methods of the invention include FAM, TET, JOE, Yakima Yellow, HEX, Cyanine3, ATTO 550, TAMRA, ROX, Texas Red, Cyanine 3.5, LC610, LC 640, ATTO 647N, Cyanine 5, Cyanine 5.5 and ATTO 680. Quenchers that can be used with the appropriate fluorophore include TAM, BHQ1, DAB, Eclip, BHQ2 and BBQ650, optionally wherein said fluorophore is selected from HEX, Texas Red and FAM. Preferred combinations of fluorophore and quencher include FAM with BHQ1 and Texas Red with BHQ2.

Use of the Probe in a qPCR Assay

Hydrolysis probes of the invention are typically temperature gradient optimised with concentration matched negative controls. Preferably single-step PCR reactions are optimized. More preferably a standard curve is calculated. An advantage of using a specific probe that binds across the junction of the ligated sequence is that specificity for the ligated sequence can be achieved without using a nested PCR approach. The methods described herein allow accurate and precise quantification of low copy number targets. The target ligated sequence can be purified, for example gel-purified, prior to temperature gradient optimization. The target ligated sequence can be sequenced. Preferably PCR reactions are performed using about 10ng, or 5 to 15 ng, or 10 to 20ng, or 10 to 50ng, or 10 to 200ng template DNA. Forward and reverse primers are designed such that one primer binds to the sequence of one of the chromosome regions represented in the ligated DNA sequence, and the other primer binds to other chromosome region represented in the ligated DNA sequence, for example, by being complementary to the sequence.

Choice of Ligated DNA Target

The invention includes selecting primers and a probe for use in a PCR method as defined herein comprising selecting primers based on their ability to bind and amplify the ligated sequence and

selecting the probe sequence based properties of the target sequence to which it will bind, in particular the curvature of the target sequence.

Probes are typically designed/chosen to bind to ligated sequences which are juxtaposed restriction fragments spanning the restriction site. In one embodiment of the invention, the predicted curvature of possible ligated sequences relevant to a particular chromosome interaction is calculated, for example using a specific algorithm referenced herein. The curvature can be expressed as degrees per helical turn, e.g. 10.5° per helical turn. Ligated sequences are selected for targeting where the ligated sequence has a curvature propensity peak score of at least 5° per helical turn, typically at least 10°, 15° or 20° per helical turn, for example 5° to 20° per helical turn. Preferably the curvature propensity score per helical turn is calculated for at least 20, 50, 100, 200 or 400 bases, such as for 20 to 400 bases upstream and/or downstream of the ligation site. Thus in one embodiment the target sequence in the ligated product has any of these levels of curvature. Target sequences can also be chosen based on lowest thermodynamic structure free energy.

Particular Embodiments

In particular embodiments chromosome interactions in IGFBP3 are not typed/detected.

In certain embodiments chromosome interactions in any of the genes mentioned herein are not typed/detected. In one embodiment any of the following genes are not typed/detected:

BCAS1, ZNF217,TSHZ2, SUMO1P1, MIR4756, BCAS3, TBX2, C17orf82, TBX4, BCA54, LINC00651, UBE2V1, TMEM189, CEBPB, LOC284751, PTPNI, MIR645, FAM65C PARD68, ADNP, LINC00494, PREX1, ARFGEF2, CSE1L, PDE4DIP, SEC22B, NOTCH2NL NBP10, HFE2, TXNIP, POLR3GL, ANKRD34A, LIX1L, RBM8A, GNRHR2, PEX11B, ITGA10, ANKRD35, PIAS3, NUDTI7, POLR3C, RNF115, CD160, PDZK1, GPR89A, ZNF334. OCSTAMP, SLC13A3, TP53RK, SLC2A10, EYA2, MIR3616, ZMYND8, L0C100131496, DLG1, MIR4797, DLG1-AS1, BDH1, LOC220729, KIAA0226, MIR922, FYTTDl , LRCH3, IQCG, RPL35A, LMLN, ANKRD18DP, DDX59, CAMSAP2, GPR25, C1orf106, KIF21B, CACNA15, ASCLS, TMEM9, IGFN1, PKP1, TNN2, LAD1, TNNI1, PHLDA3, NCOA1, PTRHD1, CENPO, ADCY3, DNAJC27, DNAJC27-AS1, EFR3B. POMC, DNMT3A, MIR1301, DTNB, SPON2, LOC100130872, CTBP1, CTBP1-AS1, MAEA, UVSSA, CRIPAK, FAM53A, SLBP, TMEM129, TACC3, FGFR3, LETM1, WHSC1, SCARNA22, WHSC2, MIR943, C4orf48, NAT8L, POLN, HAUS3, MXD4, MIR4800, ZFYVE28, LOC402160, RNF4, LOC1OO506190, C9orf50, NTMT1, ASB6, PRRX2, PTGES, TOR1B, TOR1A, C9orf78, USP20, FNBP1, GPR107, NC51, ASS1.

In one embodiment only intrachromosomal interactions are typed/detected, and no extrachromosomal interactions (between different chromosomes) are typed/detected.

Publications

The contents of all publications mentioned herein are incorporated by reference into the present specification and may be used to further define the features relevant to the invention.

Specific Embodiments

The EpiSwitch™ platform technology detects epigenetic regulatory signatures of regulatory changes between normal and abnormal conditions at loci. The EpiSwitch™ platform identifies and monitors the fundamental epigenetic level of gene regulation associated with regulatory high order structures of human chromosomes also known as chromosome conformation signatures. Chromosome signatures are a distinct primary step in a cascade of gene deregulation. They are high order biomarkers with a unique set of advantages against biomarker platforms that utilize late epigenetic and gene expression biomarkers, such as DNA methylation and RNA profiling.

EpiSwitch™ Array Assay

The custom EpiSwitch™ array-screening platforms come in 4 densities of, 15K, 45K, 100K, and 250K unique chromosome conformations, each chimeric fragment is repeated on the arrays 4 times, making the effective densities 60K, 180K, 400K and 1 Million respectively.

Custom Designed EpiSwitch™ Arrays

The 15K EpiSwitch™ array can screen the whole genome including around 300 loci interrogated with the EpiSwitch™ Biomarker discovery technology. The EpiSwitch™ array is built on the Agilent SurePrint G3 Custom CGH microarray platform; this technology offers 4 densities, 60K, 180K, 400K and 1 Million probes. The density per array is reduced to 15K, 45K, 100K and 250K as each EpiSwitch™ probe is presented as a quadruplicate, thus allowing for statistical evaluation of the reproducibility. The average number of potential EpiSwitch™ markers interrogated per genetic loci is 50; as such the numbers of loci that can be investigated are 300, 900, 2000, and 5000.

EpiSwitch™ Custom Array Pipeline

The EpiSwitch™ array is a dual colour system with one set of samples, after EpiSwitch™ library generation, labelled in Cy5 and the other of sample (controls) to be compared/ analyzed labelled in Cy3. The arrays are scanned using the Agilent SureScan Scanner and the resultant features extracted using the Agilent Feature Extraction software. The data is then processed using the EpiSwitch™ array processing scripts in R. The arrays are processed using standard dual colour packages in Bioconductor in R: Limma *. The normalisation of the arrays is done using the normalisedWithinArrays function in Limma * and this is done to the on chip Agilent positive controls and EpiSwitch™ positive controls. The data is filtered based on the

Agilent Flag calls, the Agilent control probes are removed and the technical replicate probes are averaged, in order for them to be analysed using Limma *. The probes are modelled based on their difference between the 2 scenarios being compared and then corrected by using False Discovery Rate. Probes with Coefficient of Variation (CV) <=30% that are <=-1.1 or =>1.1 and pass the p<=0.1 FDR p-value are used for further screening. To reduce the probe set further Multiple Factor Analysis is performed using the FactorMineR package in R.

* Note: LIMMA is Linear Models and Empirical Bayes Processes for Assessing Differential Expression in Microarray Experiments. Limma is an R package for the analysis of gene expression data arising from microarray or RNA-Seq.

The pool of probes is initially selected based on adjusted p-value, FC and CV <30% (arbitrary cut off point) parameters for final picking. Further analyses and the final list are drawn based only on the first two parameters (adj. p-value; FC).

Genes Mentioned Herein

TSPYL5 – TSPY-like 5

SRD5A1 - steroid 5 alpha-reductase 1

MAP3K1 - mitogen-activated protein kinase kinase kinase 1

VAV3 - vav guanine nucleotide exchange factor 3

ATM - ATM serine/threonine kinase

SLC16A10 - solute carrier family 16 member 10

ME3 - malic enzyme 3

The invention is illustrated by the following non-limiting examples.

Example 1

Statistical Pipeline

EpiSwitch™ screening arrays are processed using the EpiSwitch™ Analytical Package in R in order to select high value EpiSwitch™ markers for translation on to the EpiSwitch™ PCR platform.

Step 1

Probes are selected based on their corrected p-value (False Discovery Rate, FDR), which is the product of a modified linear regression model. Probes below p-value <= 0.1 are selected and then further reduced

by their Epigenetic ratio (ER), probes ER have to be <=-1.1 or =>1.1 in order to be selected for further analysis. The last filter is a coefficient of variation (CV), probes have to be below <=0.3.

Step 2

The top 40 markers from the statistical lists are selected based on their ER for selection as markers for PCR translation. The top 20 markers with the highest negative ER load and the top 20 markers with the highest positive ER load form the list.

Step 3

The resultant markers from step 1, the statistically significant probes form the bases of enrichment analysis using hypergeometric enrichment (HE). This analysis enables marker reduction from the significant probe list, and along with the markers from step 2 forms the list of probes translated on to the EpiSwitch™ PCR platform.

The statistical probes are processed by HE to determine which genetic locations have an enrichment of statistically significant probes, indicating which genetic locations are hubs of epigenetic difference.

The most significant enriched loci based on a corrected p-value are selected for probe list generation. Genetic locations below p-value of 0.3 or 0.2 are selected. The statistical probes mapping to these genetic locations, with the markers from step 2, form the high value markers for EpiSwitch™ PCR translation.

Array design and processing

Array Design

1. Genetic loci are processed using the SII software (currently v3.2) to:
    a. Pull out the sequence of the genome at these specific genetic loci (gene sequence with 50kb upstream and 20kb downstream)
    b. Define the probability that a sequence within this region is involved in CCs
    c. Cut the sequence using a specific RE
    d. Determine which restriction fragments are likely to interact in a certain orientation
    e. Rank the likelihood of different CCs interacting together.
2. Determine array size and therefore number of probe positions available (x)
3. Pull out x/4 interactions.
4. For each interaction define sequence of 30bp to restriction site from part 1 and 30bp to restriction site of part 2. Check those regions aren't repeats, if so exclude and take next interaction down on the list. Join both 30bp to define probe.
5. Create list of x/4 probes plus defined control probes and replicate 4 times to create list to be created on array

6. Upload list of probes onto Agilent Sure design website for custom CGH array.
7. Use probe group to design Agilent custom CGH array.

Array Processing

1. Process samples using EpiSwitch™ Standard Operating Procedure (SOP) for template production.
2. Clean up with ethanol precipitation by array processing laboratory.
3. Process samples as per Agilent SureTag complete DNA labelling kit - Agilent Oligonucleotide Array-based CGH for Genomic DNA Analysis Enzymatic labelling for Blood, Cells or Tissues
4. Scan using Agilent C Scanner using Agilent feature extraction software.

Breast cancer overview

Age-specific incidence rates indicate that the age effects for invasive breast cancer are more similar among Asian and Western populations. In fact, the Asian breast cancer rates in recent generations are even surpassing the historically high rates in the United States, highlighting an urgent need for efficient prevention and treatment strategies among Asian populations. However, the results of a large scale 25 year study showed that mammography did not reduce breast cancer associated mortality. Early detection of breast cancer, before tumours become macroscopic, would mean that medical intervention could initiate at a stage when the cancer is more treatable.

***EpiSwitch*™ technology overview**

The *EpiSwitch*™ platform offers a highly effective means of screening, early detection, companion-diagnosis, monitoring and prognostic analysis of major diseases associated with aberrant and responsive gene expression. The major advantages of this approach is that it is non-invasive, rapid, and relies on highly stable DNA based targets as part of chromosomal signatures, rather than unstable protein/RNA molecules.

EpiSwitch™ biomarker signatures demonstrate high robustness, sensitivity and specificity in the stratification of complex disease phenotypes. This technology takes advantage of the latest breakthroughs in the science of epigenetics, monitoring and evaluation of chromosome conformation signatures as a highly informative class of epigenetic biomarkers. Current research methodologies deployed in academic environment require from 3 to 7 days for biochemical processing of cellular material in order to detect CCSs. Those procedures have limited sensitivity, and reproducibility; and furthermore, do not have the benefit of the targeted insight provided by the *EpiSwitch*™ *Analytical Package* at the design stage.

EpiSwitch™ Analytical Package

The EpiSwitch™ platform technology detects changes in the higher order structure of human chromosomes as part of the main epigenetic framework of regulation. Juxtaposing distant sites in the chromosome forms a specific type of biomarker - regulatory chromosome conformation signatures. One of the biggest challenges in this process is to identify the potential sites in the gene/loci in the chromosome that forms part of the higher order structure. This is performed by use of proprietary pattern recognition software that identifies the potential sites within a given sequence. The *EpiSwitch™ Analytical Package* software, which includes a machine-learning algorithm, identifies patterns in DNA that are likely to form higher order structures of CCSs.

EpiSwitch™ Array *in silico* marker identification

CCS sites across the genome are directly evaluated by the EpiSwitch™ Array on clinical samples from testing cohorts for identification of all relevant stratifying lead biomarkers. The EpiSwitch™ Array platform is used for marker identification due to its high-throughput capacity, and its ability to screen large numbers of loci rapidly. The array used was the Agilent custom-CGH array, which allows markers identified through the *in silico* software to be interrogated.

EpiSwitch™ PCR

Potential markers identified by *EpiSwitch™ Array* are then validated either by *EpiSwitch™ PCR* or DNA sequencers (i.e. Roche 454, Nanopore MinION, etc.). The top PCR markers which are statistically significant and display the best reproducibility are selected for further reduction into the final *EpiSwitch™* Signature Set, and validated on an independent cohort of samples. *EpiSwitch™ PCR* can be performed by a trained technician following a standardised operating procedure protocol established. All protocols and manufacture of reagents are performed under ISO 13485 and 9001 accreditation to ensure the quality of the work and the ability to transfer the protocols. *EpiSwitch™ PCR* and *EpiSwitch™ Array* biomarker platforms are compatible with analysis of both whole blood and cell lines. The tests are sensitive enough to detect abnormalities in very low copy numbers using small volumes of blood.

Summary

The inventors have used epigenetic chromosomal interactions as the basis for identifying biomarkers to be used as a companion diagnostic method in breast cancer diagnosis. The *EpiSwitch™* biomarker discovery platform was developed by the inventors to detect epigenetic regulatory signature changes such as those driving phenotypic changes implicated in breast cancer. The *EpiSwitch™* biomarker discovery platform identifies CCSs which define the initial regulatory process in integrating environmental cues into the epigenetic and transcriptional machinery. As such, CCSs are the primary step in a cascade of gene regulation. The CCSs isolated by the *EpiSwitch™* biomarker discovery platform

have several well documented advantages: severe biochemical and physiological stability; their binary nature and readout; and their primary position in the eukaryotic cascade of gene regulation.

The *EpiSwitch*™ *Array* screening platform was applied in this invention and its results translated onto the *EpiSwitch*™ *PCR* platform to meet the following aims:

1. Identify *EpiSwitch*™ markers that differentiate patients with breast cancer from healthy individuals;
2. Identify *EpiSwitch*™ markers that can be developed into a test that offers standards of sensitivity, specificity or positive predictive value (PPV), in relation to current existing clinical practice.

In this breast cancer biomarker discovery project an 8x60k array was utilised, which allows for the study of up to 56,964 potential chromosome conformations in quadruplicates. Two arrays were produced using eight stage II/III breast cancer patient samples from a range of backgrounds individually tested against eight pooled healthy control patient samples. Each array contained 56,964 *EpiSwitch*™ probes. The *EpiSwitch*™ template was prepared for each of the samples. The first array was carried out on Asian breast cancer samples. The second array used European and Asian samples. Asian and European breast cancers can differ between ER+ and ER- status. Overlapping probes were found for similar cancers in multiple populations. Each of the probes were then tested for statistical quality of the data, and then analysed as described subsequently.

Blood Sample Quality Control Results

The samples used in the study were from Malaysia. Biochemical quality of blood samples suitable for the *EpiSwitch*™ *Assay* is directly affected by the extent of sample oxidation and protein denaturation, as exemplified by haemoglobin. These two parameters are the standard means of assessing blood quality prior to sample processing. Briefly, when oxygenated haemoglobin (oxyhaemoglobin) is oxidized methaemoglobin is formed, and if the globin domains are denatured methaemoglobin is converted to hemichrome. Spectral changes were used to calculate the abundance of each fraction by the quality control method described by Winterbourn (1990), Oxidative reactions of hemoglobin. Methods Enzymol. 1990; 186: 265-72, which is based on the extinction coefficient of each haemoglobin fraction. In accordance with this document, as part of the quality control for each sample, blood was diluted in PBS and analysed on a spectrophotometer (Epoch Microplate (BioTek)) at 560, 577 and 630 nm. The micromolar concentrations of each of the three haemoglobin fractions were monitored according to standard calculations: µM oxyhaemoglobin = $119*A_{577} - 39*A_{630} - 89*A_{560}$, µM methaemoglobin = $28*A_{577} + 307*A_{630} - 55*A_{560}$, µM hemichrome = $- 133*A_{577} - 114*A_{630} + 233*A_{560}$. Samples that demonstrated an oxyhaemoglobin:methaemoglobin ratio ≥ 0.75 were cleared on quality control and considered suitable for *EpiSwitch™* processing. 11 samples failed haemoglobin QC (samples BrCaMa132, BrCaMa136, BrCaMa137, BrCaMa147, BrCaMa164, BrCaMa165, BrCaMa166, BrCaMa167, BrCaMa168,

BrCaMa169, and BrCaMa170) and were excluded on the basis of their biochemical state of oxidation and denaturation.

Sample ID	Client ID	Basic annotation	OD 577nm	OD 630nm	OD 560nm	QC: nM oxYes-Hb est	QC: µM met-Hb est	QC: oxYes/met-Hb ratio	Development Cohort	Independent Validation Cohort	QC: Outliers
BrCaMa- 002	022 - Site 01	BrCa Control	1.924	0.296	1.243	1067.85	763.79	1.398	Yes		
BrCaMa- 005	013 - Site 01	BrCa Control	2.602	0.205	1.572	1617.35	493.31	3.279	Yes		
BrCaMa- 006	024 - Site 01	BrCa Control	1.887	0.269	1.204	1069.06	691.99	1.545	Yes		
BrCaMa- 007	015 - Site 01	BrCa Control	2.384	0.196	1.44	1478.92	477.24	3.099	Yes		
BrCaMa- 012	019 - Site 01	BrCa Control	1.861	0.341	1.232	985.12	890.35	1.106	Yes		
BrCaMa- 014	020 - Site 01	BrCa Control	1.851	0.284	1.192	1031.05	734.56	1.404	Yes		
BrCaMa- 015	008 - Site 01	BrCa Control	2.752	0.25	1.681	1681.29	613.51	2.74	Yes		
BrCaMa- 016	007 - Site 01	BrCa Control	2.56	0.225	1.557	1572.92	551.2	2.854		Yes	
BrCaMa- 017	009 - Site 01	BrCa Control	2.492	0.203	1.504	1547.75	493.77	3.135		Yes	
BrCaMa- 018	010 - Site 01	BrCa Control	2.246	0.182	1.353	1397.59	443.47	3.151		Yes	
BrCaMa- 019	038 - Site 01	BrCa	1.756	0.326	1.158	931.88	855.6	1.089	Yes		
BrCaMa- 020	037 - Site 01	BrCa	1.68	0.317	1.114	884.11	830.89	1.064	Yes		
BrCaMa- 021	031 - Site 01	BrCa Control	2.074	0.271	1.318	1189.35	687.79	1.729		Yes	
BrCaMa- 022	032 - Site 01	BrCa Control	2.767	0.216	1.67	1722.19	519.38	3.316		Yes	
BrCaMa- 023	030 - Site 01	BrCa Control	1.566	0.235	1	881.89	609.93	1.446		Yes	
BrCaMa- 024	035 - Site 01	BrCa	2.18	0.32	1.402	1221.62	821.7	1.487	Yes		
BrCaMa- 025	036 - Site 01	BrCa	1.844	0.326	1.212	988.54	850.54	1.162	Yes		
BrCaMa- 026	034 - Site 01	BrCa Control	2.021	0.391	1.351	1050.11	1023.2	1.026		Yes	
BrCaMa- 027	033 - Site 01	BrCa Control	1.761	0.208	1.101	1034.58	526.09	1.967		Yes	
BrCaMa- 028	026 - Site 01	BrCa Control	1.485	0.32	1.003	749.68	846.55	0.886	Yes		
BrCaMa- 029	025 - Site 01	BrCa Control	1.595	0.234	1.022	897.21	602.88	1.488	Yes		
BrCaMa- 030	027 - Site 01	BrCa Control	2.044	0.326	1.321	1129.53	846.59	1.334	Yes		
BrCaMa- 031	029 - Site 01	BrCa Control	1.944	0.379	1.296	1012.11	995.05	1.017	Yes		
BrCaMa- 032	028 - Site 01	BrCa	1.634	0.26	1.056	903.22	674.92	1.338		Yes	
BrCaMa- 036	005 - Site 01	BrCa	2.678	0.241	1.629	1643.02	593.76	2.767		Yes	
BrCaMa- 039	001 - Site 04	BrCa	1.986	0.346	1.304	1067.84	901.1	1.185	Yes		
BrCaMa- 040	002 - Site 04	BrCa	1.635	0.245	1.056	910.26	629.15	1.447	Yes		
BrCaMa- 041	003 - Site 04	BrCa	1.847	0.365	1.235	956.43	958.46	0.998	Yes		
BrCaMa- 042	007 - Site 04	BrCa	1.874	0.28	1.213	1041.29	717.17	1.452	Yes		
BrCaMa- 043	004 - Site 04	BrCa Control	2.452	0.208	1.494	1507.1	503.42	2.994	Yes		
BrCaMa- 044	005 - Site 04	BrCa Control	1.802	0.424	1.256	861.18	1115.44	0.772	Yes		
BrCaMa- 045	006 - Site 04	BrCa Control	1.733	0.376	1.18	865.43	990.56	0.874	Yes		
BrCaMa- 046	008 - Site 04	BrCa Control	1.888	0.329	1.241	1013.92	856.12	1.184	Yes		

BrCaMa- 050	039 - Site 01	BrCa	1.537	0.312	1.034	787.09	819.5	0.96		Yes	
BrCaMa- 051	040 - Site 01	BrCa	1.951	0.316	1.265	1072.6	820.65	1.307		Yes	
BrCaMa- 055	044 - Site 01	BrCa Control	2.984	0.217	1.803	1861.66	510.06	3.65	Yes		
BrCaMa- 056	045 - Site 01	BrCa Control	2.545	0.235	1.555	1552.95	578.8	2.683	Yes		
BrCaMa- 057	046 - Site 01	BrCa Control	2.455	0.202	1.484	1521.91	491.34	3.097	Yes		
BrCaMa- 058	047 - Site 01	BrCa Control	1.507	0.292	1.003	786.78	766.75	1.026	Yes		
BrCaMa- 059	048 - Site 01	BrCa Control	2.69	0.236	1.638	1651.24	576.82	2.863	Yes		
BrCaMa- 060	049 - Site 01	BrCa	1.863	0.341	1.238	982.16	887.61	1.107		Yes	
BrCaMa- 061	050 - Site 01	BrCa	2.119	0.36	1.397	1137.88	930.17	1.223		Yes	
BrCaMa- 062	051 - Site 01	BrCa	1.752	0.334	1.165	917.77	875.19	1.049		Yes	
BrCaMa- 063	052 - Site 01	BrCa	1.758	0.331	1.169	922.52	865.46	1.066		Yes	
BrCaMa- 064	053 - Site 01	BrCa	1.898	0.297	1.222	1055.21	771.13	1.368		Yes	
BrCaMa- 065	054 - Site 01	BrCa	1.599	0.227	1.02	906.48	583.61	1.553		Yes	
BrCaMa- 066	055 - Site 01	BrCa	1.5	0.286	0.996	787.02	750.22	1.049	Yes		
BrCaMa- 067	001 - Site 02	BrCa	1.821	0.36	1.227	934.56	940.23	0.994	Yes		
BrCaMa- 068	002 - Site 02	BrCa	2.476	0.231	1.51	1512.45	571.95	2.644	Yes		
BrCaMa- 069	003 - Site 02	BrCa	2.461	0.452	1.644	1289.15	1172.52	1.099	Yes		
BrCaMa- 070	004 - Site 02	BrCa	2.156	0.377	1.425	1150.36	977.32	1.177	Yes		
BrCaMa- 071	005 - Site 02	BrCa	1.898	0.346	1.262	1000.5	899.56	1.112	Yes		
BrCaMa- 072	006 - Site 02	BrCa	1.807	0.411	1.24	886.44	1085.73	0.816	Yes		
BrCaMa- 073	007 - Site 02	BrCa	1.839	0.378	1.247	931.16	989.53	0.941	Yes		
BrCaMa- 074	008 - Site 02	BrCa	1.767	0.317	1.176	932.46	821.15	1.136	Yes		
BrCaMa- 075	009 - Site 02	BrCa	2.141	0.343	1.398	1169.8	883.59	1.324		Yes	
BrCaMa- 076	010 - Site 02	BrCa	2.148	0.409	1.438	1116.79	1066.17	1.047		Yes	
BrCaMa- 077	011 - Site 02	BrCa	1.721	0.344	1.156	884.99	902.16	0.981		Yes	
BrCaMa- 078	012 - Site 02	BrCa	2.142	0.448	1.454	1080.2	1175.42	0.919		Yes	
BrCaMa- 079	013 - Site 02	BrCa	1.888	0.281	1.213	1057.56	724.16	1.46		Yes	
BrCaMa- 080	014 - Site 02	BrCa	1.97	0.301	1.264	1101.95	780.47	1.412		Yes	
BrCaMa- 081	015 - Site 02	BrCa	2.128	0.312	1.373	1188.67	798.53	1.489	Yes		
BrCaMa- 082	016 - Site 02	BrCa	1.978	0.332	1.303	1064.67	856.43	1.243	Yes		
BrCaMa- 083	017 - Site 02	BrCa	2.025	0.389	1.357	1050.31	1014.88	1.035	Yes		
BrCaMa- 084	018 - Site 02	BrCa	2.048	0.362	1.358	1087.32	937.88	1.159	Yes		
BrCaMa- 085	019 - Site 02	BrCa	2.216	0.388	1.457	1188.99	1010.29	1.177	Yes		
BrCaMa- 086	020 - Site 02	BrCa	1.549	0.354	1.066	756.51	934.2	0.81	Yes		
BrCaMa- 087	021 - Site 02	BrCa	2.064	0.366	1.363	1100.35	951.89	1.156	Yes		
BrCaMa- 088	022 - Site 02	BrCa	1.734	0.362	1.172	879.2	952.26	0.923	Yes		
BrCaMa- 089	023 - Site 02	BrCa	2.063	0.431	1.393	1047.11	1134.66	0.923	Yes		
BrCaMa- 090	024 - Site 02	BrCa	1.966	0.429	1.349	971.62	1125.56	0.863	Yes		
BrCaMa- 091	025 - Site 02	BrCa	1.697	0.309	1.124	898.56	805.59	1.115	Yes		
BrCaMa- 093	001 - Site 02	BrCa Control	1.539	0.295	1.025	804.11	772.82	1.04	Yes		
BrCaMa- 094	002 - Site 02	BrCa Control	1.634	0.311	1.089	853.96	813.34	1.05	Yes		

BrCaMa- 095	003 - Site 02	BrCa Control	1.381	0.313	0.947	678.49	826.74	0.821		Yes	
BrCaMa- 096	004 - Site 02	BrCa Control	1.545	0.286	1.021	818.32	749.07	1.092		Yes	
BrCaMa- 097	005 - Site 02	BrCa Control	1.817	0.297	1.177	998.87	773.2	1.292		Yes	
BrCaMa- 098	006 - Site 02	BrCa Control	1.753	0.375	1.194	877.16	985.39	0.89		Yes	
BrCaMa- 099	007 - Site 02	BrCa Control	2.129	0.336	1.381	1173.38	868.09	1.352		Yes	
BrCaMa- 100	008 - Site 02	BrCa Control	1.693	0.274	1.102	927.03	709.12	1.307		Yes	
BrCaMa- 101	009 - Site 02	BrCa Control	0.988	0.147	0.634	554.13	379.23	1.461		Yes	
BrCaMa- 102	010 - Site 02	BrCa Control	2.017	0.274	1.279	1155.06	702.49	1.644		Yes	
BrCaMa- 103	011 - Site 02	BrCa Control	2.011	0.287	1.283	1139.29	738.52	1.543	Yes		
BrCaMa- 104	012 - Site 02	BrCa Control	1.853	0.356	1.231	970.64	934.71	1.038	Yes		
BrCaMa- 105	013 - Site 02	BrCa Control	1.802	0.379	1.214	916.11	1000.39	0.916	Yes		
BrCaMa- 106	014 - Site 02	BrCa Control	1.943	0.271	1.244	1099.32	691.81	1.589	Yes		
BrCaMa- 107	015 - Site 02	BrCa Control	1.933	0.288	1.248	1077.23	739	1.458	Yes		
BrCaMa- 108	016 - Site 02	BrCa Control	1.774	0.374	1.209	889.19	979.95	0.907	Yes		
BrCaMa- 109	017 - Site 02	BrCa Control	1.774	0.316	1.166	950.08	825.54	1.151	Yes		
BrCaMa- 110	018 - Site 02	BrCa Control	1.639	0.31	1.087	862.08	812.77	1.061	Yes		
BrCaMa- 111	019 - Site 02	BrCa Control	2.169	0.399	1.435	1148.35	1043	1.101	Yes		
BrCaMa- 112	020 - Site 02	BrCa Control	2.017	0.359	1.324	1081.86	938.69	1.153	Yes		
BrCaMa- 113	021 - Site 02	BrCa Control	1.817	0.323	1.189	978.05	846.42	1.156	Yes		
BrCaMa- 114	022 - Site 02	BrCa Control	1.716	0.296	1.13	920.9	767.7	1.2	Yes		
BrCaMa- 115	023 - Site 02	BrCa Control	1.609	0.242	1.038	896.51	622.56	1.44	Yes		
BrCaMa- 116	024 - Site 02	BrCa Control	2.054	0.348	1.349	1107.93	901.53	1.229	Yes		
BrCaMa- 117	025 - Site 02	BrCa Control	1.956	0.362	1.304	1025.9	941.82	1.089	Yes		
BrCaMa- 118	056 - Site 01	BrCa	1.153	0.203	0.755	620.95	530.8	1.17	Yes		
BrCaMa- 119	057 - Site 01	BrCa	1.79	0.326	1.185	948.31	850.27	1.115	Yes		
BrCaMa- 120	058 - Site 01	BrCa	1.383	0.286	0.933	703.86	752.11	0.936	Yes		
BrCaMa- 121	059 - Site 01	BrCa	1.043	0.171	0.672	576.4	447.41	1.288	Yes		
BrCaMa- 122	060 - Site 01	BrCa	1.834	0.316	1.198	993	824.74	1.204	Yes		
BrCaMa- 123	061 - Site 01	BrCa	1.782	0.315	1.168	958.21	823.61	1.163	Yes		
BrCaMa- 124	062 - Site 01	BrCa	1.166	0.184	0.754	644.72	476.66	1.353	Yes		
BrCaMa- 125	063 - Site 01	BrCa	1.638	0.293	1.075	878.2	766.9	1.145	Yes		
BrCaMa- 126	064 - Site 01	BrCa	1.542	0.274	1.011	828.33	716.89	1.155	Yes		
BrCaMa- 127	065 - Site 01	BrCa	1.349	0.352	0.943	628.76	939.71	0.669		Yes	
BrCaMa- 129	009 - Site 04	BrCa	1.727	0.31	1.139	920.52	808.81	1.138		Yes	
BrCaMa- 130	010 - Site 04	BrCa Control	2.016	0.303	1.308	1116.75	775.29	1.44		Yes	
BrCaMa- 131	001 - Site 03	BrCa	2.063	0.379	1.386	1073.62	978.87	1.097	Yes		
BrCaMa- 132	002 - Site 03	BrCa Control	1.719	0.428	1.211	800.9	1129.23	0.709			
BrCaMa- 133	003 - Site 03	BrCa Control	1.825	0.322	1.221	959.48	827.99	1.159	Yes		
BrCaMa- 134	004 - Site 03	BrCa	1.855	0.383	1.256	940.24	1004.41	0.936		Yes	
BrCaMa- 135	005 - Site 03	BrCa Control	2.024	0.402	1.365	1036.93	1050.11	0.987	Yes		
BrCaMa- 136	Site 3_006	BrCa	0.134	0.11	0.136	-4.48	300.42	-0.015			

BrCaMa- 137	Site 3_007	BrCa	0.139	0.116	0.141	-5.32	317.49	-0.017			
BrCaMa- 138	008 - Site 03	BrCa Control	1.308	0.514	1.021	447.37	1382.67	0.324		Yes	
BrCaMa- 139	009 - Site 03	BrCa Control	1.595	0.532	1.188	633.25	1426.44	0.444		Yes	
BrCaMa- 140	010 - Site 03	BrCa	0.217	0.069	0.155	93.37	187.34	0.498		Yes	
BrCaMa- 141	026 - Site 02	BrCa	1.715	0.397	1.182	834.04	1048.89	0.795	Yes		
BrCaMa- 142	027 - Site 02	BrCa	1.626	0.276	1.063	881.23	717.95	1.227	Yes		
BrCaMa- 143	028 - Site 02	BrCa	0.218	0.062	0.153	99.07	167.23	0.592	Yes		
BrCaMa- 144	029 - Site 02	BrCa	1.704	0.341	1.142	878.39	895.89	0.98	Yes		
BrCaMa- 145	030 - Site 02	BrCa	1.721	0.311	1.143	909.43	808	1.126	Yes		
BrCaMa- 146	031 - Site 02	BrCa	1.96	0.385	1.317	1010.12	1006.4	1.004	Yes		
BrCaMa- 147	032 - Site 02	BrCa	0.227	0.071	0.162	98.26	192.43	0.511			
BrCaMa- 148	033 - Site 02	BrCa	1.502	0.317	1.018	757.73	833.85	0.909	Yes		
BrCaMa- 149	035 - Site 02	BrCa	1.7	0.336	1.141	876.47	879.97	0.996	Yes		
BrCaMa- 150	036 - Site 02	BrCa	1.955	0.339	1.283	1052.37	882.48	1.193	Yes		
BrCaMa- 151	037 - Site 02	BrCa	1.66	0.333	1.112	855.85	875.51	0.978	Yes		
BrCaMa- 152	038 - Site 02	BrCa	2.434	0.491	1.637	1248.04	1288.54	0.969	Yes		
BrCaMa- 153	039 - Site 02	BrCa	1.77	0.324	1.175	934.19	844.03	1.107	Yes		
BrCaMa- 154	040 - Site 02	BrCa	1.871	0.35	1.247	980.16	912.53	1.074	Yes		
BrCaMa- 155	041 - Site 02	BrCa	1.694	0.363	1.159	842.78	951.28	0.886	Yes		
BrCaMa- 156	042 - Site 02	BrCa	1.828	0.371	1.231	935.04	973.76	0.96	Yes		
BrCaMa- 157	043 - Site 02	BrCa	1.75	0.337	1.17	909.77	881.09	1.033	Yes		
BrCaMa- 158	044 - Site 02	BrCa	1.851	0.356	1.238	962.03	930.3	1.034	Yes		
BrCaMa- 159	045 - Site 02	BrCa	1.77	0.321	1.171	938.92	837.02	1.122	Yes		
BrCaMa- 160	046 - Site 02	BrCa	1.67	0.347	1.126	849.83	913.59	0.93	Yes		
BrCaMa- 161	047 - Site 02	BrCa	0.18	0.06	0.131	74.21	162.55	0.457		Yes	
BrCaMa- 162	048 - Site 02	BrCa	1.846	0.393	1.257	924.74	1032.04	0.896	Yes		
BrCaMa- 163	049 - Site 02	BrCa	1.63	0.436	1.161	736.37	1156.37	0.637		Yes	
BrCaMa- 164	050 - Site 02	BrCa	1.08	0.75	1.028	77.78	2039.5	0.038		Yes	
BrCaMa- 165	Site 2_051	BrCa	0.135	0.098	0.125	11.18	269.91	0.041			
BrCaMa- 166	Site 2_052	BrCa	0.578	0.388	0.567	31.87	1041.15	0.031			
BrCaMa- 167	053 - Site 02	BrCa	1.021	0.692	0.974	78.25	1874.62	0.042		Yes	
BrCaMa- 168	054 - Site 02	BrCa	0.899	0.63	0.849	68.5	1718.87	0.04		Yes	
BrCaMa- 169	055 - Site 02	BrCa	1.799	1.162	1.754	126.57	3106.36	0.041		Yes	
BrCaMa- 170	056 - Site 02	BrCa	1.497	0.939	1.431	141.63	2514.84	0.056		Yes	
BrCaMa- 171	026 - Site 02	BrCa Control	1.495	0.373	1.048	700.86	987.31	0.71			Yes
BrCaMa- 172	027 - Site 02	BrCa Control	1.92	0.448	1.328	928.16	1182.56	0.785		Yes	Yes
BrCaMa- 173	028 - Site 02	BrCa Control	2.1	0.392	1.392	1107.24	1025.84	1.079		Yes	Yes
BrCaMa- 174	029 - Site 02	BrCa Control	2.125	0.335	1.377	1172.57	866.1	1.354			Yes
BrCaMa- 175	030 - Site 02	BrCa Control	1.935	0.41	1.313	974.18	1078.35	0.903			Yes
BrCaMa- 176	031 - Site 02	BrCa Control	1.812	0.314	1.186	978.28	819.04	1.194		Yes	Yes
BrCaMa- 177	032 - Site 02	BrCa Control	1.821	0.344	1.213	953.26	898.81	1.061		Yes	Yes

BrCaMa- 178	033 - Site 02	BrCa Control	1.617	0.324	1.08	836.67	853.44	0.98			Yes
BrCaMa- 179	034 - Site 02	BrCa Control	1.429	0.255	0.942	762.68	664.87	1.147			Yes
BrCaMa- 180	035 - Site 02	BrCa Control	1.44	0.217	0.921	809.28	562.84	1.438		Yes	Yes
BrCaMa- 181	036 - Site 02	BrCa Control	1.882	0.358	1.253	984.79	936.87	1.051		Yes	Yes
BrCaMa- 182	037 - Site 02	BrCa Control	1.752	0.329	1.16	924.17	862.59	1.071		Yes	Yes
BrCaMa- 183	038 - Site 02	BrCa Control	2	0.363	1.321	1062.74	947.86	1.121		Yes	Yes
BrCaMa- 184	039 - Site 02	BrCa Control	1.852	0.289	1.196	1026.73	747.99	1.373		Yes	Yes
BrCaMa- 185	040 - Site 02	BrCa Control	1.618	0.361	1.103	802.96	954.66	0.841			Yes
BrCaMa- 186	041 - Site 02	BrCa Control	1.762	0.325	1.166	932.29	849.81	1.097			Yes
BrCaMa- 187	042 - Site 02	BrCa Control	1.818	0.309	1.186	987.37	805.37	1.226			Yes
BrCaMa- 188	043 - Site 02	BrCa Control	1.935	0.318	1.255	1061.68	827.81	1.283			Yes
BrCaMa- 189	044 - Site 02	BrCa Control	1.723	0.297	1.127	931.51	774.38	1.203			Yes
BrCaMa- 190	045 - Site 02	BrCa Control	1.875	0.36	1.248	980.13	943.8	1.038			Yes
BrCaMa- 191	046 - Site 02	BrCa Control	1.784	0.267	1.146	998.89	688.91	1.45			Yes
BrCaMa- 192	047 - Site 02	BrCa Control	1.742	0.413	1.202	842.13	1094.57	0.769			Yes
BrCaMa- 193	048 - Site 02	BrCa Control	1.872	0.309	1.217	1024.04	803.44	1.275			Yes
BrCaMa- 194	049 - Site 02	BrCa Control	1.616	0.257	1.046	891.87	666.17	1.339			Yes
BrCaMa- 195	050 - Site 02	BrCa Control	1.265	0.209	0.823	691.37	543.18	1.273			Yes
BrCaMa- 196	051 - Site 02	BrCa Control	1.601	0.358	1.096	790.13	944.54	0.837			Yes
BrCaMa- 197	052 - Site 02	BrCa Control	1.661	0.24	1.061	938.7	618.33	1.518			Yes
BrCaMa- 198	053 - Site 02	BrCa Control	1.546	0.272	1.015	830.31	709.67	1.17			Yes
BrCaMa- 199	054 - Site 02	BrCa Control	1.505	0.314	1.019	761.58	824.93	0.923			Yes
BrCaMa- 200	055 - Site 02	BrCa Control	1.939	0.309	1.256	1069.06	800.75	1.335			Yes
BrCaMa- 201	Site 05-001	BrCa	1.077	0.083	0.645	675.655	201.895	3.347		Yes	
BrCaMa- 202	Site 05-002	BrCa Control	0.985	0.084	0.592	612.213	208.01	2.943	Yes		
BrCaMa- 203	Site 05-003	BrCa	0.858	0.079	0.52	527.805	196.073	2.692		Yes	
BrCaMa- 204	Site 05-004	BrCa	0.929	0.088	0.563	570.093	218.958	2.604	Yes		
BrCaMa- 205	Site 05-005	BrCa	0.82	0.072	0.494	507.638	177.965	2.852	Yes		
BrCaMa- 206	Site 05-006	BrCa	0.821	0.071	0.494	509.685	174.75	2.917	Yes		
BrCaMa- 207	Site 05-007	BrCa	0.993	0.085	0.599	615.535	210.445	2.925	Yes		
BrCaMa- 208	Site 05-008	BrCa Control	0.846	0.079	0.512	521.188	196.62	2.651	Yes		
BrCaMa- 209	Site 05-009	BrCa Control	0.534	0.059	0.327	320.603	150.523	2.13	Yes		
BrCaMa- 210	Site 05-010	BrCa Control	0.854	0.074	0.515	528.775	182.28	2.901	Yes		
BrCaMa- 211	Site 05-011	BrCa Control	0.806	0.079	0.492	490.5	198.583	2.47	Yes		
BrCaMa- 212	Site 05-012	BrCa	1.13	0.083	0.673	712.738	199.248	3.577	Yes		
BrCaMa- 213	Site 05-013	BrCa Control	1.005	0.085	0.605	623.435	210.09	2.967	Yes		
BrCaMa- 214	Site 05-014	BrCa	1.233	0.086	0.732	781.85	204.985	3.814		Yes	
BrCaMa- 215	Site 05-015	BrCa Control	1.081	0.093	0.655	667.788	227.38	2.937	Yes		
BrCaMa- 216	Site 05-016	BrCa	1.112	0.092	0.667	693.89	225.483	3.077		Yes	
BrCaMa- 217	Site 05-017	BrCa Control	0.973	0.082	0.585	605.285	201.03	3.011	Yes		
BrCaMa- 218	Site 05-018	BrCa Control	1.088	0.087	0.65	682.81	214.438	3.184	Yes		

BrCaMa- 219	Site 05-019	BrCa Control	1.018	0.082	0.609	637.258	202.665	3.144	Yes		
BrCaMa- 220	Site 05-020	BrCa	1.451	0.098	0.861	921.855	231.848	3.976	Yes		
ISH- 1008	N/A	BrCa	2.358	0.269	1.464	1398.15	680.87	2.053	Yes		
MM- 5013	N/A	BrCa control	2.528	0.365	1.617	1426.84	939.04	1.519	Yes		
PAH- 1004	N/A	BrCa	2.06	0.312	1.334	1142.46	800.94	1.426	Yes		
PAH- 1007	N/A	BrCa	1.921	0.232	1.214	1115.05	582.42	1.915	Yes		

Table 1. Oxy/met-Hb ratio thresholds of the samples and their usage within the statistical processes.

Epigenetic profiling of all the processed samples included a second quality control for the outliers. Shipment 122 (site 2 batch 2 controls) demonstrated fundamentally different distribution and quality from all the other sites and shipments. According to the standard practice of outlier control, 30 samples from Site 2 batch 2 (shipment 122) were excluded from the development of the test.

EpiSwitch™ Array Results

- Both data sets produced many significant probes;
- **Array 1, BCa1 4185** significant *EpiSwitch*™ markers identified in the analysis of breast cancer versus healthy controls;
- **Array 2, BCa2 4856** significant *EpiSwitch*™ markers identified in the analysis of breast cancer versus healthy controls;
- However there was an overlap between both analyses of **2116** significant probes consistent between the 2 studies (see Figure 1)

All data was originally taken and all saturated probes were removed. They were then normalised to even up the data between the channels. All of the four replicates for each data set were then combined together, and the co-efficient of variation was determined. The 2116 probes were narrowed down using normalised correlation values to rank the most changed genes on the array. Enrichment analysis was used to find the most differentially expressed genes above that of random chance. Altogether there were 138 markers from the combined BCa1 and BCa2 arrays that showed differential up-regulated or down-regulated expression. The top 80 *EpiSwitch*™ markers (see Appendix I) including 41 markers from array 1 and 39 markers from array 2 were taken on for validation with the *EpiSwitch*™ *PCR* assay to stratify between breast cancer and healthy controls.

EpiSwitch™ PCR platform and marker verification

Primers were designed using the Integrated DNA Technologies (IDT) software (and Primer3web version 4.0.0 software if required) from markers identified on the microarray. Primer testing was carried out on each primer set. Each set was tested on a pooled subset of samples to ensure that appropriate primers could study the potential interactions. If the primer testing was successful then the primer sets were taken through to screening.

168 samples were used. These samples were split into 2 sets: 118 patient samples (68 BrCa & 50 Control) were used in marker reduction and model development, and the remaining 50 samples (31 BrCa & 19 Control) were used as an independent cohort to validate the final model developed from the initial 118 patient set. 30 Control samples from site 2, shipment 122 (defined as batch 2) were not used in the final patient set as they proved to be outliers in quality control procedures.

Primer Screen

This test was used to eliminate non-specific primers, and to determine whether the primers are enabling the detection of the 3C conformational looping. All extracted blood samples were diluted from 1:2 – 1:64. Initial results were produced in a binary format; i.e. '1'- yes, a band is present at the correct size or '0' – no, a band is not present at the correct size. All read-outs by EpiSwitch™ *PCR* were performed in presence of and with >95% accuracy of detecting both positive and negative controls.

Screen 1

51 primer sets successfully passed the primer testing stage and were tested on the 8 BrCa and 8 control blood samples. In the first screen the samples were matched to those used on the array.

BrCa Sample ID	Patient ID	Control Sample ID	Patient ID
BrCaMa050	039 site 1	BrCaMa057	046 site 1
BrCaMa051	040 site 1	BrCaMa058	047 site 1
BrCaMa060	049 site 1	BrCaMa055	044 site 1
BrCaMa061	050 site 1	BrCaMa056	045 site 1
BrCaMa062	051 site 1	BrCaMa096	004 site 2
BrCaMa064	053 site 1	BrCaMa097	005 site 2
BrCaMa089	023 site 2	BrCaMa043	004 site 4
BrCaMa041	003 site 4	BrCaMa045	006 site 4

Table 2. Samples used on Array 1 and in further PCR validation.

Screen 2

Primer sets showing differentiation were then screened with a further 12 BrCa and 12 control blood samples. A 1:2 to 1:64 dilution series was used to identify the range of assay sensitivity. Results from screen 1 and 2 were merged together to give a full representation of all the 20 samples used. A further 24 BrCa and 24 controls and finally the remainder of the samples were tested.

Screen 3

The final 20 BrCa and 20 control samples were then screened using the most informative three dilutions, covering the sensitive range of detection for the assay for each primer set. 13 markers in total were used in the final 20 samples screen. The results from screen 3 were merged together with the 90 BrCa and 90 control samples to give a full representation of 100 samples used for both BrCa and controls. These were then tested for efficacy in differentiating BrCa patients from control samples. A chi-square test (Fisher's exact) was produced to give the final markers.

PROBES	OUTERS	INNERS
MELK_9_36577630_36579243_36637050_36643005_RF	PRMR-2/4	PRMR-1/3
ATM_11_108118137_108126372_108155279_108156687_RF	PRMR-54/56	PRMR-53/55
CDC6_17_38421089_38423079_38467677_38474960_FR	PRMR-90/92	PRMR-89/91
CDC6_17_38421089_38423079_38451196_38457050_FF	PRMR-102/104	PRMR-101/103
SLC16A10_6_111441989_111447305_111492951_111498421_FR	PRMR-114/80	PRMR-113/115
TSPYL5_8_98276431_98282736_98316421_98318720_FF	PRMR-130/132	PRMR-129/131
MAP3K1_5_56102259_56110500_56140227_56144076_FF	PRMR-162/164	PRMR-161/163
ME3_11_86300063_86304401_86420537_86426200_FR	PRMR-174/176	PRMR-173/175
SRD5A1_5_6634973_6639025_6667775_6669711_RF	PRMR-178/180	PRMR-177/179
VAV3_1_108148303_108158073_108220200_108227533_RF	PRMR-186/188	PRMR-185/187
FOXC1_6_1577253_1581989_1604206_1605973_FR	PRMR-198/200	PRMR-197/199
NF1_17_29477103_29483764_29651799_29657368_FF	PRMR-262/264	PRMR-261/263
MSH3_5_80021913_80025030_80153948_80159012_RF	PRMR-302/304	PRMR-301/303

Table 3. The final markers and primer sets.

Marker Reduction 13 Primer Combinations with 3 dilution factors, 39 Markers

To reduce the finally selected 13 locations and 39 markers to a working classifying model, the GLMNET package with the R statistical language was used. GLMNET performs penalized (elastic-net penalty) regression modelling allowing ridge or lasso regression (which omits co-linearity of markers). Multivariate logistic regression analysis using lasso regression was performed on patient set 1. [See Figure 3]

Gene	Marker	GLMNET
SRD5A1_5	PRMR.177.179_2	0.233358596
NF1_17	PRMR.261.263_4	0.145129097
TSPYL5_8	PRMR.129.131_2	0.04597074
ME3_11	PRMR.173.175_4	0.019318541
VAV3_1	PRMR.185.187_8	-0.008248717
ATM_11	PRMR.53.55_32	-0.029412806
MAP3K1_5	PRMR.161.163_8	-0.045528058
SLC16A10_6	PRMR.113.115_4	-0.0174300311

Table 4. The 13-marker set with their 3 dilutions were reduced down to an 8 marker set using the 118 patients from set 1; the GLMNET coefficients for the markers are shown in the table above. The top 4 markers are ones that trend with the BrCa phenotype and the bottom four markers in blue are ones that trend with the Control phenotype.

Logistic regression analysis

Logistic regression analysis was carried out using the Waikato to Environment for Knowledge Analysis (WEKA) software version 3.6.12. Using this analysis the classification function of sensitivity and specificity was established for patient set 1 (118 patients, 68 BrCa and 50 Control), with the 8 markers identified by the GLMNET analysis.

	95% Confidence Interval (CI)	
Sensitivity	85.71%	57.2%-98.2%
Specificity	80.00%	44.4%-97.2%
PPV	85.71%	57.2%-98.2%
NPV	80.00%	44.4%-97.2%

Table 5. Above shows the model test statistics for the 118 patient, 8 marker model. The classification was based on 80% Training (94 known samples) and 20% Test (24 blinded samples) analysis. The AUC for this model is 0.832.

Model Validation

The 8 marker logistic model was then tested on patient set 2, (31 BrCa & 19 control), these patients were not used to reduce the markers and are an independent data set.

	95% Confidence Interval (CI)	
Sensitivity	83.3%	35.9%-99.6%
Specificity	100.0%	39.8%-100.0%
PPV	100.0%	47.8%-100.0%
NPV	80.0%	28.4%-99.5%

Table 6. The table above shows the model test statistics for the 8-marker model on the independent 50 patient set. The classification was based on 80% Training (40 known samples) and 20% Test (10 blinded samples) analysis. The AUC for this model is 0.98.

Principal Components Analysis (PCA) is an exploratory multivariate statistical technique for simplifying complex data sets. Given m observations on n variables, the goal of PCA is to reduce the dimensionality of the data matrix by finding r new variables, where r is less than n. Termed principal components, these r new variables together account for as much of the variance in the original n variables as possible while remaining mutually uncorrelated and orthogonal. Each principal component is a linear combination of the original variables, and so it is often possible to ascribe meaning to what the components represent. Principal components analysis has been used in a wide range of biomedical problems, including the analysis of microarray data in search of outlier genes as well as the analysis of other types of expression data.

Patient	Dim.1	Dim.2	Dim.3	Dim.4	Dim.5
MM5013	-1.800071608	0.492729305	0.6019709	0.438989393	1.26872044
BrCaMa219	-0.785966577	0.47223154	1.51829263	-1.895977084	-3.11799252
BrCaMa218	0.173347221	1.548046183	-0.91822436	0.767768358	-0.14166967
BrCaMa217	0.802477895	1.191180105	0.33952978	-0.318075596	1.27316931
BrCaMa215	1.113161481	1.960102724	-0.36790426	1.04353295	-0.62147173
BrCaMa213	0.350909675	1.263048827	-0.49646041	0.70813229	0.19512773
BrCaMa211	-0.120178677	1.753308317	1.88817334	-2.596145659	-1.63044319
BrCaMa210	-0.89241577	0.371232864	-1.64136315	-0.663666676	-0.27700782
BrCaMa209	-0.137336365	0.779123564	-0.21079032	-0.593840188	1.75297137
BrCaMa208	0.595430617	0.127397803	-0.49637741	-0.378540735	-1.98291708
BrCaMa202	-0.894770738	3.078346899	1.37016687	1.491976975	0.74357301
BrCaMa135	1.076504256	0.322159869	0.51996917	0.657857571	-0.69418208
BrCaMa133	-0.130163964	1.068286761	-1.51280699	-0.328266016	-1.09360727
BrCaMa117	1.261218517	1.40847458	-0.1264967	-1.07870931	-0.49536775
BrCaMa116	0.809650296	1.480343302	-0.96248689	-0.052501424	-1.57340933
BrCaMa115	0.321404256	0.996418039	-0.6768168	-1.354473902	-0.01556569
BrCaMa114	0.624915441	1.476177461	-0.08223417	-0.258439528	0.93637191
BrCaMa113	1.224561292	-0.229468275	0.76137673	-1.464384689	-0.56807811
BrCaMa112	0.802477895	1.191180105	0.33952978	-0.318075596	1.27316931
BrCaMa111	-0.440847549	0.299364142	-0.80537296	-1.689874562	0.80103376
BrCaMa110	0.802477895	1.191180105	0.33952978	-0.318075596	1.27316931
BrCaMa109	-0.89241577	0.371232864	-1.64136315	-0.663666676	-0.27700782
BrCaMa108	-1.375844377	2.883584833	0.35382028	0.455578669	-0.54516199
BrCaMa107	0.624915441	1.476177461	-0.08223417	-0.258439528	0.93637191
BrCaMa106	0.350909675	1.263048827	-0.49646041	0.70813229	0.19512773
BrCaMa105	-3.181468802	0.837563041	2.61664094	-1.078446956	0.133645
BrCaMa104	-0.89241577	0.371232864	-1.64136315	-0.663666676	-0.27700782
BrCaMa103	1.838756062	1.019213766	0.64852532	0.993258231	-1.51078154
BrCaMa094	-0.137336365	0.779123564	-0.21079032	-0.593840188	1.75297137
BrCaMa093	0.321404256	0.996418039	-0.6768168	-1.354473902	-0.01556569
BrCaMa059	0.321404256	0.996418039	-0.6768168	-1.354473902	-0.01556569

BrCaMa058	-0.440847549	0.299364142	-0.80537296	-1.689874562	0.80103376
BrCaMa057	-0.89241577	0.371232864	-1.64136315	-0.663666676	-0.27700782
BrCaMa056	-4.427149215	2.652861113	4.48326822	-0.294602271	0.68209028
BrCaMa055	-1.822244743	0.644197879	1.20929708	-3.207310911	-0.33404168
BrCaMa046	0.595430617	0.127397803	-0.49637741	-0.378540735	-1.98291708
BrCaMa045	-0.588904585	0.850992286	-1.04678051	0.432367698	0.67492979
BrCaMa044	0.04739849	0.783289405	-1.09104305	-0.387902084	-0.75680988
BrCaMa043	-3.936548207	0.429672342	1.18606812	-1.148273444	-1.89633418
BrCaMa031	0.321404256	0.996418039	-0.6768168	-1.354473902	-0.01556569
BrCaMa030	-0.89241577	0.371232864	-1.64136315	-0.663666676	-0.27700782
BrCaMa029	0.183332507	2.233067739	2.48275597	-1.500111286	-0.67850559
BrCaMa028	0.173347221	1.548046183	-0.91822436	0.767768358	-0.14166967
BrCaMa015	0.809650296	1.480343302	-0.96248689	-0.052501424	-1.57340933
BrCaMa014	0.624915441	1.476177461	-0.08223417	-0.258439528	0.93637191
BrCaMa012	0.624915441	1.476177461	-0.08223417	-0.258439528	0.93637191
BrCaMa007	1.261218517	1.40847458	-0.1264967	-1.07870931	-0.49536775
BrCaMa006	0.321404256	0.996418039	-0.6768168	-1.354473902	-0.01556569
BrCaMa005	-0.440847549	0.299364142	-0.80537296	-1.689874562	0.80103376
BrCaMa002	-2.735068436	3.076949995	1.76116414	2.584442624	-0.07747531
PAH1010-BRCA	2.155416969	-0.944242691	1.21265768	-0.457038415	-1.12641136
PAH1007-BRCA	1.429822388	-0.003353733	0.19622809	-0.406763696	-0.23710155
PAH1004-BRCA	0.038439907	-0.343541552	-1.1900822	0.343679598	-0.83534107
ISH1008-BRCA	0.667570582	-0.70040763	0.06767194	-0.742164356	0.57949791
BrCaMa220	0.490008128	-0.415410274	-0.35409201	-0.682528288	0.24270051
BrCaMa212	1.703848748	-0.872373969	0.37666749	0.569169471	-2.20445294
BrCaMa207	0.341951092	0.13621787	-0.59549956	1.439713972	0.11659654
BrCaMa206	0.27343348	0.938758261	3.12354749	1.147076052	0.85377359
BrCaMa205	-0.723811899	-1.040595449	-1.31863835	0.008278938	-0.01874161
BrCaMa204	0.001782682	-1.981484407	-0.30220877	-0.041995781	-0.90805142
BrCaMa162	1.733333573	0.476405689	0.79081073	0.689270678	0.71483606
BrCaMa160	0.031267506	-0.632704749	0.11193447	0.078105426	2.01123757
BrCaMa159	1.429822388	-0.003353733	0.19622809	-0.406763696	-0.23710155
BrCaMa158	-0.420300714	-0.560836027	-0.72405572	1.104313312	0.93319599
BrCaMa157	0.971081767	-0.220648208	0.66225457	0.353870018	1.53143551
BrCaMa156	-2.590781534	-1.994913652	3.36121269	-1.552810435	-1.44933621
BrCaMa155	0.490008128	-0.415410274	-0.35409201	-0.682528288	0.24270051
BrCaMa154	-0.272243678	-1.112464171	-0.48264816	-1.017928948	1.05929997
BrCaMa153	2.155416969	-0.944242691	1.21265768	-0.457038415	-1.12641136
BrCaMa152	0.764034488	-1.28443051	-0.17365262	0.293404879	-1.72465088
BrCaMa151	-0.578109831	1.114890442	1.93429922	0.041680329	1.12794319
BrCaMa150	-0.272243678	-1.112464171	-0.48264816	-1.017928948	1.05929997
BrCaMa149	0.341951092	0.13621787	-0.59549956	1.439713972	0.11659654

BrCaMa148	-2.083035957	-0.847230287	0.08870551	2.137142893	0.44894507
BrCaMa146	-2.083035957	-0.847230287	0.08870551	2.137142893	0.44894507
BrCaMa145	-0.272243678	-1.112464171	-0.48264816	-1.017928948	1.05929997
BrCaMa144	-0.417627116	-1.376062704	1.65545519	2.362632767	-0.9201668
BrCaMa143	0.756862087	-1.573593707	1.12836406	0.027830707	1.12192776
BrCaMa142	-0.898700755	-1.57082477	0.63910861	1.326234461	-2.2089018
BrCaMa141	0.031267506	-0.632704749	0.11193447	0.078105426	2.01123757
BrCaMa131	-0.272243678	-1.112464171	-0.48264816	-1.017928948	1.05929997
BrCaMa126	-2.386547141	-1.326989709	-0.50587713	1.04110852	-0.50299254
BrCaMa125	-0.723811899	-1.040595449	-1.31863835	0.008278938	-0.01874161
BrCaMa124	-2.386547141	-1.326989709	-0.50587713	1.04110852	-0.50299254
BrCaMa123	1.733333573	0.476405689	0.79081073	0.689270678	0.71483606
BrCaMa122	1.696676347	-1.161537166	1.67868416	0.303595299	0.6421257
BrCaMa121	0.453350903	-2.053353129	0.53378142	-1.068203667	0.16999016
BrCaMa120	1.733333573	0.476405689	0.79081073	0.689270678	0.71483606
BrCaMa119	-0.272243678	-1.112464171	-0.48264816	-1.017928948	1.05929997
BrCaMa118	0.031267506	-0.632704749	0.11193447	0.078105426	2.01123757
BrCaMa091	0.453350903	-2.053353129	0.53378142	-1.068203667	0.16999016
BrCaMa090	0.667570582	-0.70040763	0.06767194	-0.742164356	0.57949791
BrCaMa089	-0.723811899	-1.040595449	-1.31863835	0.008278938	-0.01874161
BrCaMa088	-0.272243678	-1.112464171	-0.48264816	-1.017928948	1.05929997
BrCaMa087	1.519113893	-0.87653981	1.25692021	0.363231367	0.30532831
BrCaMa086	0.031267506	-0.632704749	0.11193447	0.078105426	2.01123757
BrCaMa085	0.031267506	-0.632704749	0.11193447	0.078105426	2.01123757
BrCaMa084	0.667570582	-0.70040763	0.06767194	-0.742164356	0.57949791
BrCaMa083	-0.928046291	-1.708519392	2.54845146	-2.585640016	-0.96508529
BrCaMa082	-1.624295336	-0.629935812	-0.37732097	1.37650918	-1.31959199
BrCaMa081	-2.386547141	-1.326989709	-0.50587713	1.04110852	-0.50299254
BrCaMa074	0.453350903	-2.053353129	0.53378142	-1.068203667	0.16999016
BrCaMa073	0.764034488	-1.28443051	-0.17365262	0.293404879	-1.72465088
BrCaMa072	0.341951092	0.13621787	-0.59549956	1.439713972	0.11659654
BrCaMa071	-3.767944336	-0.982155972	1.50879291	-0.47632783	-1.63806798
BrCaMa070	0.793519312	0.064349148	0.24049063	0.413506086	1.19463812
BrCaMa069	-0.869215931	-0.222045112	1.05325185	1.446335667	0.71038719
BrCaMa068	1.733333573	0.476405689	0.79081073	0.689270678	0.71483606
BrCaMa067	-1.624295336	-0.629935812	-0.37732097	1.37650918	-1.31959199
BrCaMa066	0.756862087	-1.573593707	1.12836406	0.027830707	1.12192776
BrCaMa042	-1.624295336	-0.629935812	-0.37732097	1.37650918	-1.31959199
BrCaMa041	-0.723811899	-1.040595449	-1.31863835	0.008278938	-0.01874161
BrCaMa040	-0.723811899	-1.040595449	-1.31863835	0.008278938	-0.01874161
BrCaMa039	0.038439907	-0.343541552	-1.1900822	0.343679598	-0.83534107
BrCaMa025	-0.272243678	-1.112464171	-0.48264816	-1.017928948	1.05929997
BrCaMa024	2.458928153	-0.464483269	1.80724031	0.638995959	-0.17447375
BrCaMa020	-2.083035957	-0.847230287	0.08870551	2.137142893	0.44894507

BrCaMa019	-0.272243678	-1.112464171	-0.48264816	-1.017928948	1.05929997
BrCaMa101	-0.166821189	-0.569656094	-0.62493357	-0.713941395	-1.16631763
BrCaMa138	0.173347221	1.548046183	-0.91822436	0.767768358	-0.14166967
BrCaMa016	0.321404256	0.996418039	-0.6768168	-1.354473902	-0.01556569
BrCaMa017	0.04739849	0.783289405	-1.09104305	-0.387902084	-0.75680988
BrCaMa018	-0.130163964	1.068286761	-1.51280699	-0.328266016	-1.09360727
BrCaMa021	-0.89241577	0.371232864	-1.64136315	-0.663666676	-0.27700782
BrCaMa022	0.173347221	1.548046183	-0.91822436	0.767768358	-0.14166967
BrCaMa023	0.04739849	0.783289405	-1.09104305	-0.387902084	-0.75680988
BrCaMa026	0.809650296	1.480343302	-0.96248689	-0.052501424	-1.57340933
BrCaMa027	0.624915441	1.476177461	-0.08223417	-0.258439528	0.93637191
BrCaMa095	-0.130163964	1.068286761	-1.51280699	-0.328266016	-1.09360727
BrCaMa096	0.284747031	-0.641524816	0.21105663	-1.740149281	-0.08827605
BrCaMa097	-3.349263206	1.828267955	1.87401555	0.126799705	0.86522812
BrCaMa098	-0.588904585	0.850992286	-1.04678051	0.432367698	0.67492979
BrCaMa099	-0.588904585	0.850992286	-1.04678051	0.432367698	0.67492979
BrCaMa100	1.113161481	1.960102724	-0.36790426	1.04353295	-0.62147173
BrCaMa102	0.321404256	0.996418039	-0.6768168	-1.354473902	-0.01556569
BrCaMa130	1.113161481	1.960102724	-0.36790426	1.04353295	-0.62147173
BrCaMa139	-0.440847549	0.299364142	-0.80537296	-1.689874562	0.80103376
BrCaMa036	2.007359933	-0.392614547	0.97125012	1.665203845	-1.25251533
BrCaMa080	-0.723811899	-1.040595449	-1.31863835	0.008278938	-0.01874161
BrCaMa032	2.458928153	-0.464483269	1.80724031	0.638995959	-0.17447375
BrCaMa050	-2.386547141	-1.326989709	-0.50587713	1.04110852	-0.50299254
BrCaMa051	0.216002362	-0.628538908	-0.76831825	0.28404353	-0.49854367
BrCaMa060	-0.723811899	-1.040595449	-1.31863835	0.008278938	-0.01874161
BrCaMa061	-0.861883648	0.19605425	1.84093442	-0.137358446	-0.68168151
BrCaMa062	0.941596942	-1.569427866	0.24811133	0.233768811	-1.38785348
BrCaMa063	0.756862087	-1.573593707	1.12836406	0.027830707	1.12192776
BrCaMa064	-0.420300714	-0.560836027	-0.72405572	1.104313312	0.93319599
BrCaMa065	2.155416969	-0.944242691	1.21265768	-0.457038415	-1.12641136
BrCaMa075	1.281765352	0.548274411	-0.04517946	1.715478564	-0.36320552
BrCaMa076	-1.143221697	-0.435173746	0.63902561	2.412907486	-0.03085699
BrCaMa077	0.305293867	-1.501724985	0.29237387	1.054038593	0.04388618
BrCaMa078	1.733333573	0.476405689	0.79081073	0.689270678	0.71483606
BrCaMa079	0.216002362	-0.628538908	-0.76831825	0.28404353	-0.49854367
BrCaMa127	-0.420300714	-0.560836027	-0.72405572	1.104313312	0.93319599
BrCaMa129	-0.723811899	-1.040595449	-1.31863835	0.008278938	-0.01874161
BrCaMa134	0.978254168	0.068514989	-0.6397621	0.61944419	-1.31514313
BrCaMa140	-0.723811899	-1.040595449	-1.31863835	0.008278938	-0.01874161
BrCaMa161	0.341951092	0.13621787	-0.59549956	1.439713972	0.11659654
BrCaMa163	2.458928153	-0.464483269	1.80724031	0.638995959	-0.17447375
BrCaMa164	-0.420300714	-0.560836027	-0.72405572	1.104313312	0.93319599
BrCaMa167	0.341951092	0.13621787	-0.59549956	1.439713972	0.11659654

BrCaMa168	2.458928153	-0.464483269	1.80724031	0.638995959	-0.17447375
BrCaMa169	-0.420300714	-0.560836027	-0.72405572	1.104313312	0.93319599
BrCaMa170	0.453350903	-2.053353129	0.53378142	-1.068203667	0.16999016
BrCaMa201	1.519113893	-0.87653981	1.25692021	0.363231367	0.30532831
BrCaMa203	-0.272243678	-1.112464171	-0.48264816	-1.017928948	1.05929997
BrCaMa214	0.031267506	-0.632704749	0.11193447	0.078105426	2.01123757
BrCaMa216	1.703848748	-0.872373969	0.37666749	0.569169471	-2.20445294

Table 7. Shows factor analysis data (principle component analysis) for the 8-marker BrCa model validated signature using all the 168 samples used in the analysis: development (118 patients) and validation (50 patients) steps of the project.

CONCLUSION

Quality control procedure identified and excluded shipment 122 (site 2 controls) as samples fundamentally different in their profiles and quality from all other samples from other sites and shipments. Chromosome conformation analysis and logistic regression of the results by the *EpiSwitch™* methodology has developed a signature of 8 biomarkers that stratified 118 samples of breast cancer patients and healthy controls with cross-validation results of 85.7% sensitivity, 80% specificity, 85.7% PPV and 80% NPV. Independent cohort validation on 50 samples demonstrated 83.3% sensitivity, 100% specificity, 100% PPV and 80% NPV of the biomarkers.

Appendix I: Leading marker List

	Array 1
NUMBER	PROBES
Marker 1	MELK_9_36577630_36579243_36637050_36643005_RF
Marker 2	TPRG1_3_188933689_188940214_188962938_188970637_FF
Marker 3	LYPD6_2_150146782_150153111_150236512_150246806_RR
Marker 4	KCNE4_2_223978296_223985382_224015903_224024643_FR
Marker 5	SYT9_11_7410026_7412890_7469239_7478268_RF
Marker 6	NOS1AP_1_162209626_162215852_162277389_162286110_RF
Marker 7	STK32B_4_5251609_5261154_5459392_5462470_FF
Marker 8	CENPK_5_64812989_64817647_64878910_64881785_RF
Marker 9	ATM_11_108055477_108058111_108208085_108223747_FF
Marker 10	AR_X_66911452_66916150_66961257_66967450_FF
Marker 11	MAPT_17_43962855_43965625_44076167_44084076_FR
Marker 12	CCNG2_4_78068534_78075153_78309908_78315095_FR
Marker 13	ADCY1_7_45624230_45629168_45722424_45731328_FF
Marker 14	ATM_11_108118137_108126372_108155279_108156687_RF
Marker 15	ESR1_6_152307023_152319013_152333402_152336355_FR
Marker 16	FMNL2_2_153432680_153440869_153479856_153483982_FF
Marker 17	MAP3K1_5_56069013_56071773_56102259_56110500_RF

Marker 18	SKP2_5_36136526_36142109_36155505_36160932_FR
Marker 19	SLC16A10_6_111430971_111434623_111492951_111498421_RR
Marker 20	SLC16A10_6_111393624_111400094_111492951_111498421_RR
Marker 21	SLC16A10_6_111388697_111391406_111492951_111498421_FR
Marker 22	CDC6_17_38421089_38423079_38467677_38474960_FR
Marker 23	NOS1AP_1_162189941_162197873_162209626_162215852_FR
Marker 24	SOX11_2_5786050_5796562_5820335_5823500_RF
Marker 25	CDC6_17_38421089_38423079_38451196_38457050_FF
Marker 26	SLC16A10_6_111430971_111434623_111492951_111498421_FR
Marker 27	BLVRA_7_43784657_43787628_43835273_43842181_FR
Marker 28	SLC16A10_6_111441989_111447305_111492951_111498421_FR
Marker 29	MAP3K1_5_56102259_56110500_56137105_56140227_FR
Marker 30	NOS1AP_1_162189941_162197873_162354198_162360018_FR
Marker 31	SLC16A10_6_111438349_111441989_111492951_111498421_FR
Marker 32	TSPYL5_8_98276431_98282736_98316421_98318720_FF
Marker 33	PCM1_8_17764504_17769874_17830373_17837849_FR
Marker 34	CDC6_17_38421089_38423079_38457050_38462370_FR
Marker 35	NOS1AP_1_162247113_162253340_162264341_162270934_FR
Marker 36	TSPYL5_8_98276431_98282736_98295938_98301017_FR
Marker 37	ESR1_6_152082003_152085698_152307023_152319013_RF
Marker 38	BARD1_2_215635297_215642717_215688320_215695844_RF
Marker 39	ESR1_6_152082003_152085698_152307023_152319013_FF
Marker 40	MAP3K1_5_56102259_56110500_56140227_56144076_FF
Marker 41	SCUBE2_11_9094735_9101051_9144362_9152463_RF
	ARRAY 2
Marker 42	SYBU_8_110644489_110652424_110667554_110675383_FR
Marker 43	ME3_11_86300063_86304401_86420537_86426200_FR
Marker 44	SRD5A1_5_6634973_6639025_6667775_6669711_RF
Marker 45	SYTL2_11_85446267_85449759_85489426_85497695_FF
Marker 46	VAV3_1_108148303_108158073_108220200_108227533_RF
Marker 47	FOXC1_6_1577253_1581989_1622941_1624186_FR
Marker 48	SYTL2_11_85458295_85462105_85489426_85497695_FF
Marker 49	FOXC1_6_1577253_1581989_1604206_1605973_FR
Marker 50	FOXC1_6_1577253_1581989_1616641_1619635_FF
Marker 51	FOXC1_6_1577253_1581989_1608642_1611166_FF
Marker 52	AR_X_66736338_66750729_66911452_66916150_FR
Marker 53	FOXC1_6_1577253_1581989_1608642_1611166_FR
Marker 54	FOXC1_6_1577253_1581989_1621017_1622239_FF
Marker 55	AR_X_66875649_66881776_66911452_66916150_RF
Marker 56	FOXC1_6_1577253_1581989_1606219_1607879_FR
Marker 57	FOXC1_6_1577253_1581989_1622941_1624186_FF
Marker 58	FOXC1_6_1577253_1581989_1612413_1614478_FF
Marker 59	FMNL2_2_153385935_153395520_153444403_153446929_FR
Marker 60	GFRA1_10_117851659_117860183_117872774_117878186_RR
Marker 61	FOXC1_6_1577253_1581989_1606219_1607879_FF
Marker 62	RERG_12_15275463_15281772_15426692_15434723_FF

Marker 63	MSH3_5_80104716_80118379_80153948_80159012_FF
Marker 64	GPR126_6_142730628_142735943_142754471_142757840_FR
Marker 65	NF1_17_29477103_29483764_29651799_29657368_FF
Marker 66	AR_X_66750729_66754087_66950367_66956132_FF
Marker 67	FMNL2_2_153328638_153335686_153385935_153395520_RF
Marker 68	GFRA1_10_117891959_117898614_117911689_117919592_RR
Marker 69	NOSTRIN_2_169646544_169651214_169732611_169738179_RF
Marker 70	ADCY1_7_45638428_45640651_45722424_45731328_FF
Marker 71	CCNG2_4_78068534_78075153_78338468_78342587_RR
Marker 72	TPRG1_3_188814108_188822963_188962938_188970637_FF
Marker 73	GFRA1_10_117891959_117898614_117944517_117949325_RR
Marker 74	DACH1_13_71994847_72006255_72288568_72291811_RR
Marker 75	MSH3_5_80021913_80025030_80153948_80159012_RF
Marker 76	NOSTRIN_2_169599544_169606207_169732611_169738179_RF
Marker 77	FMNL2_2_153193445_153196492_153385935_153395520_RF
Marker 78	TPRG1_3_188823929_188830326_188962938_188970637_RF
Marker 79	BMPR1A_10_88534921_88537932_88549709_88557473_RF
Marker 80	PTPRT_20_40761966_40770575_40995945_41003669_FR

Table 8. 80 markers identified via *EpiSwitch*™ technology which stratify breast cancer from control samples.

Probe	GeneLocus	Probe_ Count_ Total	Probe_ Count_ Sig	HyperG_Stats	FDR_HyperG	Percent_ Sig	logFC	AveExpr	t
17_29477103_29483764_29651799_29657368_FF	NF1	139	8	0.957207644	1	5.76	0.239382	0.239382	5.889898
8_98276431_98282736_98316421_98318720_FF	TSPYL5	23	6	0.017130277	0.3203372	26.09	-0.38273	-0.38273	-12.2759
5_6634973_6639025_6667775_6669711_RF	SRD5A1	13	2	0.350038364	1	15.38	-0.32108	-0.32108	-6.71927
5_56102259_56110500_56140227_56144076_FF	MAP3K1	43	9	0.01691209	0.3203372	20.93	-0.34444	-0.34444	-9.94973
1_108148303_108158073_108220200_108227533_RF	VAV3	170	7	0.997311958	1	4.12	-0.46122	-0.46122	-7.26115
11_108118137_108126372_108155279_108156687_RF	ATM	54	11	0.010710258	0.3194381	20.37	0.436205	0.436205	12.70358
6_111441989_111447305_111492951_111498421_FR	SLC16A10	58	9	0.091353207	0.7687041	15.52	0.436205	0.436205	12.70358
11_86300063_86304401_86420537_86426200_FR	ME3	144	14	0.491902034	1	9.72	-0.40222	-0.40222	-3.89629

							Probe sequence
P.Value	adj.P.Val	B	FC	FC_1	LS	Loop detected	**60 mer**
0.000342	0.003906	0.413061	1.180487	1.180487	1	CTL	ATTTCTTTCTTCTTCCCATTTTCTAAAATCGATTTTTAAATTAAAGGTACAAGTTAAGGC
1.55E-06	0.000361	5.940348	0.766984	-1.30381	-1	BrCa	GGATGGAGGAAGAGGAGGAATTCAAGACTCGAACTAAACAAAAAGGAGATGATCCTGGGT
0.000138	0.002522	1.36779	0.80047	-1.24927	-1	BrCa	AGCTCAAATTCTTTTACTAATTGTTACATCGAAAGTTCAAAATTAAATTTTAAACGTTTT
7.79E-06	0.000846	4.341083	0.787612	-1.26966	-1	BrCa	CCAAAGACAGCCAAGGAAAAACTAAAGATCGAAAGTTTTTATTACTTCCAAATTAGTAAA
7.98E-05	0.002061	1.943909	0.726373	-1.3767	-1	BrCa	AATTTAGAGGAACTCTATATAAACAACATCGAAACTTTGCTTCATGCACAAAATTTAAAA
1.19E-06	0.000329	6.197546	1.353041	1.353041	1	CTL	TTGGAGGGAAAAGTAATTACGTTCAACTTCGACTGTATTCTACAAAGTGCTGGGATTACA
1.19E-06	0.000329	6.197546	1.353041	1.353041	1	CTL	ATACTCATCATAAATGTCAGATTTATAATCGAGATCACAGTGAGCTGAGATTGCACCACT
0.004417	0.017168	-2.28251	0.756691	-1.32154	-1	BrCa	AGGATCTCATGATGCTTTGAATACTTTCTCGATACCTTATTATAAAATCAGCTTTGTGTT

Probe Location					4 kb Sequence Location				
Chr	**Start1**	**End1**	**Start2**	**End2**	**Chr**	**Start1**	**End1**	**Start2**	**End2**
17	29483735	29483764	29657339	29657368	17	29479765	29483764	29653369	29657368
8	98282707	98282736	98318691	98318720	8	98278737	98282736	98314721	98318720
5	6634973	6635002	6669682	6669711	5	6634973	6638972	6665712	6669711
5	56110471	56110500	56144047	56144076	5	56106501	56110500	56140077	56144076
1	108148303	108148332	108227504	108227533	1	108148303	108152302	108223534	108227533
11	108118137	108118166	108156658	108156687	11	108118137	108122136	108152688	108156687
6	111447276	111447305	111492951	111492980	6	111443306	111447305	111492951	111496950
11	86304372	86304401	86420537	86420566	11	86300402	86304401	86420537	86424536

The three sections of Table 9 above provide information on the final 8 markers for marker set 1.

Probe	**PCR-Primer1_ID**	**PCR_Primer1**	**PCR-Primer2_ID**	**PCR_Primer2**	**GLMNET**
17_29477103_29483764_29651799_29657368_FF	PRMR-261	TGTAGTAGTTACCCTGTTGTTG	PRMR-263	GCCTCACAGTGCTCTTATG	0.1451291
8_98276431_98282736_98316421_98318720_FF	PRMR-129	GTGCTTTGTAAACCATGAAGTG	PRMR-131	TCGTGGGCATATGACTGAG	0.0459707
5_6634973_6639025_6667775_6669711_RF	PRMR-177	GGCATTGCTTTGCCTTATC	PRMR-179	CAACTTCCTTGGGTGTAGAG	0.2333586
5_56102259_56110500_56140227_56144076_FF	PRMR-161	CGCTATATGTGGTTCTGTACG	PRMR- 163	CTTCTCTAAAGGGAGATTTGGG	-0.0455281
1_108148303_108158073_108220200_108227533_RF	PRMR-185	TGTTGAGCAAGATGGATAGC	PRMR-187	ATATTCAGGATGGAACCCAAG	-0.0082487
11_108118137_108126372_108155279_108156687_RF	PRMR-53	TCCAGAGGTTATGGAATTTGAG	PRMR-55	AAGAAACAGACTGGGCTTG	-0.0294128
6_111441989_111447305_111492951_111498421_FR	PRMR-113	ACTCAAATACTGCTCTACACTG	PRMR-115	AAGGAAGTTAAGCCCTATGC	-0.01743
11_86300063_86304401_86420537_86426200_FR	PRMR-173	ACCCTCCTTCACTCACATAG	PRMR-175	GCACCTAATCTACCTAACATCAC	0.0193185

Table 10. PCR Primers for the 8 markers

Example 2

Oxford BioDynamics (OBD) is a healthcare service company offering a novel patented platform technology in the field of aberrant gene expression and epigenetics. The patented *EpiSwitch™* platform technology detects epigenetic regulatory signature changes. The *EpiSwitch™* biomarker discovery platform identifies Chromosome Conformation Signatures (CCSs), which define the initial regulatory process in integrating environmental cues into the epigenetic and transcriptional machinery. As such, CCSs are the primary step in a cascade of gene regulation.

The CCSs isolated by the *EpiSwitch™* biomarker discovery platform have several advantages:

- → Severe biochemical and physiological stability;
- → Their binary nature and readout;
- → Their primary position in the eukaryotic cascade of gene regulation.

Specific conformation signatures at loci either exist or are absent due to the regulatory epigenetic control settings associated with pathology or treatment. CCSs have mild off-rates and when representing a particular phenotype or pathology, they will only change with a physiologically signalled transition to a new phenotype or as a result of external intervention. In addition, the measurement of these events is binary, and so this read out is in stark contrast to the continuum readout of varying levels of DNA methylation, histone modifications and most of the non-coding RNAs. The continuum read-out for most of the molecular biomarkers used to date offers a challenge to data analysis, in that the magnitude of change for particular biomarkers varies greatly from patient to patient, causing problems for classification statistics, which are used to stratify patients. These classification statistics and inference approaches are better-suited using biomarkers that are absent of magnitude and offer just a "yes or no" binary score of phenotypic differences signifying that *EpiSwitch™* CCS biomarkers are an excellent resource for potential diagnostic, prognostic and predictive biomarkers.

OBD has consistently observed highly disseminating *EpiSwitch™* markers in all its developed applications, with high concordance to the primary and secondary affected tissues and strong validation results. *EpiSwitch™* biomarker signatures demonstrated high robustness and high sensitivity and specificity in the stratification of complex disease phenotypes. The OBD technology takes advantage of the latest breakthroughs in the science of epigenetics and offers a unique and the only industrial-quality ISO certified platform for the discovery, monitoring and evaluation of chromosome conformation signatures, as a highly informative class of epigenetic biomarkers.

The *EpiSwitch™* technology offers a highly effective means of screening; early detection; companion diagnostic; monitoring and prognostic analysis of major diseases associated with aberrant and responsive gene expression. A major advantage of the OBD approach is that it is non-invasive, rapid, and relies on

highly stable DNA based targets as part of chromosomal signatures, rather than unstable protein/RNA molecules.

Technology overview

CCSs form a stable regulatory framework of epigenetic controls and access to genetic information across the whole genome of the cell. Changes in CCSs reflect early changes in the mode of regulation and gene expression well before the results manifest themselves as obvious abnormalities. A simple way of thinking of CCSs is that they are topological arrangements where different distant regulatory parts of the DNA are brought in close proximity to influence each other's function. These connections are not done randomly; they are highly regulated and are well recognised as high level regulatory mechanisms with significant biomarker stratification power. In the fast developing field of applied epigenetics, CCSs offer significant advantages against alternative biomarker platforms. As a new biomarker entity, discovery, monitoring and validation of CCSs requires a technology acceptable to the industry for its performance on quality, stability, sensitivity, reproducibility, cost and time of operational turnover.

DNA that is likely to form higher order structures of CCSs across the genome are directly evaluated by the *EpiSwitch™* Array on clinical samples from testing cohorts for identification of all relevant stratifying lead biomarkers. Following the *EpiSwitch™* Array screening, the pools of statistically significant stratifying biomarkers normally exceed 300 leads. A number of leads are then translated into the *EpiSwitch™* CR. The minimal signature of stratifying biomarkers (<15) undergoes standard validation and, once confirmed Validated signatures contain binary CCSs which are either present, or absent as conditional biomarkers of epigenetic regulation in patients with specific pathology. The OBD technology takes advantage of the latest breakthroughs in the science of epigenetics and offers a unique and the only industrial-quality ISO certified platform for the discovery, monitoring and evaluation of chromosome conformation signatures.

***EpiSwitch™* Assay**

Proprietary biochemical processing of clinical samples offer quick and effective (<4 hrs) conversion of epigenetic CCS biomarkers into sequence based analytes which are then read by the *EpiSwitch™* Array (a modified version of Agilent CGH array platform), *EpiSwitch™* PCR or DNA sequencers i.e. Roche 454, Nanopore MinION, etc

EpiSwitch™ *Array Analysis*

The *EpiSwitch*™ array platform is used for marker identification due to its high-throughput, and ability to screen large numbers of loci rapidly. The array used in this project is the Agilent custom-CGH array, which allows OBD to interrogate the markers identified through the *in silico* software.

The project was to be carried out using an array with samples from group 1 (stage I, II, III & IV) using a 15K *EpiSwitch* ™ array, but to increase the scope of the analysis the samples were used in collaboration with different ethnicity to increase the breadth of data sourced from the array. So instead we used two 8x60k array, which allows the study of up to 56,964 potential chromosome conformations in quadruplicate, so a 60k array was used in this project. This can be used to look at the chromosomal conformation signatures in up to 14,000 probes in four replicates. Two arrays were produced using 8 stage II/III breast cancer patient samples from a range of backgrounds individually tested against 8 pooled healthy control patient samples. The *EpiSwitch*™ template was prepared for each of the samples. The first array was carried out on Asian breast cancer samples procured by OBD. The second array used Polish cohort and an independent Asian samples cohort. Asian and European breast cancers can differ between ER+ and ER- status, as well as in prevalence of other subtypes and epigenetic profiles. Overlapping probes were found for similar cancers in multiple populations.

The main outcomes of the analysis were:

- Both data sets produced a lot of significant probes;
- **Array 1, BrCa1 4185** significant *EpiSwitch*™ markers identified in the analysis of breast cancer versus healthy controls;
- **Array 2, BrCa2 4856** significant *EpiSwitch*™ markers identified in the analysis of breast cancer versus healthy controls;
- There was an overlap between both analyses of **2116** significant probes consistent between the 2 studies.

Figure 1 shows a comparison of significant probes from BrCa1 (table 11) and BrCa2 (table 12, Polish cohort included) arrays. The probes adjusted p-value <0.05.

All data was originally taken and all saturated probes were removed. Normalisation occurred to even up the data between the channels. All the four replicates for each data set were then combined together and co-efficient of variation was determined. The 2116 probes were narrowed down using normalised correlation values to rank the most changed genes on the array. Enrichment analysis was used to find the most differentially expressed genes above that of random chance. So altogether there were 138 markers from the combined BrCa1 and BrCa2 arrays that showed differential up-regulated or down-regulated expression. The samples used in the array were matched as closely as possible in ages, array, age range 33-68 years, array 2 32 to 65 years.

Sample ID	Patient ID	Ethnicity	Age	Type	Stage	Pathology	ER	PR	HER
BrCaMa050	039 site 1	Indian	46	IDC	T3, N1, 0	IIIA	+	+	-

BrCaMa051	040 site 1	Malaysian	47	IDC	T3, N2, 0	IIIA	N/A	N/A	N/A
BrCaMa060	049 site 1	Chinese	68	IDC	4c, N2, 0	IIIB	N/A	N/A	N/A
BrCaMa061	050 site 1	Indian	59	IDC	4a, 0,0	IIIB	N/A	N/A	N/A
BrCaMa062	051 site 1	Malaysian	33	IDC	T3, 0, 0	IIB	N/A	N/A	N/A
BrCaMa064	053 site 1	Malaysian	50	IDC	4c, N1,0	IIIB	(-)	(-)	(-)
BrCaMa089	023 site 2	Indian	66	IDC	Ct4, N+, 0	IIIB	(-)	(-)	+
BrCaMa041	003 site 4	Indian	48	ILC	T2 ,0, 0	III	+	+	(-)

Table 11, Samples used on BrCa array 1.

Sample ID	Patient ID	Ethnicity	Age	Type	Stage	Pathology			ER	PR	HER
						NG	G	Mi			
ISH1008	24925/14	Chinese	45	BC	T3, X, X				N/A	N/A	N/A
PAH1004	0491895	Chinese	65	BC	T2, N1,0				+	+	2+
PAH1007	0488720	Indian	55	BC	T2,N1, 0				+	+	2+
PAH1008	0494750	Chinese	54	BC	T2, N3, 1				+	+	(-)
10782	10782	European	49	ILC	B5	-	X	Luminal A	+	+	(-)
10892	10892	European	62	IC NST	B5b	2	2	3/10	+	+	+
11015	11015	European	32	IC NST	B5	3	3	36/10	(-)	(-)	(-)
11081	11081	European	54	IC NST	B5	3	X	Necrosis	(-)	(-)	+

Table 12, Samples used on the BrCa array 2.

Screen1, EpiSwitch™ marker validation

The *EpiSwitch*™ PCR assay is a molecular biology test that can be performed by a trained technician following a standardised operating procedure protocol. All protocols and reagent manufacture are performed according to ISO 13485 and 9001 specifications to ensure the quality of the work and the ability to transfer the protocols.

Primers were designed using the Integrated DNA Technologies (IDT) software (and Primer3web version 4.0.0 software if required) from markers identified from the microarray. Sample quality control was carried out using MMP1 primers on singlicate extracted samples. All samples showed a positive result for MMP1, allowing the samples to continue onto the *EpiSwitch*™ PCR. All extracted blood samples were diluted from 1:2 – 1:64 and nested PCR carried out. Initial results were produced in a binary format, i.e. '1'- yes, a band is present at the correct size or '0' – no, a band is not present at the correct size.

After statistical analysis the top 80 *EpiSwitch*™ markers including 41 markers from array 1 and 39 markers from array 2 were taken on for validation with the *EpiSwitch*™ PCR assay to stratify between breast cancer samples.

After the first round of screening on 8 BrCa and 8 control samples the markers were reduced to 51, the second round used a further 36 BrCa and 36 control samples the markers were reduced to 13 good markers (table 13) that were able to stratify between BrCa and control patients.

PROBES	OUTERS	INNERS
MELK_9_36577630_36579243_36637050_36643005_RF	OBD116-2/4	OBD116-1/3

ATM_11_108118137_108126372_108155279_108156687_RF	OBD116-54/56	OBD116-53/55
CDC6_17_38421089_38423079_38467677_38474960_FR	OBD116-90/92	OBD116-89/91
CDC6_17_38421089_38423079_38451196_38457050_FF	OBD116-102/104	OBD116-101/103
SLC16A10_6_111441989_111447305_111492951_111498421_FR	OBD116-114/80	OBD116-113/115
TSPYL5_8_98276431_98282736_98316421_98318720_FF	OBD116-130/132	OBD116-129/131
MAP3K1_5_56102259_56110500_56140227_56144076_FF	OBD116-162/164	OBD116-161/163
ME3_11_86300063_86304401_86420537_86426200_FR	OBD116-174/176	OBD116-173/175
SRD5A1_5_6634973_6639025_6667775_6669711_RF	OBD116-178/180	OBD116-177/179
VAV3_1_108148303_108158073_108220200_108227533_RF	OBD116-186/188	OBD116-185/187
FOXC1_6_1577253_1581989_1604206_1605973_FR	OBD116-198/200	OBD116-197/199
NF1_17_29477103_29483764_29651799_29657368_FF	OBD116-262/264	OBD116-261/263
MSH3_5_80021913_80025030_80153948_80159012_RF	OBD116-302/304	OBD116-301/303

Table 13, Good markers used for evaluation by PCR on Gliwice samples

Screen 2, EpiSwitch ™ PCR validation on the Gliwice Samples

Screening was carried out using the 13 good markers on the 50 Gliwice samples and 22 control samples, using a 1:2 to 1:64 dilution series. See table 18 in appendix for the binary data results. After screening was carried out the binary results were tested for efficacy in differentiating the BrCa from control samples by the use of a chi-square test (Fisher's exact) was produced to give the final markers.

The results of the 13 markers were then assessed using GLMNET and Bayes logistic Modelling statistics. 10 markers (table 4) were then highlighted having good scores

Marker	Estimate	Std. Error	z_value	Pr(>|z|)	Glmnet_0.5
OBD116.301.303_2	3.592231	1.494287	2.404	0.0162	-0.266166
OBD116.185.187_16	2.135415	1.532293	1.394	0.1634	-0.032431
OBD116.53.55_8	-1.78499	1.341226	-1.331	0.1832	0.1394873
OBD116.161.163_64	3.616204	2.872291	1.259	0.208	-0.267241
OBD116.197.199_8	-1.87868	1.491999	-1.259	0.208	0.1215176
OBD116.129.131_4	1.560365	1.479932	1.054	0.2917	0
OBD116.173.175_2	-1.39826	1.491721	-0.937	0.3486	0.0080034
OBD116.89.103_8	-1.24015	1.35114	-0.918	0.3587	0.0081371
OBD116.177.179_8	-1.20884	1.323655	-0.913	0.3611	0
OBD116.113.87_4	1.615246	2.000497	0.807	0.4194	-0.051646

Table 14 Markers showing good GLMNET scores

Additional statistical analysis reduced the markers further; using a classification random tree with a 66% training set and a 34% test set, in which 24 samples were used.

Correctly classified instances were 19 (79.1667%), incorrectly classified instances were 5 (20.8333%), and this gave a Kappa statistic of 0.5 and a mean absolute error of 0.2322. The mean absolute error was 0.4656, with the relative absolute error being 55.2934%, the root squared error was 108.4286%

Detailed Accuracy by class

TP Rate	FP Rate	Precision	Recall	F-Measure	ROC Area	Class
0.75	0	1	0.75	0.857	0.925	BrCa
1	0.25	0.444	1	0.615	0.925	Control
0.792	0.042	0.907	0.792	0.817	0.925	

Confusion Matrix

a	b	
15	5	a= BrCa
0	4	b= Control

The final 8 markers were produced using GLMNET

Marker	Estimate	Std. Error	z_value	Pr(>|z|)	Glmnet_0.5
OBD116.301.303_2	3.592231	1.494287	2.404	0.0162	-0.266166
OBD116.185.187_16	2.135415	1.532293	1.394	0.1634	-0.032431
OBD116.53.55_8	-1.78499	1.341226	-1.331	0.1832	0.1394873
OBD116.197.199_8	-1.87868	1.491999	-1.259	0.208	0.1215176
OBD116.129.131_4	1.560365	1.479932	1.054	0.2917	0
OBD116.173.175_2	-1.39826	1.491721	-0.937	0.3486	0.0080034
OBD116.89.103_8	-1.24015	1.35114	-0.918	0.3587	0.0081371
OBD116.177.179_8	*-1.20884*	*1.323655*	*-0.913*	*0.3611*	*0*

Table 15 the final 8 markers

Independent classification of samples

The final stage was to test the markers stratification on an independent cohort of 25 samples using logistic modelling and 5 fold cross validation.

True positive rate	0.836

Num true positives	8.36
False positive rate	0.09
Num_ false positives	0.44
True negative rate	0.91
Num true negatives	4.08
False negative rate	0.164
Num false negatives	1.64
IR precision	0.953505
IR recall	0.836
F measure	0.887459
Area under ROC	0.9026

This shows that in the independent cohort validation the classifier based on the selected markers that were capable of 83.6% sensitivity and 91.0% specificity with a ROC value of 0.903. This means that the performance of the binary classifier is of a high standard, the highest ROC being1 and the lowest being 0.5.

Conclusion

The aim of the study was to determine the epigenetic changes in whole blood of women with breast cancer or predisposed to breast cancer then to use the biomarkers for diagnostic stratification.

A 60K *EpiSwitch*^{TM} array was developed to look at 56964 potential chromosome interactions in relation to the diagnosis of breast cancer patients from control patients.

Two arrays were produced the first having Asian BrCa and control patient samples, the second having both Asian and Polish BrCa samples and controls, this was to allow us to see if there were any similar markers between the two arrays. This would ultimately allow a greater depth of markers to be found between different ethnic groups. After analysis of the arrays 4185 and 4856 probes were found with an overlap of 2116 significant probes. Correction normalisation of the probes was carried out and 138 potential markers were found that could be used to determine a diagnosis of breast cancer from control patients. Further statistical reductions were carried out to produce the top 80 markers that went onto PCR screening. After several rounds of screening 13 markers showed robustness in their screening abilities, each having p-values >0.3. These 13 markers were then used to screen the 50 BrCa samples from the Memorial Cancer Center and Institute of Oncology, Gliwice Branch (IOG) plus 22 control patient samples. After the serial dilution nested PCR screen was carried out, the binary read out was then analysed to determine which markers were able to distinguish between BrCa and controls, 8 markers were finally narrowed down, please see table 16.

Marker	Probe
OBD116.53.55_8	ATM_11_108118137_108126372_108155279_108156687_RF
OBD116.89.103_8	CDC6_17_38421089_38423079_38451196_38457050_FF
OBD116.129.131_4	TSPYL5_8_98276431_98282736_98316421_98318720_FF
OBD116.173.175_2	ME3_11_86300063_86304401_86420537_86426200_FR
OBD116.177.179_8	SRD5A1_5_6634973_6639025_6667775_6669711_RF
OBD116.185.187_16	VAV3_1_108148303_108158073_108220200_108227533_RF
OBD116.197.199_8	FOXC1_6_1577253_1581989_1604206_1605973_FR
OBD116.301.303_2	*MSH3_5_80021913_80025030_80153948_80159012_RF*

Table 16 showing the final markers produced

The final stage in the analysis was to determine if the 8 markers could be used for diagnosis of breast cancer patients from control patients. An independent subset of 25 samples was used to run logistic modelling to determine if the markers could predict the samples correctly. Out of the 25 samples the markers showed 83.6% sensitivity and 91.0% specificity with a ROC value of 0.903.

The markers found by the *EpiSwitch*™ screen also show interesting features in cancer diagnosis. Ataxia telangiectasia mutated kinase (ATM) plays key role in DNA damage response, loss of function may lead to cancer development; they are also linked to signalling pathways in sustained tumour growth. ATM promotes tumourigencity of HER2 (Human epidermal growth factor receptor 2) in a positive breast cancer cell line. ATM participates in a trimeric compound with HSP90 (heat shock protein) and HER2 and has been identified in several tumours. Significant risk of breast cancer is associated with CHEK2, PALB2 and TP53, plus moderate risk with mutations in ATM.

The gene SLC16A10, Solute carrier family 16 (aromatic amino acid transporter), member 10, is involved in system T which is a Na+ independent transporter system that selectively transports aromatic amino acids it is known to be strongly expressed in kidney, liver and intestines. Among its related pathways are the transport of glucose and other sugars, bile salts and organic acids, metal ions and amine compounds, protein digestion and absorption. GO annotations related to this gene include *transporter activity*. The uniporter TAT1 (Slc16a10) is required to equilibrate the concentration of AAAs across specific membranes.

Vav3 is an oncogene that plays a significant role in prostate cancer tumorigenesis, it is also expressed and up regulated in breast cancer. Vav proteins are guanine nucleotide exchange factors for GTPases of the Rho family. They are involved in cell signalling and tumorigenesis. Vav3 enhances cell growth and proliferation. Breast and prostate cancers are hormone independent tumours whose growth is mediated by their respective hormone receptors. Vav3 is epigenetically regulated in the development of breast cancer.

MSH3, MutS homolog 3 has been associated with several different types of cancer such as colorectal, breast, prostate, bladder, thyroid, ovarian and oesophageal cancers. Mismatch repair pathways are involved in cell cycle regulation, apoptosis and DNA damage. In humans there are 7 mismatch repair genes, there has been 180 SNPs reported for the MSH3 gene. Loss of MSH3 protein expression is associated with colorectal cancer and a polymorphism rs26279G is associated with the risk of breast cancer.

FOXC1, Forkhead box C1 is a transcription factor involved in the development of mesoderm, brain and eye during embryogenesis, it may be a key diagnostic marker for basal like breast cancer. Elevated levels of FOXC1 predict poor survival in cancers such as lung and hepatocellular carcinoma. FOXC1 protein is exclusively expressed in basal cell. FOXC1 has been identified as a Smoothhead (SMO)-independent activator of Hedgehog signalling via direct interaction of Gli2 transcription factor.

These results show a very robust and specific set of markers, monitored as epigenetic deregulation at the level of 3D chromatin architecture of specific genetic loci, that can help stratify the breast cancer patient samples from control samples with a high degree of reliability.

Appendix II

Sample No	Patient's age at Diagnosis (yrs)	Clinical Diagnosis	NG	G	IM	Cat	ER	PR	HER2
			Histopathology						
10692	47.75	carcinoma infiltrans	3	3	IM: 21 mitotic figures/10HPF	B5	+++	+++	(-)
10693	66.91	Invasive ductal carcinoma	3	3	IM: 41/10 HPF. Type luminal B (HER 2 negative)	B5b	+	+	
10695	29.87	Invasive carcinoma of NST	3	3	IM: 25/10 HPF	B5b	+	+	+
10698	58.06	Invasive carcinoma NST	2	3	IM: 26 mitotic figures / 10 HPF Type: luminal B (HER-2 negative)	B5	+++	(-)	+
10715	49.85	carcinoma lobulare infiltrans	2	2	IM: 3 mitotic figures /10HPF. Type: luminal A	B5	+++	++	+
10717	67.58	Invasive lobular carcinoma	2	2	IM: 2/10 HPF		+++	+++	(-)
10726	37.39	Invasive carcinoma NST	3	3	IM 18/10 HPF. DCIS NG2 in single canals. Type: luminal B (HER 2 positive)	B5	++	+++	+++
10731	43.03	Invasive carcinoma	3	3	IM: 20/10 HPF. Wide necrosis	B5	+++	(-)	+++
10732	45.65	Invasive lobular carcinoma	2		LCIS. DCIS comedo type with microcalcfications. Type: luminal A	B5	+++	+++	+
10752	62.68	Invasive carcinoma NST		2	IM: 4/10 HPF	B5	+++	+++	++
10754	67.93	carcinoma ductale infiltrans	2	3	IM: 39 mitotic figures /10HPF Type: Luminal B (HER-2 negative)	B5	+++	+++	+
10764	34.31	Invasive carcinoma NST		3	IM: 18/10 HPF Triple negative (ductal).	B5b	(-)	(-)	(-)
10775	50.36	Invasive carcinoma NST		3	Severe lymphocyte infiltrates in microenvironment IM: 45 mitotic figures /10HPF Triple negative.	B5	+	(-)	+
10782	49.09	carcinoma lobulare infiltrans		X	LCIS Widening LCIS in adenoma region (adenosis). Type: luminal A	B5	+++	+++	(-)
10791	63.86	Invasive carcinoma	3	2	Invasive ductal carcinoma Type: luminal B (HER-2 negative).		+++	+++	+
10794	33.94	Invasive carcinoma NST et invasive micropapillary carcinoma	2	3	IM: 34 mitotic figures /10HPF	B5	+++	+	+++
10830	56.67	carcinoma ductale invasivum		2	IM: 11/10HPF		+++	+	
10832	45.39	Intraductal carcinoma in situ (CDIS) visible taking approx 10% of the tumour	2	2	Examined tissue biopsy of mixed type: approx. 90% - Carcinoma mucinosum; 10% - IM: 14/10HPF.		+++	++	+
10836	32.31	Carcinoma ductale invasivum NST	2	2	IM 9/10 HPF.				+
10853	62.82	Invasive carcinoma with low grade DCIS. NST	2	1	IM: 2/10 HPF. Type: luminal B HER 2 negative)	B5b	+++	+++	(-)
10855	38.75	Invasive carcinoma NST	3	3	IM:29 mitotic figures /10HPF Type: Luminal B (HER-2 negative)	B5	+++	+	(-)
10861	45.69	Invasive carcinoma NST	2	2	IM: 6/10 HPF Type: luminal B; (HER 2 positive).	B5b	+++	++	+++

10865	63.80	Invasive carcinoma NST	3	3	IM: 11/10 HPF. Triple negative (ductal). Type: basal-like	B5b	(-)	(-)	(-)
10876	47.10	Invasive carcinoma NST	3	3	IM: 37/10 HPF	B5b	+++	(-)	(-)
10883	48.65	Invasive carcinoma NST	3	3	IM: 31 mitotic figures / 10HPF Severe lymphocyte infiltrates in microenvironment. Type: Luminal B (HER-2 negative)	B5	+++	+++	(-)
10885	45.37	Invasive carcinoma NST	3	3	IM 44/10 HPF	B5b	(-)	(-)	++
10891	64.11	Invasive carcinoma	3	3	IM: 24/10 HPF. Severe lymphocyte infiltrates in microenvironment Triple negative	B5	(-)	(-)	(-)
10892	62.44	Invasive carcinoma NST	2	2	IM: 3/10 HPF. Type: luminal B (HER 2 negative).	B5b	+++	+++	+
10903	64.13	Invasive lobular carcinoma	3	3	Mitotic activity: 16 mitotic figures / 10 hpf. Type: luminal B (HER2 negative)	B5	+++	+++	+
10915	48.03	Invasive carcinoma	2	2	IM: 1/10 HPF Type: luminal B; HER 2 negative.	B5	+++	+++	+
10942	46.33	Invasive carcinoma	3	3	IM: 58 mitotic figures / 10 HPF Type: luminal B (HER-2 positive).	B5	+	+	+++
10947	52.78	NST	3	3	IM: 34/10 HPF Type: luminal B; HER 2 positive.	B5	+	(-)	+++
10955	68.82	Invasive carcinoma NST	2	2	IM: 3/10 HPF.Type: luminal B (HER 2 negative). Invasive carcinoma	B5b	+++	+	+
10963	65.42	invasive lobular carcinoma - tubulo- lobular variant	2	2	IM: 4/10 HPF A mixed tumour differentiating towards lobular and ductal carcinoma.	B5	+++	(-)	++
11015	32.48	Invasive carcinoma NST	3	3	IM:36/10 HPF	B5	(-)	(-)	(-)
11035	59.62	Invasive carcinoma NST	1		(diam. 2 mm). DCIS NG-2. DCIS	B5	+++	+	+
11036	47.37	Invasive carcinoma NST	2	1	IM: 2/10 HPF. DCIS NG2. Type: luminal B - HER 2 negative.	B5	+++	+++	+
11053	63.00	carcinoma ductale infiltrans	2	2	NG-2, G-2. DCIS NG-2, cribriform type with necrosis. Cat: B5.	B5			
11059	39.45	Invasive carcinoma NST	2	1	IM: 1/10 HPF. Multiple microcalcifications around tumour site. Type: luminal A		+++	+++	+
11081	54.28	Invasive carcinoma NST	3	X	DCIS with focal necrosis	B5	(-)	(-)	+++
11083	59.55	Invasive carcinoma NST	3	2	IM: 16 mitotic figures /10HPF DCIS NG-2 Type: Luminal B		+++	++	++
11097	36.19	Carcinoma ductale invasivum		2	Tumour diameter 1cm. Desmoplasia in microenvironment.		+++	+++	++
11099	44.00	Invasive carcinoma NST	3	3	IM: 59 mitotic figures / 10 HPF. Type: triple negative.	B5	(-)	(-)	+
11122	60.62	Invasive carcinoma NST	2		(B2) et DCIS NG-2, solid type with microcalcifications.	B5	+++	+	
11136	57.50	Invasive carcinoma NST	2	1	IM: 5 mitotic figures / 10 HPF	B5	+++	++	++
11153	43.38	Invasive carcinoma NST	2	2	IM 6/10 HPF	B5b	+++	++	+

11180	42.56	Invasive carcinoma NST	3	2	pT1c NO(sn) IM 8/10 HPF		+++	+++	(-)
11187	44.19	Invasive carcinoma	2	2	IM: 16/10 HPF. DCIS NG 2 cribriform type.	B5	+++	+++	(-)
11217	52.04	Invasive carcinoma of NST differentiation		3	index mit. IM [/10HPF] : 14/10		(-)	(-)	(-)
11245	35.35	Invasive carcinoma NST	3	3	IM: 13/10 HPF	B5	+++	+++	++

Table 17, BCa samples from Maria Sklodowska-Curie Memorial Cancer Center and Institute of Oncology, Gliwice Branch (IOG)

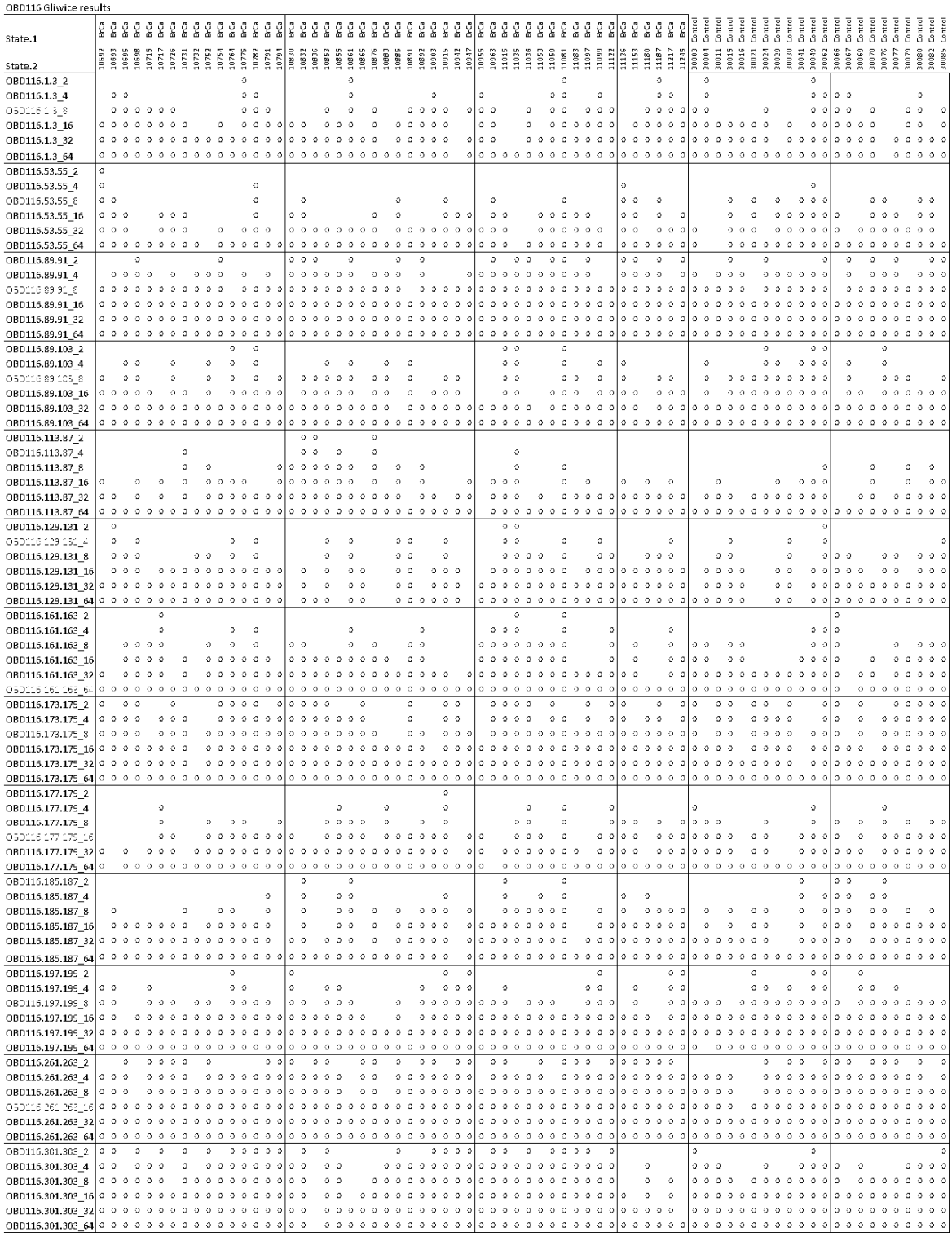

Table 18 Binary analysis results using the top 13 markers. Darker results showing a p-value > 0.3. Lighter results show best dilution.

5

Example 3

The work described in the Example concerns 13 nested markers which are typed by polymerase chain reaction (PCR) and qPCR (please see the Table 19 below). These markers were developed to differentiate patients with breast cancer from non-malignant individuals.

No	3C interactions identified by nested PCR
1	ATM_11_108118137_108126372_108155279_108156687_RF
2	CDC6_17_38421089_38423079_38451196_38457050_FF
3	CDC6_17_38421089_38423079_38467677_38474960_FR
4	FOXC1_6_1577253_1581989_1604206_1605973_FR
5	MAP3K1_5_56102259_56110500_56140227_56144076_FF
6	ME3_11_86300063_86304401_86420537_86426200_FR
7	MELK_9_36577630_36579243_36637050_36643005_RF
8	MSH3_5_80021913_80025030_80153948_80159012_RF
9	NF1_17_29477103_29483764_29651799_29657368_FF
10	SLC16A10_6_111441989_111447305_111492951_111498421_FR
11	SRD5A1_5_6634973_6639025_6667775_6669711_RF
12	TSPYL5_8_98276431_98282736_98316421_98318720_FF
13	VAV3_1_108148303_108158073_108220200_108227533_RF

The overview of the qPCR development is:

- nested PCR primers

- single-step SYBR PCR (temperature gradient optimised)

- gel purification

- fluorometer measurement, sequencing, homology and genomic mapping check

- hydrolysis probe optimisation

- std curve testing with patient samples.

The work was to identify epigenetic changes that can be used to discriminate breast cancer patients from non-malignant material using small volume blood samples.

Blood samples from the Asian cohort, which includes blood samples from the original test evaluation work, were to be used to validate the qPCR probe assays according to the MIQE (Minimum Information for the Publication of Quantitative Real-Time PCR Experiments) guidelines.

Each marker qPCR probe and individual detection assay was developed and tested across a temperature gradient on the representative pools of samples (4x4) to meet the following MIQE–compliant criteria for quality detection:

1. Specificity: the predicted PCR amplicon was validated with sequencing.
2. Linear standard curve ($R^2 > 0.98$).
3. Efficiency (E), E > 90%.
4. Genomic non-specific cross reaction controls used with all assays.

The requirement was that at least 70% of the nested marker would be developed for detection using hydrolysis probes with the performance of the assays meeting the four criteria described above.

EpiSwitch™ qPCR assay development data

CCS biomarkers were confirmed by nested PCR. All development PCRs were made using the QIAgility. 3C sample templates at 10 ng per well were screened using a single step temperature gradient PCR and SYBR based detection, with concentration matched negative controls. 10 interactions were identified and sequenced. The sequencing data was submitted to ENSEMBL and the genomic location of each of the predicted 3C interactions was confirmed using ENSEMBL Blat and the Needleman-Wunsch algorithm. The hydrolysis probes were designed for the junction region of each confirmed interaction and optimized by temperature gradient. All samples were positive for a stable independent 3C interaction (MMP1). All assays were tested with n = 8 patient samples (4 = breast cancer, 4 = non-malignant), standard curves and concentration matched negative controls.

The primer data from the development process including the original sequencing electropherograms and is presented in a clear easy to check format for each qPCR assay. The assays are in alphabetical order. Standards at 10^6 copies were used as a positive control during the probe temperature gradient optimisation. Patient samples were tested with curves between 1-10^6 copies. Any variations in analysis of the standard curves during the patient screening are noted for each assay described in the report.

EpiSwitch™ Summary of qPCR assays

ATM_11_108118137_108126372_108155279_108156687_RF

i. The 3C template amplified at single step.

ii. Lab Chip image. Comments: the amplicon is visible in paraformaldehyde fixed samples only (10 ng of template). The single-step PCR product is of expected size, 472 bp.

iii. ENSEMBL BLAT (Figure 1) after direct sequencing of the sample PCR product. Comments: the good quality sequencing (forward and reverse primers) has 100% homology to the predicted 3C interaction.

iv. The performance of the quantitative PCR assay standards. The standard curve is linear from 10^{2}-10^{6} copies. $R^2 = 0.996$.

v. One amplicon doubles to produce two = 100% efficient. Efficiency of assay = 91.7% (> 90% MIQE guideline).

vi. The assay shows profound copy number differences (Table 20) between the patient (n = 8) subsets (C01-C12 = breast cancer, D01 to D12 = non-malignant). SQ = starting quantity, copies in 20 ng of template. NaN = 0 copies.

ENSEMBL BLAT after direct sequencing of the sample PCR product.

This PCR product was sequenced and mapped to chromosome 11q22.3. The two 3C fragments are ligated at Taq I (TCGA). Above the sequence traces are the ENSEMBL BLAT mapping data (sequence homologies in red).

Table 19

Well	Fluor	Target	Content	Sample	Cq	Starting Quantity (SQ)
C01	FAM	032B 20 ng	Unkn	032B 20 ng	41.04	0.42
C02	FAM	032B 20 ng	Unkn	032B 20 ng	39.89	0.89
C03	FAM	032B 20 ng	Unkn	032B 20 ng	37.82	3.42
C04	FAM	063B 20 ng	Unkn	063B 20 ng	37.92	3.19
C05	FAM	063B 20 ng	Unkn	063B 20 ng	36.8	6.64
C06	FAM	063B 20 ng	Unkn	063B 20 ng	36.78	6.72
C07	FAM	065A 20 ng	Unkn	065A 20 ng	37.5	4.21
C08	FAM	065A 20 ng	Unkn	065A 20 ng	37.23	5.01
C09	FAM	065A 20 ng	Unkn	065A 20 ng	37.33	4.68
C10	FAM	124B 20 ng	Unkn	124B 20 ng	37.21	5.07

C11	FAM	124B 20 ng	Unkn	124B 20 ng	37.33	4.69
C12	FAM	124B 20 ng	Unkn	124B 20 ng	NaN	NaN
D01	FAM	005B 20 ng	Unkn	005B 20 ng	38.6	2.06
D02	FAM	005B 20 ng	Unkn	005B 20 ng	NaN	NaN
D03	FAM	005B 20 ng	Unkn	005B 20 ng	37.12	5.4
D04	FAM	007B 20 ng	Unkn	007B 20 ng	NaN	NaN
D05	FAM	007B 20 ng	Unkn	007B 20 ng	NaN	NaN
D06	FAM	007B 20 ng	Unkn	007B 20 ng	NaN	NaN
D07	FAM	17B 20 ng	Unkn	17B 20 ng	37.98	3.07
D09	FAM	17B 20 ng	Unkn	17B 20 ng	38.63	2.01
D10	FAM	022B 20 ng	Unkn	022B 20 ng	NaN	NaN
D11	FAM	022B 20 ng	Unkn	022B 20 ng	NaN	NaN
D12	FAM	022B 20 ng	Unkn	022B 20 ng	NaN	NaN
E07	FAM	Gen Neg Ctrl	Neg Ctrl	Gen Neg Ctrl	NaN	NaN
E08	FAM	Gen Neg Ctrl	Neg Ctrl	Gen Neg Ctrl	NaN	NaN
E09	FAM	No fix 20 ng	Neg Ctrl	No fix 20 ng	NaN	NaN
E10	FAM	No fix 20 ng	Neg Ctrl	No fix 20 ng	NaN	NaN
E11	FAM	ATE Ext	NTC	ATE Ext	NaN	NaN
E12	FAM	ATE Ext	NTC	ATE Ext	NaN	NaN
F01	FAM	ATE Post-PCR	NTC	ATE Post-PCR	NaN	NaN
F02	FAM	ATE Post-PCR	NTC	ATE Post-PCR	NaN	NaN
F03	FAM	Water	NTC	Water	NaN	NaN
F04	FAM	Water	NTC	Water	NaN	NaN = 0 copies

CDC6_17_38421089_38423079_38451196_38457050_FF

i. The 3C template amplified at single step.

ii. Lab Chip image. Comments: the band is visible in paraformaldehyde fixed samples only (10 ng of template). Single-step PCR product of expected size, 428 bp.

iii. ENSEMBL BLAT after direct sequencing of the sample PCR product. Comments: the good quality sequencing (forward and reverse primers) has 100% homology to the predicted 3C interaction.

iv. The performance of the quantitative PCR assay standards. The standard curve is linear from 10^{1}-10^{6}copies. R^2 = 0.99.

v. One amplicon doubles to produce two = 100% efficient. Efficiency 90.7% (> 90% MIQE guideline).

vi. The assay shows profound copy number differences (Table 2) between the patient (n = 8) subsets (C01-C12 = breast cancer, D01-D12 = non-malignant). SQ = starting quantity, copies in 20 ng of template. NaN = 0 copies.

Table 20

Well	Fluor	Target	Content	Sample	Cq	Starting Quantity (SQ)
C01	FAM	032B 20 ng	Unkn	032B 20 ng	39.63	1.93
C02	FAM	032B 20 ng	Unkn	032B 20 ng	38.14	5.05
C03	FAM	032B 20 ng	Unkn	032B 20 ng	38.80	3.30
C04	FAM	063B 20 ng	Unkn	063B 20 ng	37.18	9.40
C05	FAM	063B 20 ng	Unkn	063B 20 ng	37.35	8.40
C06	FAM	063B 20 ng	Unkn	063B 20 ng	37.41	8.10
C07	FAM	065A 20 ng	Unkn	065A 20 ng	36.32	16.34
C08	FAM	065A 20 ng	Unkn	065A 20 ng	36.36	15.92
C09	FAM	065A 20 ng	Unkn	065A 20 ng	36.75	12.41
C10	FAM	124B 20 ng	Unkn	124B 20 ng	34.76	44.71
C11	FAM	124B 20 ng	Unkn	124B 20 ng	NaN	NaN
C12	FAM	124B 20 ng	Unkn	124B 20 ng	NaN	NaN
D01	FAM	005B 20 ng	Unkn	005B 20 ng	36.24	17.24
D02	FAM	005B 20 ng	Unkn	005B 20 ng	36.14	18.42
D03	FAM	005B 20 ng	Unkn	005B 20 ng	35.53	27.29
D04	FAM	007B 20 ng	Unkn	007B 20 ng	36.57	13.97
D05	FAM	007B 20 ng	Unkn	007B 20 ng	37.49	7.68
D06	FAM	007B 20 ng	Unkn	007B 20 ng	36.60	13.68
D07	FAM	17B 20 ng	Unkn	17B 20 ng	36.01	19.97
D08	FAM	17B 20 ng	Unkn	17B 20 ng	37.43	8.01
D09	FAM	17B 20 ng	Unkn	17B 20 ng	36.75	12.38
D10	FAM	022B 20 ng	Unkn	022B 20 ng	NaN	NaN
D11	FAM	022B 20 ng	Unkn	022B 20 ng	39.06	2.80
D12	FAM	022B 20 ng	Unkn	022B 20 ng	36.75	12.41
E07	FAM	Gen Neg Ctrl	Neg Ctrl	Gen Neg Ctrl	NaN	NaN
E08	FAM	Gen Neg Ctrl	Neg Ctrl	Gen Neg Ctrl	NaN	NaN
E09	FAM	No fix 20 ng	Neg Ctrl	No fix 20 ng	NaN	NaN
E10	FAM	No fix 20 ng	Neg Ctrl	No fix 20 ng	NaN	NaN
E11	FAM	ATE Ext	NTC	ATE Ext	NaN	NaN
E12	FAM	ATE Ext	NTC	ATE Ext	NaN	NaN
F01	FAM	ATE Post-PCR	NTC	ATE Post-PCR	NaN	NaN
F02	FAM	ATE Post-PCR	NTC	ATE Post-PCR	NaN	NaN
F03	FAM	Water	NTC	Water	NaN	NaN
F04	FAM	Water	NTC	Water	NaN	NaN = 0 copies

FOXC1_6_1577253_1581989_1604206_1605973_FR.

i. The 3C template amplified at single step.

ii. Lab Chip image. Comments: the band is visible in paraformaldehyde fixed samples only (10 ng of template). Single-step PCR product of expected size, 208 bp.

iii. ENSEMBL BLAT after direct sequencing of the sample PCR product. Comments: the good quality sequencing (forward and reverse primers) has 100% homology to the predicted 3C interaction.

iv. The performance of the quantitative PCR assay standards. The standard curve is linear from 10^{1}-10^{6}copies. $R^2 = 0.992$.

v. One amplicon doubles to produce two = 100% efficient. The efficiency of this assay was 101.6%, (> 90% MIQE guideline).

vi. The assay shows profound copy number differences (Table 21) between the patient (n = 8) subsets (C01-C12 = breast cancer, D01-D12 = non-malignant). SQ = starting quantity, copies in 20 ng of template. NaN = 0 copies.

ENSEMBL BLAT after direct sequencing of the sample PCR product.

The Lab Chip image of the FOXC1 well B7 208 bp single step amplification (inner primers). This PCR product was sequenced and mapped to chromosome 6p.

Table 21

Well	Fluor	Target	Content	Sample	Cq	Starting Quantity (SQ)
C01	FAM	032B 20 ng	Unkn	032B 20 ng	36.16	3.05
C02	FAM	032B 20 ng	Unkn	032B 20 ng	NaN	NaN
C03	FAM	032B 20 ng	Unkn	032B 20 ng	35.80	3.92
C04	FAM	063B 20 ng	Unkn	063B 20 ng	38.13	0.77
C05	FAM	063B 20 ng	Unkn	063B 20 ng	NaN	NaN
C06	FAM	063B 20 ng	Unkn	063B 20 ng	37.49	1.19
C07	FAM	065A 20 ng	Unkn	065A 20 ng	33.48	19.86
C08	FAM	065A 20 ng	Unkn	065A 20 ng	35.45	4.99
C09	FAM	065A 20 ng	Unkn	065A 20 ng	37.30	1.36
C10	FAM	142 20 ng	Unkn	142 20 ng	34.89	7.39
C11	FAM	142 20 ng	Unkn	142 20 ng	34.05	13.35
C12	FAM	142 20 ng	Unkn	142 20 ng	NaN	NaN
D01	FAM	005B 20 ng	Unkn	005B 20 ng	34.05	13.38
D02	FAM	005B 20 ng	Unkn	005B 20 ng	33.72	16.81
D03	FAM	005B 20 ng	Unkn	005B 20 ng	33.52	19.37
D04	FAM	007B 20 ng	Unkn	007B 20 ng	34.87	7.54
D05	FAM	007B 20 ng	Unkn	007B 20 ng	35.17	6.08

D06	FAM	007B 20 ng	Unkn	007B 20 ng	33.62	18.02
D07	FAM	17B 20 ng	Unkn	17B 20 ng	33.57	18.72
D08	FAM	17B 20 ng	Unkn	17B 20 ng	35.18	6.04
D09	FAM	17B 20 ng	Unkn	17B 20 ng	34.50	9.71
D10	FAM	022B 20 ng	Unkn	022B 20 ng	35.58	4.57
D11	FAM	022B 20 ng	Unkn	022B 20 ng	33.97	14.13
D12	FAM	022B 20 ng	Unkn	022B 20 ng	34.97	7.00
E07	FAM	Gen Neg Ctrl	Neg Ctrl	Gen Neg Ctrl	NaN	NaN
E08	FAM	Gen Neg Ctrl	Neg Ctrl	Gen Neg Ctrl	NaN	NaN
E09	FAM	No fix 20 ng	Neg Ctrl	No fix 20 ng	NaN	NaN
E10	FAM	No fix 20 ng	Neg Ctrl	No fix 20 ng	NaN	NaN
E11	FAM	ATE Ext	NTC	ATE Ext	NaN	NaN
E12	FAM	ATE Ext	NTC	ATE Ext	NaN	NaN
F01	FAM	ATE Post-PCR	NTC	ATE Post-PCR	NaN	NaN
F02	FAM	ATE Post-PCR	NTC	ATE Post-PCR	NaN	NaN
F03	FAM	Water	NTC	Water	NaN	NaN
F04	FAM	Water	NTC	Water	NaN	NaN = 0 copies

MAP3K1_5_56102259_56110500_56140227_56144076_FF

i. The 3C template amplified at single step.

ii. Lab Chip image. Comments: the amplicon is visible in paraformaldehyde fixed samples only (10 ng of template). The single-step PCR product is of expected size, 495 bp.

iii. ENSEMBL BLAT after direct sequencing of the sample PCR product. Comments: the good quality sequencing (forward and reverse primers) has 100% homology to the predicted 3C interaction.

iv. The performance of the quantitative PCR assay standards. The standard curve is linear from 10^{2}-10^{6} copies. $R^2 = 0.999$.

v. One amplicon doubles to produce two = 100% efficient. Efficiency of assay = 91.9 % (> 90% MIQE guideline).

vi. Copy number differences (Table 22) between the patient (n = 8) subsets (C01-C12 = breast cancer, D01-D12 = non-malignant). SQ = starting quantity, copies in 20 ng of template. NaN = 0 copies.

ENSEMBL BLAT after direct sequencing of the sample PCR product.

This PCR product was sequenced and mapped to chromosome 5q11.2.

Table 22

Well	Fluor	Target	Content	Sample	Cq	Starting Quantity (SQ)
C01	FAM	032B 20 ng	Unkn	032B 20 ng	NaN	NaN
C02	FAM	032B 20 ng	Unkn	032B 20 ng	38.94	3.68
C03	FAM	032B 20 ng	Unkn	032B 20 ng	40.24	1.58
C04	FAM	063B 20 ng	Unkn	063B 20 ng	38.22	5.87
C05	FAM	063B 20 ng	Unkn	063B 20 ng	37.85	7.48
C06	FAM	063B 20 ng	Unkn	063B 20 ng	38.05	6.58
C07	FAM	065A 20 ng	Unkn	065A 20 ng	38.01	6.75
C08	FAM	065A 20 ng	Unkn	065A 20 ng	37.72	8.14
C09	FAM	065A 20 ng	Unkn	065A 20 ng	39.02	3.50
C10	FAM	142 20 ng	Unkn	124B 20 ng	37.40	10.02
C11	FAM	142 20 ng	Unkn	124B 20 ng	38.74	4.19
C12	FAM	142 20 ng	Unkn	124B 20 ng	39.94	1.92
D01	FAM	005B 20 ng	Unkn	005B 20 ng	42.07	0.48
D02	FAM	005B 20 ng	Unkn	005B 20 ng	37.03	12.77
D03	FAM	005B 20 ng	Unkn	005B 20 ng	38.12	6.29
D04	FAM	007B 20 ng	Unkn	007B 20 ng	39.94	1.92
D05	FAM	007B 20 ng	Unkn	007B 20 ng	39.27	2.97
D06	FAM	007B 20 ng	Unkn	007B 20 ng	37.04	12.66
D07	FAM	17B 20 ng	Unkn	17B 20 ng	36.62	16.75
D08	FAM	17B 20 ng	Unkn	17B 20 ng	38.11	6.33
D09	FAM	17B 20 ng	Unkn	17B 20 ng	38.06	6.54
D10	FAM	022B 20 ng	Unkn	022B 20 ng	37.38	10.18
D11	FAM	022B 20 ng	Unkn	022B 20 ng	38.85	3.90
D12	FAM	022B 20 ng	Unkn	022B 20 ng	NaN	NaN
E07	FAM	Gen Neg Ctrl	Neg Ctrl	Gen Neg Ctrl	NaN	NaN
E08	FAM	Gen Neg Ctrl	Neg Ctrl	Gen Neg Ctrl	NaN	NaN
E09	FAM	No fix 20 ng	Neg Ctrl	No fix 20 ng	NaN	NaN
E10	FAM	No fix 20 ng	Neg Ctrl	No fix 20 ng	NaN	NaN
E11	FAM	ATE Ext	NTC	ATE Ext	NaN	NaN
E12	FAM	ATE Ext	NTC	ATE Ext	NaN	NaN
F01	FAM	ATE Post-PCR	NTC	ATE Post-PCR	NaN	NaN
F02	FAM	ATE Post-PCR	NTC	ATE Post-PCR	NaN	NaN
F03	FAM	Water	NTC	Water	NaN	NaN
F04	FAM	Water	NTC	Water	NaN	NaN = 0 copies

ME3_11_86300063_86304401_86420537_86426200_FR

i. The 3C template amplified at single step.

ii. Lab Chip image. Comments: the amplicon is visible in paraformaldehyde fixed samples only (10 ng of template). The single-step PCR product is of expected size, 291 bp.

iii. ENSEMBL BLAT after direct sequencing of the sample PCR product. Comments: the good quality sequencing (forward and reverse primers) has 100% homology to the predicted 3C interaction.

iv. The performance of the quantitative PCR assay standards. The standard curve is linear from 10^{2}-10^{6} copies. R^2 = 0.998.

v. One amplicon doubles to produce two = 100% efficient. Efficiency of assay = 96.8 % (> 90% MIQE guideline).

vi. The assay differences (Table 5) between the patient (n = 8) subsets (C01-C12= breast cancer, D01-D12 = non-malignant). SQ = starting quantity, copies in 20 ng of template. NaN = 0 copies.

Table 23

Well	Fluor	Target	Content	Sample	Cq	Starting Quantity (SQ)
C01	FAM	032B 20 ng	Unkn	032B 20 ng	NaN	NaN
C02	FAM	032B 20 ng	Unkn	032B 20 ng	NaN	NaN
C03	FAM	032B 20 ng	Unkn	032B 20 ng	40.29	0.63
C04	FAM	063B 20 ng	Unkn	063B 20 ng	40.51	0.54
C05	FAM	063B 20 ng	Unkn	063B 20 ng	41.88	0.21
C06	FAM	063B 20 ng	Unkn	063B 20 ng	41.21	0.34
C07	FAM	065A 20 ng	Unkn	065A 20 ng	40.75	0.46
C08	FAM	065A 20 ng	Unkn	065A 20 ng	41.84	0.22
C09	FAM	065A 20 ng	Unkn	065A 20 ng	41.59	0.26
C10	FAM	142 20 ng	Unkn	142 20 ng	NaN	NaN
C11	FAM	142 20 ng	Unkn	142 20 ng	NaN	NaN
C12	FAM	142 20 ng	Unkn	142 20 ng	NaN	NaN
D01	FAM	005B 20 ng	Unkn	005B 20 ng	42.62	0.13
D02	FAM	005B 20 ng	Unkn	005B 20 ng	39.44	1.11
D03	FAM	005B 20 ng	Unkn	005B 20 ng	NaN	NaN
D04	FAM	007B 20 ng	Unkn	007B 20 ng	NaN	NaN
D05	FAM	007B 20 ng	Unkn	007B 20 ng	NaN	NaN
D06	FAM	007B 20 ng	Unkn	007B 20 ng	43.48	0.07
D07	FAM	17B 20 ng	Unkn	17B 20 ng	NaN	NaN
D08	FAM	17B 20 ng	Unkn	17B 20 ng	41.34	0.31
D09	FAM	17B 20 ng	Unkn	17B 20 ng	40.22	0.66
D10	FAM	022B 20 ng	Unkn	022B 20 ng	NaN	NaN
D11	FAM	022B 20 ng	Unkn	022B 20 ng	NaN	NaN
D12	FAM	022B 20 ng	Unkn	022B 20 ng	NaN	NaN
E07	FAM	Gen Ctrl	Neg Ctrl	Gen Ctrl	NaN	NaN
E08	FAM	Gen Ctrl	Neg Ctrl	Gen Ctrl	NaN	NaN
E09	FAM	No fix 20 ng	Neg Ctrl	No fix 20 ng	NaN	NaN
E10	FAM	No fix 20 ng	Neg Ctrl	No fix 20 ng	NaN	NaN
E11	FAM	ATE Ext	NTC	ATE Ext	NaN	NaN
E12	FAM	ATE Ext	NTC	ATE Ext	NaN	NaN
F01	FAM	ATE Post-PCR	NTC	ATE Post-PCR	NaN	NaN
F02	FAM	ATE Post-PCR	NTC	ATE Post-PCR	NaN	NaN = 0 copies

MELK_9_36577630_36579243_36637050_36643005_RF

i. The 3C template amplified at single step.

ii. Lab Chip image. Comments: the amplicon is visible in paraformaldehyde fixed samples only (10 ng of template). The single-step PCR product is of expected size, 265 bp.

iii. ENSEMBL BLAT after direct sequencing of the sample PCR product. Comments: the good quality sequencing (forward and reverse primers) has 100% homology to the predicted 3C interaction.

iv. The performance of the quantitative PCR assay standards. The standard curve is linear from 10^{2}-10^{6} copies. $R^2 = 0.995$.

v. One amplicon doubles to produce two = 100% efficient. Efficiency of assay = 91.3% (> 90% MIQE guideline).

vi. The assay differences (Table 24) between the patient (n = 8) subsets (C01-C12 = breast cancer, D01-D12 = non-malignant). SQ = starting quantity, copies in 20 ng of template. NaN = 0 copies.

ENSEMBL BLAT after direct sequencing of the sample PCR product.

This PCR product was sequenced and mapped to chromosome 9p13.2.

Table 24

Well	Fluor	Target	Content	Sample	Cq	Starting Quantity (SQ)
C01	FAM	032B 20 ng	Unkn	032B 20 ng	NaN	NaN
C02	FAM	032B 20 ng	Unkn	032B 20 ng	38.94	3.68
C03	FAM	032B 20 ng	Unkn	032B 20 ng	40.24	1.58
C04	FAM	063B 20 ng	Unkn	063B 20 ng	38.22	5.87
C05	FAM	063B 20 ng	Unkn	063B 20 ng	37.85	7.48
C06	FAM	063B 20 ng	Unkn	063B 20 ng	38.05	6.58
C07	FAM	065A 20 ng	Unkn	065A 20 ng	38.01	6.75
C08	FAM	065A 20 ng	Unkn	065A 20 ng	37.72	8.14
C09	FAM	065A 20 ng	Unkn	065A 20 ng	39.02	3.5
C10	FAM	142 20 ng	Unkn	124B 20 ng	37.4	10.02
C11	FAM	142 20 ng	Unkn	124B 20 ng	38.74	4.19
C12	FAM	142 20 ng	Unkn	124B 20 ng	39.94	1.92
D01	FAM	005B 20 ng	Unkn	005B 20 ng	42.07	0.48
D02	FAM	005B 20 ng	Unkn	005B 20 ng	37.03	12.77
D03	FAM	005B 20 ng	Unkn	005B 20 ng	38.12	6.29
D04	FAM	007B 20 ng	Unkn	007B 20 ng	39.94	1.92
D05	FAM	007B 20 ng	Unkn	007B 20 ng	39.27	2.97
D06	FAM	007B 20 ng	Unkn	007B 20 ng	37.04	12.66
D07	FAM	17B 20 ng	Unkn	17B 20 ng	36.62	16.75

D08	FAM	17B 20 ng	Unkn	17B 20 ng	38.11	6.33
D09	FAM	17B 20 ng	Unkn	17B 20 ng	38.06	6.54
D10	FAM	022B 20 ng	Unkn	022B 20 ng	37.38	10.18
D11	FAM	022B 20 ng	Unkn	022B 20 ng	38.85	3.9
D12	FAM	022B 20 ng	Unkn	022B 20 ng	NaN	NaN
E07	FAM	Gen Neg Ctrl	Neg Ctrl	Gen Neg Ctrl	NaN	NaN
E08	FAM	Gen Neg Ctrl	Neg Ctrl	Gen Neg Ctrl	NaN	NaN
E09	FAM	No fix 20 ng	Neg Ctrl	No fix 20 ng	NaN	NaN
E10	FAM	No fix 20 ng	Neg Ctrl	No fix 20 ng	NaN	NaN
E11	FAM	ATE Ext	NTC	ATE Ext	NaN	NaN
E12	FAM	ATE Ext	NTC	ATE Ext	NaN	NaN
F01	FAM	ATE Post-PCR	NTC	ATE Post-PCR	NaN	NaN
F02	FAM	ATE Post-PCR	NTC	ATE Post-PCR	NaN	NaN
F03	FAM	Water	NTC	Water	NaN	NaN
F04	FAM	Water	NTC	Water	NaN	NaN = 0 copies

MSH3_5_80021913_80025030_80153948_80159012_RF

i. The 3C template amplified at single step.

ii. Lab Chip image. Comments: the amplicon is visible in paraformaldehyde fixed samples only (10 ng of template). The single-step PCR product is of expected size, 207 bp.

iii. ENSEMBL BLAT after direct sequencing of the sample PCR product. Comments: the good quality sequencing (forward and reverse primers) has 100% homology to the predicted 3C interaction.

iv. The performance of the quantitative PCR assay standards. The standard curve is linear from 10^{2}-10^{6} copies. $R^2 = 0.99$.

v. One amplicon doubles to produce two = 100% efficient. Efficiency of assay = 97.1% (> 90% MIQE guideline).

vi. The assay differences (Table 25) between the patient (n = 8) subsets (C01-C12 = breast cancer, D01-D12 = non-malignant). SQ = starting quantity, copies in 20 ng of template. NaN = 0 copies.

ENSEMBL BLAT after direct sequencing of the sample PCR product.

This PCR product was sequenced and mapped to chromosome 5q14.1.

Table 25

Well	Fluor	Target	Content	Sample	Cq	Starting Quantity (SQ)
C01	FAM	032B 20 ng	Unkn	032B 20 ng	NaN	NaN
C02	FAM	032B 20 ng	Unkn	032B 20 ng	NaN	NaN
C03	FAM	032B 20 ng	Unkn	032B 20 ng	NaN	NaN

C04	FAM	063B 20 ng	Unkn	063B 20 ng	NaN	NaN
C05	FAM	063B 20 ng	Unkn	063B 20 ng	38.99	7.08
C06	FAM	063B 20 ng	Unkn	063B 20 ng	NaN	NaN
C07	FAM	065A 20 ng	Unkn	065A 20 ng	NaN	NaN
C08	FAM	065A 20 ng	Unkn	065A 20 ng	NaN	NaN
C09	FAM	065A 20 ng	Unkn	065A 20 ng	NaN	NaN
C10	FAM	142 20 ng	Unkn	142 20 ng	NaN	NaN
C11	FAM	142 20 ng	Unkn	142 20 ng	NaN	NaN
C12	FAM	142 20 ng	Unkn	142 20 ng	41.30	1.48
D01	FAM	005B 20 ng	Unkn	005B 20 ng	NaN	NaN
D02	FAM	005B 20 ng	Unkn	005B 20 ng	NaN	NaN
D03	FAM	005B 20 ng	Unkn	005B 20 ng	NaN	NaN
D04	FAM	007B 20 ng	Unkn	007B 20 ng	NaN	NaN
D05	FAM	007B 20 ng	Unkn	007B 20 ng	41.01	1.80
D06	FAM	007B 20 ng	Unkn	007B 20 ng	NaN	NaN
D07	FAM	17B 20 ng	Unkn	17B 20 ng	NaN	NaN
D08	FAM	17B 20 ng	Unkn	17B 20 ng	NaN	NaN
D09	FAM	17B 20 ng	Unkn	17B 20 ng	NaN	NaN
D10	FAM	022B 20 ng	Unkn	022B 20 ng	39.09	6.63
D11	FAM	022B 20 ng	Unkn	022B 20 ng	NaN	NaN
D12	FAM	022B 20 ng	Unkn	022B 20 ng	NaN	NaN
E07	FAM	Gen Neg Ctrl	Neg Ctrl	Gen Neg Ctrl	NaN	NaN
E08	FAM	Gen Neg Ctrl	Neg Ctrl	Gen Neg Ctrl	NaN	NaN
E09	FAM	No fix 20 ng	Neg Ctrl	No fix 20 ng	NaN	NaN
E10	FAM	No fix 20 ng	Neg Ctrl	No fix 20 ng	NaN	NaN
E11	FAM	ATE Ext	NTC	ATE Ext	NaN	NaN
E12	FAM	ATE Ext	NTC	ATE Ext	NaN	NaN
F01	FAM	ATE Post PCR	NTC	ATE Post PCR	NaN	NaN
F02	FAM	ATE Post PCR	NTC	ATE Post PCR	NaN	NaN
F03	FAM	Water	NTC	Water	NaN	NaN
F04	FAM	Water	NTC	Water	NaN	NaN = 0 copies

NF1_17_29477103_29483764_29651799_29657368_FF

i. The 3C template amplified at single step.

ii. Lab Chip image. Comments: the amplicon of expected size is visible in paraformaldehyde fixed samples only (10 ng of template). The single-step PCR product is of expected size, 401 bp.

iii. ENSEMBL BLAT after direct sequencing of the sample PCR product. Comments: the good quality sequencing (forward and reverse primers) has 100% homology to the predicted 3C interaction.

iv. The performance of the quantitative PCR assay standards. The standard curve is linear from 10^{2}-10^{6} copies. $R^2 = 0.987$.

v. One amplicon doubles to produce two = 100% efficient. Efficiency of assay = 99% (> 90% MIQE guideline).

vi. The assay shows differences (Table 26) between the patient (n = 8) subsets (C01-C12 = breast cancer, D01-D12 = non-malignant). SQ = starting quantity, copies in 20 ng of template. NaN = 0 copies.

Table 26

Well	Fluor	Target	Content	Sample	Cq	Starting Quantity (SQ)
C01	FAM	032B 20 ng	Unkn	032B 20 ng	NaN	NaN
C02	FAM	032B 20 ng	Unkn	032B 20 ng	NaN	NaN
C03	FAM	032B 20 ng	Unkn	032B 20 ng	38.29	3.69
C04	FAM	063B 20 ng	Unkn	063B 20 ng	38.94	2.36
C05	FAM	063B 20 ng	Unkn	063B 20 ng	36.45	13.11
C06	FAM	063B 20 ng	Unkn	063B 20 ng	39.37	1.76
C07	FAM	065A 20 ng	Unkn	065A 20 ng	41.96	0.30
C08	FAM	065A 20 ng	Unkn	065A 20 ng	NaN	NaN

C09	FAM	065A 20 ng	Unkn	065A 20 ng	37.18	7.92
C10	FAM	142 20 ng	Unkn	142 20 ng	NaN	NaN
C11	FAM	142 20 ng	Unkn	142 20 ng	NaN	NaN
C12	FAM	142 20 ng	Unkn	142 20 ng	41.77	0.34
D01	FAM	005B 20 ng	Unkn	005B 20 ng	38.43	3.36
D02	FAM	005B 20 ng	Unkn	005B 20 ng	NaN	NaN
D03	FAM	005B 20 ng	Unkn	005B 20 ng	NaN	NaN
D04	FAM	007B 20 ng	Unkn	007B 20 ng	NaN	NaN
D05	FAM	007B 20 ng	Unkn	007B 20 ng	40.95	0.60
D06	FAM	007B 20 ng	Unkn	007B 20 ng	NaN	NaN
D07	FAM	17B 20 ng	Unkn	17B 20 ng	37.66	5.73
D08	FAM	17B 20 ng	Unkn	17B 20 ng	NaN	NaN
D09	FAM	17B 20 ng	Unkn	17B 20 ng	NaN	NaN
D10	FAM	022B 20 ng	Unkn	022B 20 ng	38.33	3.59
D11	FAM	022B 20 ng	Unkn	022B 20 ng	NaN	NaN
D12	FAM	022B 20 ng	Unkn	022B 20 ng	NaN	NaN
E07	FAM	Gen Ctrl	Neg Ctrl	Gen Ctrl	NaN	NaN
E08	FAM	Gen Ctrl	Neg Ctrl	Gen Ctrl	NaN	NaN
E09	FAM	No fix 20 ng	Neg Ctrl	No fix 20 ng	NaN	NaN
E10	FAM	No fix 20 ng	Neg Ctrl	No fix 20 ng	NaN	NaN
E11	FAM	ATE Ext	NTC	ATE Ext	NaN	NaN
E12	FAM	ATE Ext	NTC	ATE Ext	NaN	NaN
F01	FAM	ATE Post PCR	NTC	ATE Post PCR	NaN	NaN
F02	FAM	ATE Post PCR	NTC	ATE Post PCR	NaN	NaN
F03	FAM	Water	NTC	Water	NaN	NaN
F04	FAM	Water	NTC	Water	NaN	NaN = 0 copies

SRD5A1_5_6634973_6639025_6667775_6669711_RF

i. The 3C template amplified at single step.

ii. Lab Chip image. Comments: the amplicon is visible in paraformaldehyde fixed samples only (10 ng of template). The single-step PCR product is of expected size, 219 bp.

iii. ENSEMBL BLAT after direct sequencing of the sample PCR product. Comments: the good quality sequencing (forward and reverse primers) has 100% homology to the predicted 3C interaction.

iv. The performance of the quantitative PCR assay standards. The standard curve is linear from 10^{2}-10^{6} copies. R^2 = 0. 997.

v. One amplicon doubles to produce two = 100% efficient. Efficiency of assay = 95.5% (> 90% MIQE guideline).

TSPYL5_8_98276431_98282736_98316421_98318720_FF

i. The 3C template amplified at single step.

ii. Lab Chip image. Comments: the amplicon is visible in paraformaldehyde fixed samples only (10 ng of template). The single-step PCR product is of expected size, 507 bp.

iii. ENSEMBL BLAT after direct sequencing of the sample PCR product. Comments: the good quality sequencing (forward and reverse primers) has 100% homology to the predicted 3C interaction.

iv. The performance of the quantitative PCR assay standards. The standard curve is linear from 10^{2}-10^{6} copies. R^2 = 0.998.

v. One amplicon doubles to produce two = 100% efficient. Efficiency of assay =94.2% (> 90% MIQE guideline).

Conclusions

1. The 3C markers ATM, FOXC1 and TSPYL1 produced single-step products for both sets of primers.

2. ATM copy number is increased in breast cancer (n = 4, Table 1). Samples in row C (malignant late stage disease breast cancer) differ from row D (non-malignant early stage) with a p-value of 0.009037772.
3. CDC6_FF copy number is reduced in breast cancer (n = 4, Table 2).
4. FOXC1_FR copy number is reduced in breast cancer. Row C differs from row D with a p-value of 0.004112668.

Name of the predicted interaction : ATM_11_108118137_108126372_108155279_108156687_RF

Sequence of the probe. TACGTTCAACTTCGACTGTATTCTACAA

Sequence of forward primer. GCAAGTTCCTTAGTTGCTTAG

Sequence of reverse primer. CAACCATCATCACTAATTCTGG (RC), CCAGAATTAGTGATGATGGTTG.

Position of PCR sequence BLAT.

Genomic Location	Overlapping Gene(s)	Orientation	Query start	Query end	Query ori	Length	Score	E-val	%ID
11:108285708-108285962	ATM	Forward	3	257	Forward	255 [Sequence]	495	2.80E-140	100.00 [Alignment]
11:108247409-108247583	ATM	Forward	254	428	Forward	175 [Sequence]	338	4.40E-93	100.00 [Alignment]
4:55715682-55715761		Forward	345	424	Forward	80 [Sequence]	97	2.30E-20	81.25 [Alignment]
1:10700828-10700899	CASZ1	Reverse	345	419	Forward	75 [Sequence]	85	6.20E-17	81.33 [Alignment]

Please note the first two rows are the true homologies.

>ATM_11_108118137_108126372_108155279_108156687_RF

Underlined = forward, Double underlined = reverse, Dotted Underlined= Taq I.

AAGGGATAAGTAACCAAACTTGGTCAATATTAGATAAACTTCAAGGGACCTTTTTTTTTTTTAGTTTCCTAGTT
ATCTATATTGAACCAAGAAATGGAACAGCAAGTTCCTTAGTTGCTTAGGTGGACCTATTCAGAACTGGTTGTAA
GTCTGCAGTCTGAAGGGAAATGGTGAGCAGAGGACTCCTTTCCCAAAGACAGCTGGAACAGAAATAGGCACTCC
AGAGGTTATGGAATTTGAGAGAGATACTCAGCCTCTAGCCACTCCCATTCAATCTCCCAGCTTAGTCTTCTGAG
CATTCTTAATCTTACTATTCTTTTCTTAATGTATTCAAACCAAAAGACAGCAATTTTTAGAGCCTGAATAGGTT
TTGGAGGGAAAAGTAATTACGTTCAACTTCGACTGTATTCTACAAAGTGCTGGGATTACAGGCATGAGCCACCA
AGCCCAGTCTGTTTCTTTTTTGCAATTAAGCTAGAGTTCACATAGCATAAAATTCACGATTTTGAGTTGTACAT
TTCAGTGGTTTTTAGTATTTTTACTATGTTGTACAACCATCATCACTAATTCTGGAAACTTTTTTTATTTTATT
TTTATTTTTTTGAGATGGAGTCTTGCTCTGTCACCCAGGCTGGAGTGCAGTGGCACAATCTCCGCTCACTGCAA
CCTCCCTTTCCCGGGTTCAAGTGATTCTCCTGCATCAGCCTCCCGAGTAGCTGGGACTACAGGTGCCTGCCACC
ACGCCCAGCTAATTTTTGTTTTTTTAGTAGAGACTGGGTTTCACCATATTGGCCAGCCTA

Name of the predicted interaction : CDC6_17_38421089_38423079_38451196_38457050_FF									
Sequence of the probe. AAAGAGAATGTGATCGATTTCTAAAATACT									
Sequence of forward primer. GGGTTCAAGAATGCAGGAATAG									
Sequence of reverse primer. GTATAGTCACATGGTGGCAA (RC), TTGCCACCATGTGACTATAC									
Genomic Position of PCRs									
Genomic Location	Overlapping Gene(s)	Orientation	Query start	Query end	Query ori	Length	Score	E-val	%ID
17:40266532-40266830 [Sequence]	WIPF2	Reverse	43	341	Forward	299 [Sequence]	576	9.90E-165	99.33 [Alignment]
17:40300754-40300800 [Sequence]	CDC6	Forward	1	47	Forward	47 [Sequence]	91	1.60E-18	100.00 [Alignment]

>CDC6_17_38421089_38423079_38451196_38457050_FF

AGGTAAGTTAAAGACCAAGAACTGGCATTGGTCTTAGTATCATGGGACCCTTTTGAGTAGTTTCAGTGGAGTGG
TGGAGGGTGAAAGTGAAAGCTTAATTGGAGTGGGTTCAAGAATGCAGGAATAGGAGGAGAGAAATTGGAGATAG
CAATATAGAAATCTCTTAAAGAGTTCGCTGTAAAGTCCAGGAGAGAGGGGTGAAGATAAGTGAAGTGATTGTTG
GACGAAGATGTGGGGTTGAGAGTTGTTTTTTTCCCATCCCAAGATGGGAGACCTATTTGTATGCTGATGGAATG
AGTAGCATGAAACTTAGGAGAGAGGGAAAAAATTGAATCAGAAGAGAGGGAACAGATTGCCTGAATAATGACCT
GGAGGAGGCCAGAGAAAAGAGAATGTGATCGATTTCTAAAATACTGTGTGTGTGTATGTATGATGAGACAGATG
ATACTAAGTTTGAGCTAAAGGAAATTCAAGTATAGTCACATGGTGGCAAAGCAGAGGTTTTAAATCTCTAACCA
GAGGCCAAAGGATGAGAGATAATGCTATTCTCTTAAGGATGTCAAAATAATGTGGGATGACTTGAAAAGTAGGG
TTACCCTTTCTCTGGGCCAAATAGTGAGCTGTTTTGTCCTATGGAATGTAATTTAATGTCAGAGGAACAAAACC

CACCTCATGAAAGGACCAGAGAACTACTGTATTTTTTTTTGGGACAGGATCTCTGTCACTCAGGCTGGAGTACA
GTGGCACTATCATGGCTCACTGCAGCCTTGGCTTCCTGGGTTCAAGTGATCCTCCTGCCT

Name of the predicted interaction : FOXC1_6_1577253_1581989_1604206_1605973_FR.
Sequence of the probe: AACCGGTTTCGATGCTGTTGTGCCT
Sequence of forward primer GGGACACACGTTAGTCAAG
Sequence of reverse primer CTGGAAGGAATGCGTAGC (RC), GCTACGCATTCCTTCCAG.

Position of PCR sequence. BLAT.

Genomic Location	Overlapping Gene(s)	Orientation	Query start	Query end	Query ori	Length	Score	E-val	%ID
6:1603970-1604208 [Sequence]		Forward	157	395	Forward	239 [Sequence]	465	3.90E-131	100.00 [Alignment]
6:1581597-1581752 [Sequence]		Forward	1	156	Forward	156 [Sequence]	307	9.60E-84	100.00 [Alignment]

>FOXC1_6_1577253_1581989_1604206_1605973_FR

CGCCGTCCCAGCAGCGCCCCATCTCACCAACTCCCACCTTCATGTGTGGCCGCCCACCTAGAGCCATGCCTGAA
GCCACTGTCCCTGACCACAAAGCTTTTGGCTGATAGGAAGCATGACAGCACTGGGGCCCTACACTGGAAGCGGG
ACCGTCCAGAGAAGAAGACTGCGCACAGGGATCGGGAGCTGGGACACACGTTAGTCAAGGTGTACGAGGGAGGA
ATCACCGCCATGTGGAGCCACTACTCGGGGAGGACGTGGGCCACCCGGAGCTCAGTGACAGTACTCCCGGGAGT
GTACATCGTTGGTAATGTCCACGACAGTGTCCCTGCCTGTGACCCAATAATTTCCCATCCAGGGACACACTTCA
CAGAAATGCACGCACAGGCACAACAGCATCGAAACCGGTTCTTTGGAGGCTCAGTTTTTGGTAATTAAGACTAA
GGAGTGTTTAAAAAACAGAAGTACATTTTCCTGGAAACCAGCAGTCTTTATTTGCAACTTTTATTGGCAAACCT
GGCTGCCAGTAAATACATTCCTTGGCATCTCCCACAATGTAATTCACTGGATGGAGCGGCCTTGCTTTTTCTGT
AACGTGTACGTCAATTAAAAGGGCCGCCTGGAAGGAATGCGTAGCGGTGGCTGAAAGCCCCAGTCTCGGGTCAC
CTCCCTCCACTCCAGGAACAAAAGCGTCCGTGGTCTGTGCCTGGAAGTCTGAGAGGGTCTCCCCGATGGGGCTG
TTCCCGCCCGGACCCTGAGGGATGAGAGTTGCAGCCTAGAAAACCAGGTGCCAGGCCCTG

Name of the predicted interaction : MAP3K1_5_56102259_56110500_56140227_56144076_FF
Sequence of the probe AAAACTAAAGATCGAAAGTTTTTATTACTTC

Sequence of forward primer GTGACATTACCGAGCACTTC

Sequence of reverse primer GTAACTCAAACTCAGTGTGCT (RC), AGCACACTGAGTTTGAGTTAC

Position of PCR sequence. BLAT.

Genomic Location	Gene(s)	Orientation	Query start	Query end	Query ori	Length	Score	E-val	%ID
5:56848104-56848251 [Sequence]	MAP3K1, AC008937.2	Reverse	62	209	Forward	148 [Sequence]	284	1.10E-76	100.00 [Alignment]
5:56814611-56814675 [Sequence]		Forward	1	65	Forward	65 [Sequence]	127	2.00E-29	100.00 [Alignment]

>MAP3K1_5_56102259_56110500_56140227_56144076_FF

AAACCAGCTGGAGGAAAGGAAAGGAAGGAAGAAATAAACGCAACACAGAAGTTCTCCTCAGTTGACAAAAGGTC
AAAAATCATTAACGTGTAAATGTTGCTTTTTCCATCCCAAAGCACCTTCTCACGTAGAGTCCAGGGACTAGGAG
GACTCACAACGCAGCGATGGGCAGCCAGGCCCTGCAGGAGTGGGGACAGAGGGAACCCGGCCGGTGGCCCGACC
CTGCAGGGAAGAAGGACGTGCGGCGAGAAGCATCGGATTCGGGGAGGGCCGGGACCTGGCCGAGGGTGACATTA
CCGAGCACTTCCTGGCACAGCGCTGGTCCCCTCCCCAAACGCGCTATATGTGGTTCTGTACGGGACTGCCTTTC
CCAAAGACAGCCAAGGAAAAACTAAAGATCGAAAGTTTTTATTACTTCCAAATTAGTAAATTTGTAACAATCAT
CAGGCAACTAACTATAATAAGAGGGAATTTACAAAAGACAGAGAGCTACTAGTCAGTATCAAATCATTCTTAAA
AGTGGCAACTCTGTATCAATTTTTTTTTTGCAGTCAATTACCTTTGACTCAGTCTATAAAGTACATGCCCAAAT
CTCCCTTTAGAGAAGAAAAGTGAATCAAAAAGAAAAATGTATATTAACTGTACAGTTCTCCTATACTAAATGTT
CTTACATGCTCAAAATGTATGAATATATTTAAAGCAACTGATCCTCTATTGAATACTGAATAAACTTGAAGGGA
TTTCTAAGTAAATTATTACTGGTAACTCAAACTCAGTGTGCTATAAATTTCAGACACCAC

Name of the predicted interaction : ME3_11_86300063_86304401_86420537_86426200_FR

Sequence of the probe. AATAAGGTATCGAGAAAGTATTCAAAGCA

Sequence of forward primer. ACCCTCCTTCACTCACATAG

Sequence of reverse primer. GTGATGTTAGGTAGATTAGGTGC (RC), GCACCTAATCTACCTAACATCAC

Position of PCR sequence BLAT.

Genomic Location	Overlapping Gene(s)	Orientation	Query start	Query end	Query ori	Length	Score	E-val	%ID
11:86593191-86593357 [Sequence]	RP11-317J19.1, ME3	Reverse	78	244	Forward	167 [Sequence]	325	4.40E-89	100.00 [Alignment]
11:86709494-86709570 [Sequence]	CTD-2005H7.1	Reverse	1	77	Forward	77 [Sequence]	148	7.00E-36	100.00 [Alignment]
14:59850307-59850375 [Sequence]	RTN1	Forward	1	69	Forward	69 [Sequence]	108	7.70E-24	88.41 [Alignment]
2:119300691-119300754 [Sequence]		Forward	1	64	Forward	64 [Sequence]	100	2.60E-21	89.06 [Alignment]

Please note the first two rows are the true homologies.

>ME3_11_86300063_86304401_86420537_86426200_FR

TAACCTTCCATAGGCCTCAGCTCCCTTATCTATTAACCTGGTGAAATGCAGACCCCTCTGCATGGGGTTACAAG
GTTTCAGCATGACTGGGTATGAAAAGAGAACAAAGAAGCTTCCTGGAGATGACTGTGGCCTTGGCTCACTGCCA
GGAAAATGACTCATTTCTGTATGCCAGGGTTATAGTTCACTGTTACCCTGACAAATGAATGTGGAAGACCCATG
ATTTCCTCCACCCTCCTTCACTCACATAGTAAAAGTTAGCTACTGCCTGCAACATACCAGGCACCGTACAACAC
GAAACTGTAGGCTCCCCCTCCAGGAAGTGACAATGTCATTCCTAACCTGTTGGAATTTTAACACCTGTCATAAA
AGGATCTCATGATGCTTTGAATACTTTCTCGATACCTTATTATAAAATCAGCTTTGTGTTGAATGATTTTTCCC
AGCTGTAGACTAATGTAAATGTTCTGAGCATGTTTAAGGTAGGCTAGTCTAAGCTGTGATGTTAGGTAGATTAG
GTGCATTTAAATGCATTTTCAATGATATTTTAAATTTGCAGTGGGTTTATCAGGATGTTACTCCAAGATGCTCC
TCCAAGGTGAGGGGCATCTGTGTTTTAGTCAGTGAAAATGTCTTGCAAAACTGAAGATAAAATAAATACAGTTA
GTCACACTTCACTTGCACTATAAGAAATTCTAAAGAAAAATTCTTCAAATTGAAGGAATATAATAACATAAATT
TATATCTACAGGAAGGAATAAAGAGCAAAGAAATGATAAACAAATCGCTTAAAGTGTTTA

Name of the predicted interaction : MELK_9_36577630_36579243_36637050_36643005_RF

Sequence of the probe. TGTAGTTTATTCACCTCGACTAGATTTTA

Sequence of forward primer. ATGCTTGCTGGAATATGCTTAC

Sequence of reverse primer. CAGCTTCGCTTGTTACCCAG (RC), CTGGGTAACAAGCGAAGCTG

Position of PCR sequence BLAT.

Genomic Location	Overlapping Gene(s)	Orientation	Query start	Query end	Query ori	Length	Score	E-val	%ID
9:36642807-36643010 [Sequence]	MELK	Reverse	13	215	Forward	204 [Sequence]	368	5.60E-102	98.04 [Alignment]

Genomic Location	Overlapping Gene(s)	Orientation	Query start	Query end	Length	Score	E-val	%ID
9:36642851-36642992 [Sequence]	MELK	Forward	1	142	142 [Sequence]	270	1.30E-72	100.0 [Alignment]

MELK_9_36577630_36579243_36637050_36643005_RF

AGTAGAGATGGGGTTTCACCATGCTGGCCAGGCCAGTCTCAAACTCCTGACCTCAGGTGATCTGCCCGCCCCAG
CCTCCCAAAATGCTAGAATTACAGGTGTGAACTATTGTGCCCGGCATTGTACAACCGAACTTTAACAACAGTTG
CTCAGATGATGATGGGGATAAAGAGTTGGGAAAGAGCACATCTTCTTGAAATGCTTGCTGGAATATGCTTACTT
CTTAAAAGATTATAGAGAATATTGATTCTTCCCCAAGAAATTGACAGATTCATGTTTTACATAATGATATTTGA
TTGTATAAAGTAATTATGCTGATTTTAAAATGTGAAAACATTGAATATATTTGTAATTTTTTGTTAATAAAGTG
CATAATTTTTTTTTGTAGTTTATTCACCTCGACTAGATTTTAATTTTTAATTTTTATTTATTTTTTGAGACAC
AGCTTCGCTTGTTACCCAGGCTGGAGTGCAGTGGCATGATCTCGGCTCACCGCAACCTCTGCTTCCCGGGTTCA
AGTGATTCTCCTGCCTCAGCCTCCCTAGTAGCTGGGATTACAGGCATGGGCCACCACGCCTGGCTAATTTTTTA
TATTTTTAGTAGAGACGGGGTTTCTCCATGTTGGTCAGGCTGGTCTTGAACTCCCGACCTCAGGTGATCCGCCT
GCCTCAGCCTCCCAAAGTGCCGGGATTACAAGTGTGAGCCACTGCGCCTGGCTGTTTTTTATTTTTAGTAGAGA
CAAGGTCTTGCTATATTGTCCTGGCTTGTCTTGAACTCCAGGCCTCAAGCAATCCTCCTG

Name of the predicted interaction : MSH3_5_80021913_80025030_80153948_80159012_RF

Sequence of the probe. ATTCCTGGTATCGAAATATTTTAGGTAATC

Sequence of forward primer. AGGACCCATCACCTACATATA

Sequence of reverse primer. CTCTTGGCATAAACTTGGCT RC, AGCCAAGTTTATGCCAAGAG

Position of PCR sequence BLAT.

Genomic Location	Overlapping Gene(s)	Orientation	Query start	Query end	Query ori	Length	Score	E-val	%ID
5:80726093-80726216 [Sequence]	MSH3	Reverse	1	124	Forward	124 [Sequence]	239	2.50E-63	100.00 [Alignment]
5:80863153-80863195 [Sequence]	MSH3	Reverse	121	163	Forward	43 [Sequence]	84	2.10E-16	100.00 [Alignment]

MSH3_5_80021913_80025030_80153948_80159012_RF

TAACAAAAATAAACTTTAAAATGGTGGAGGTGAGTGGGGAAAAGTGAAACCTCTGCTTTACAGAATACCAACGA
ATAAATGTAGGAAGAATTTTTTAATCAACATTTAATAACGACTATAATAATAACTGATTCAGACAGCAATGATC
AATAGATTATAAAACCTTTGGATGAAAGATTGTTGGAGAAAAGGATATTCATATATCTCAAAGTGTCATGCCAC
AGGTTATTTATTAATTACAAAGGGAAAAGGTATAGTGAAGAAATCTAGTGGGTACCCTCTTCAACCAGATAATC
AAATTTGGCATCCCCAGTTATGTAAAACTGATATCACGTCCCACCTGATGTGATGCACTGGGAAGGACCCATCA
CCTACATATACATGGAATATTCCTGGTATCGAAATATTTTAGGTAATCATTATTTGTCTAATTCACATGTCCAC
AATCAGGCATTAATGTTTACTTTCATTTGTACCTCACATTCCTGCCAGTCCAGCTTATGTTAGGGTCCATTTTG
TGGATGGTGAGGTGAATAGACATTTTCCCTCTTGGCATAAACTTGGCTTCACTCTAATCTTCATCCTACTCCAT
ATGGAGGAAATTTATCTCTGTCACATGCTAGAGAGTGTTCATCATCAGCTCCCCATCACTGCTCCATTTAAGCA
TCAGTGTCTAGTTAGCATTTCCTTGCATCTAGGCATCAGTGTCTTGTTAGCATGTCTCTTTAATTTCATGATGC
CTTGGTCAAATAAAGTGTCTGAGCGTGTATCCCACTTCTTTTTATTTTTTTCTGTAAGGT

Name of the predicted interaction : NF1_17_29477103_29483764_29651799_29657368_FF
Sequence of the probe. AATTTAAAAATCGATTTTAGAAAATGGGAAGA
Sequence of forward primer. TGTAGTAGTTACCCTGTTGTTG
Sequence of reverse primer. CATAAGAGCACTGTGAGGC RC, GCCTCACAGTGCTCTTATG

Position of PCR sequence BLAT.

Genomic Location	Overlapping Gene(s)	Orientation	Query start	Query end	Query ori	Length	Score	E-val	%ID
17:31330152-31330352 [Sequence]	NF1	Reverse	54	254	Forward	201 [Sequence]	385	4.00E-107	100.00 [Alignment]
17:31156692-31156750 [Sequence]	NF1	Forward	1	59	Forward	59 [Sequence]	113	2.90E-25	100.00 [Alignment]

>NF1_17_29477103_29483764_29651799_29657368_FF

ACTTTCATTTTAATTTATTATTTCCCTTAGAAACATCTCCTATCTTTTGTGACCATGTCTCCTTTTCCAGTATG
TTTCTTGAATTAGGATTTCATAGAGCTTTTGTGGCCTACACGAATTGACCACAGTAATCCATTACACATATTTT
TCTTTAGCATCTTGTTTGAATTTACTTACGGTTGTCCCAGCCCTAAGTAGATGATAAAATATGATCTCATAGTC
CTAAAATGTGGATTGATTTTTTTATGAAGATATGTGTTTTTTCTTCCTTCTGTAACCTGTGACAGATTCTGTAG
TAGTTACCCTGTTGTTGAAACAGTTTTTCTCAAATACCAGTTTCATCAAATAATTCCACTGTTAAAAGCTCATA
ATTTCTTTCTTCTTCCCATTTTCTAAAATCGATTTTTAAATTAAAGGTACAAGTTAAGGCACACAGAAGATTAT
AGGCAGCTGACCTAGGAGAAAACACAAATGAAGTTGTTTTAAAACGTATTTTTCCTTATAGTTCCAAAATTTTT
TCATAACATACAATTTGTGATTCTGTTACAAAGTATGATCAACTATTTTTAAATTTTATGATCAGTTAGAAATA
AGATGTTATAATTCTACAGTAAAACCAAAATACCCCTTAATCATTTAGGGATTTTATAAAAAGGGACACACTTG
ATATAACCATAAGAGCACTGTGAGGCTCCTATGACAGAGGGGCGGGGTATAGGCTTTCCTAAAATACATCTCAC
TGAGACATAAAATATGAGAGGACTTATGGTCCTAATGTGGATCAATAGAAATTAAGTCAG

Name of the predicted interaction : SRD5A1_5_6634973_6639025_6667775_6669711_RF									
Sequence of the probe. TACTAATTGTTACATCGAAAGTTCAAA									
Sequence of forward primer. GGCATTGCTTTGCCTTATC									
Sequence of reverse primer. CTCTACACCCAAGGAAGTTG RC, CAACTTCCTTGGGTGTAGAG									
Position of PCR sequence BLAT.									
Genomic Location	Overlapping Gene(s)	Orientation	Query start	Query end	Query ori	Length	Score	E-val	%ID
5:6634859-6634948 [Sequence]	SRD5A1	Forward	75	164	Forward	90 [Sequence]	174	1.60E-43	100.00 [Alignment]
5:6669523-6669602 [Sequence]	SRD5A1	Forward	1	80	Forward	80 [Sequence]	153	2.10E-37	100.00 [Alignment]

>SRD5A1_5_6634973_6639025_6667775_6669711_RF

ACCACTTTTTAAGATTTATCCTGTTTGTTCTTTGTTGATTGAAACATAATAATTGTTAAAATTCTCTACAGCCT
TCTTTTTCTTCCATAGCTAATCTTCCTTCTAATAGTTTTTGCTTTCTGTTTTGCTGTTGTTGCTTTGCAAAGCT
TTCCCCTCATAGCCTGTACCTGTTATCAATATAAAATAATCTTCCTGTTGAATGCTTCATGACTTGAATTCTAC
TTTGATAAAAACATTGCCATACTGCTTTTTATCTTGATGAATTCATCTGGCATTGCTTTGCCTTATCATCTCAT
CTGGAGTTTTTAAATGCCATTTGTTTCAGTTGTCTTTAACAACATAATAAATAGACTTTGCCATTTAACAAGGT
AGCTCAAATTCTTTTACTAATTGTTACATCGAAAGTTCAAAATTAAATTTTAAACGTTTTCATTCAGGCTCTGG
AAAGCTTCACAGTTAAAAAGGATGTCTCTACACCCAAGGAAGTTGAACTCACTGGCTGTGTGACTATGGGCAGT
TTACCCAACCTTTCTGATTTGGGGTCCCACCTTAAAACACTCACTTCCCAGAGAGACAGGAAGAACTCAGTGTG

TGTTTATAAGCCTCTCTTCTTTCTCCTGGTGTCATGCATTCCAGCGAAGAGAAAGTACACAGCTCCACTACTTG
GAACCAGTGTTGTACCCAGCACAGTTTTTGGTACCTGAGTTCCCTGAAAACCAGCACCTTACCCTGTAACTGGT
GCAGTCTGTGTCCTCAGTGTGCTTTGATGACTTGCACTTTAAACAAGGGCAAGTCAACAT

Name of the predicted interaction : TSPYL5_8_98276431_98282736_98316421_98318720_FF

Sequence of the probe. AGGAATTCAAGACTCGAACTAAA

Sequence of forward primer. TTGAGTCCTGGCTCTACTAC

Sequence of reverse primer. GAACAAAGGACCAAGTATAGCT RC, AGCTATACTTGGTCCTTTGTTC

Position of PCR sequence BLAT.

Genomic Location	Overlapping Gene(s)	Orientation	Query start	Query end	Query ori	Length	Score	E-val	%ID
8:97306203-97306494 [Sequence]		Reverse	170	461	Forward	292 [Sequence]	571	4.60E-163	100.00 [Alignment]
8:97270338-97270510 [Sequence]		Forward	1	173	Forward	173 [Sequence]	335	3.10E-92	100.00 [Alignment]
18:68006367-68006398 [Sequence]		Reverse	186	217	Forward	32 [Sequence]	58	8.70E-09	96.88 [Alignment]
22:34012396-34012431 [Sequence]		Reverse	370	404	Forward	36 [Sequence]	53	3.50E-07	91.67 [Alignment]
7:82655893-82655925 [Sequence]		Forward	180	212	Forward	33 [Sequence]	49	6.90E-06	87.88 [Alignment]
2:37820936-37820959 [Sequence]		Forward	392	415	Forward	24 [Sequence]	47	1.70E-05	100.00 [Alignment]

Please note the first two rows are the true homologies.

>TSPYL5_8_98276431_98282736_98316421_98318720_FF

TAAAGAAGTTTCACATTCATATGCCAACTCAGATTGATGGGCAGCAACTGGATAATCCGCTGTGCAGAAAGTTA
AATACAGGTTCTGTGCAAAGAAGTGTCTAGATTCATAGTGCCAGACATCTGCCCTGGGCCACATGCTTACCGTC
CCATGGATGGATGGAACTTGGAATCAGAAGACCCAAGTTTGAGTCCTGGCTCTACTACTTTTGTGATTTTGGTC
ATTTAACCTCTTTGAGCCTTCTTATGGCATAGTAGTTATAATCAAGATAATATAAGTGAATGTGCTTTGTAAAC
CATGAAGTGTTGGTCACACAGATGATAGCTACTGTCTTATATTTGTCAAACCTCAGCTGAGGACCAGGTTGACA
GGATGGAGGAAGAGGAGGAATTCAAGACTCGAACTAAACAAAAAGGAGATGATCCTGGGTGGGCTTGACTTAAT
CAAGTGAAAGCCTTAAAAGCAAGAGTCTCCTGCTAGTCTAGGAAAAGCAAACAGCCCTGCTATGAATGGCCTAT
AGAAAGGGGCAGCCTCTAGGAGCATGGGCCTCAGTCATATGCCCACGAGGAACTGAATATTGCCAGCAACCATG
TGAGCATGGAAGAGGACTCTAAGCCTCTGATGAGACCACAGCCCTGGCCAATGCTTTGATTGTGGCTCTGTGAG
GCCTTGAACAAAGGACCAAGTATAGCTATGCCAGGACTTCTGAACCACGGGAATGGTGAGATAATAAATGTTTG
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>CDC6_17_38421089_38423079_38467677_38474960_FR

AGGTAAGTTAAAGACCAAGAACTGGCATTGGTCTTAGTATCATGGGACCCTTTTGAGTAGTTTCAGTGGAGTGG
TGGAGGGTGAAAGTGAAAGCTTAATTGGAGTGGGTTCAAGAATGCAGGAATAGGAGGAGAGAAATTGGAGATAG
CAATATAGAAATCTCTTAAAGAGTTCGCTGTAAAGTCCAGGAGAGAGGGGTGAAGATAAGTGAAGTGATTGTTG
GACGAAGATGTGGGGTTGAGAGTTGTTTTTTTCCCATCCCAAGATGGGAGACCTATTTGTATGCTGATGGAATG
AGTAGCATGAAACTTAGGAGAGAGGGAAAAAATTGAATCAGAAGAGAGGGAACAGATTGCCTGAATAATGACCT
GGAGGAGGCCAGAGAAAAGAGAATGTGATCGATGACCTTAATGTCAGTGTCACTGACTCTGACAAGGAGGAAAG
GACAGCGATGAGCCAGGCTGACCCCCGCCACCCCATTCCCATAGCCCCACTTTCTTCTCTCTTCTTGTTCAGAA
ATGTTTCACTTTGCCTCATCTAGCCCCTTTGGCCAGTAGGTCACATCTGGGAGCTTCTGGGGGTGCCACATGTC
TGCCTCAATCTGGGCTGTTTCCTCCCCTCCAAGATATTTCACTGTCTCTGGGCTGGGCGCCAGGACTCCTGGGT
TTCCCTGCCTGTGGTGCAGGGCTCCCCTGCAGGGCTCCAGAGAGTCGCTTAGCTGGTTTCCTTCCTCCTTTGTG
GGGAGGGCCTTCCCCTAGGGCTGGGAGGTGTCAGGAATCCCAGGTGGGACAGGGGTGGGG

>SLC16A10_6_111441989_111447305_111492951_111498421_FR

TTCAATTGCTATATAAAAAATGTAAAGTCTGTTTACTGCCTTAAACCTTCTGGTGTATTTTTATATAAAGTAAC
ACCCTTAATTCTAACTTGGCCAACAGGTAGGATGGTATTATTATTATCTTCATTGTACAGATAAGGAAACTGAG
GCTCAGATTGACTAGATCAAACAGGAGTTTTCTGGAAAACCTAGGACACAAGCCTAAATCTTTGAACTCAAATA
CTGCTCTACACTGAATTACAGTTATATACTGATTTCTGTTGTAAATTCTTAGAGAAGACAGACATAGAAATTAG
TAACTTGAGTCAGTAGCGGCTTTGTTCAAACACAGGCACATGCATATTTTATGGTATATGTTTATATCTGTGTA
ATACTCATCATAAATGTCAGATTTATAATCGAGATCACAGTGAGCTGAGATTGCACCACTGCACTCCAGCCTGG
GCAGCGGAGTGAGACCTTTTCTCAAAAAAAAAAAAAAAAAAAGGCAAAAAATTAAATTATTAGTATGGTAAAGT
TTCGTTTGGACTTAATATGAAACTCATTTCTAGAAATGATGATCATTTGCATAGGGCTTAACTTCCTTTGCTAA
GAAAATAGAGTAGTATACTAGGAGACTTCCAGAGCTGCATAGAGCTTCAGGGTCATCTACCAAGACAGACAATT
TGTTGTCATCATCAGTGTTAAACTCTAAATTATTAAGTGCTTATGTGCCAGATACTGAAGTTTATATACACTTT
CTCTAATCTTTAATAATTCTAGAAAGGTATGTGTTTGATCCATTTTCAAGATAAGAAAAC

>VAV3_1_108148303_108158073_108220200_108227533_RF

ATGAGGTTTTTTTCCAGCCTTCCTAAGGGCCTCAAAGTCATATCCAGCAGACTTGCAGGGTTCTCAGGTGAAAG
CAAATTGGAGAAATTTTTAAAATGTAATTTTGGTTTTTACTCCAACTACTTTCAACATGGATTTGTAAAAGACT
GCTAGGATCATTAAAATCAGCATTGAAGCTATGTTGAGCAAGATGGATAGCTGCACTAGAAAAGCTGTAACAAG
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ACCTTCAGGCTTTGTGTATAAGATATATATAAAACATAAATAAATTTTGTGTTTACACTTGGGTTCCATCCTGA
ATATATCTCATGATGTTTATGCAAATATTCCAAAATCTGAAAAAATCTGAAATTCAAAACACTTCCGGTCCCAA
GCATTTTGAATAAGGGATACTCAACCTATAGCTGCATTAATTGAATTAAGACAACCACATAATCTACCTGTTAA
TTTTCTCTGGAGCCTTTTCTTCTGAGCCCTCCACGCTCTTCTAATTGATACTGCTTGCTCTACTAAGCCTGTTG
AATTACTGTAGTCCTGGGACTTCTCTTTGCTCCCCTTTCCTGGCTTCTATATCTCCCTCT

A dual label hydrolysis probe was used to detect the sequenced interaction labeled with 5'-FAM/BHQ1-3'. The probe was temperature gradient optimized and designed to span the junction of the 3C fragment making the detection of the 3C product totally specific. The qPCR standard curve (10^6 copies-1 copy) was produced from the sequenced product used in the reports figures.

MMP1 copy testing as an internal control for 3C library production.

The primer set and probe used are shown in the reference sequence below. The Taq I site is highlighted. The probe spans the junction of both fragments and is specific at an annealing temperature of 66.4°C.

GGGGAGTGGATGGGATAAGGTGGAATGTTGGGTGAACTAAAAGGCCTTTAAGGCCCCTCTGAAATCCAGCATCG
AAGAGGGAAACTGCATCACAGTTGATGGAAGTCTGTTGGCCTCTTAACAAAGCTAATGCTTGCCCTTCTGGCTTAG
CTTACATAAGAACCACAAGGAATCTTTGTTGAATTGTTTCTTTCAGATCATCGGGACAACTCTCCTTTTGATGGACC
TGGAGGAAATCTTGCTCATGCTTTTCAACCAGGCCCA
MMP 1-4 2F 5'-GGGGAGTGGATGGGATAAGGTG-3'

MPP 1F 5'-TGGGCCTGGTTGAAAAGCAT -3'

MMP1F1b2 probe 5'-FAM-ATCCAGCATCGAAGAGGGAAACTGCATCA-BHQ1-3'

The forward and reverse primer and probe sequences for the hydrolysis qPCRs are describes in the previous tables.

3C library copy number testing with the internal control marker MMP1**.**

A 3C interaction with MMP-1 was used as a internal control for the EpiSwitch™ library. A dual label 5'FAM-BHQ1-3' labeled hydrolysis probe was used to detect the sequenced interaction. Samples were screened at 20 ng and copy number recorded. The 264 bp product was quantified as above, all samples were run on LabChip, prior to screening with the 3C target. The target was expressed as a MMP1 ratio for each experiment.

qPCR screening with standard curves and estimation of 3C fragment copy number.

qPCR templates were adjusted to 20 ng of 3C library DNA, and used with concentration-matched negative controls including 3C libraries derived from normal blood. Additional negative controls included patient material without formaldehdye fixation, digested and ligated library material, and normal genomic DNA. A 3C interaction MMP-1 was used as an internal control for the EpiSwitch™ library synthesis.

We have used HEX, Texas Red and FAM with matched quenchers.

Abs [nm]	Em [nm]	3' Quencher	Flurophore
FAM	495	520	TAM, BHQ1, DAB, Eclip
TET	521	536	TAM, BHQ1
JOE	520	548	TAM, BHQ1, BHQ2
Yakima Yellow	530	549	BHQ1, Eclip
HEX	535	556	TAM, BHQ1, BHQ2, Eclip, BBQ650
Cyanine3	552	570	BHQ1, BHQ2, BBQ650
ATTO 550	554	576	TAM, BHQ2
TAMRA	544	576	BHQ2
ROX	575	602	TAM, BHQ2, BBQ650
Texas Red	583	603	BHQ2, BBQ650
Cyanine3.5	588	604	BHQ2
LC 610	590	610	BHQ2
LC 640	625	640	BHQ2, BBQ650
ATTO 647N	644	669	BHQ2, BBQ650
Cyanine5	649	670	BHQ2, BBQ650
Cyanine5.5	675	694	BHQ2, BBQ650
ATTO 680	680	700	BBQ650

CLAIMS

1. A process for detecting a chromosome state which represents a subgroup in a population comprising determining whether a chromosome interaction relating to that chromosome state is present or absent within a defined disease-associated region of the genome, wherein said disease is breast cancer, and wherein a specific combination of chromosome interactions are typed in the individual comprising:

(i) at least 4, 5, 6 or 7 of the chromosome interactions represented by the following probes (Marker Set 1);

- ATTTCTTTCTTCTTCCCATTTTCTAAAATCGATTTTTAAATTAAAGGTACAAGTTAAGGC,
- GGATGGAGGAAGAGGAGGAATTCAAGACTCGAACTAAACAAAAAGGAGATGATCCTGGGT,
- AGCTCAAATTCTTTTACTAATTGTTACATCGAAAGTTCAAAATTAAATTTTAAACGTTTT,
- CCAAAGACAGCCAAGGAAAAACTAAAGATCGAAAGTTTTTATTACTTCCAAATTAGTAAA,
- AATTTAGAGGAACTCTATATAAACAACATCGAAACTTTGCTTCATGCACAAAATTTAAAA,
- TTGGAGGGAAAAGTAATTACGTTCAACTTCGACTGTATTCTACAAAGTGCTGGGATTACA,
- ATACTCATCATAAATGTCAGATTTATAATCGAGATCACAGTGAGCTGAGATTGCACCACT,
- AGGATCTCATGATGCTTTGAATACTTTCTCGATACCTTATTATAAAATCAGCTTTGTGTT;

or

(iii) at least 4, 5, 6 or 7 of the chromosome interactions represented by the following probes (Marker Set 2);

- TTGGAGGGAAAAGTAATTACGTTCAACTTCGACTGTATTCTACAAAGTGCTGGGATTACA,
- GGAGGAGGCCAGAGAAAAGAGAATGTGATCGATTTCTAAAATACTGTGTGTGTGTATGTA,
- GGATGGAGGAAGAGGAGGAATTCAAGACTCGAACTAAACAAAAAGGAGATGATCCTGGGT,
- AGGATCTCATGATGCTTTGAATACTTTCTCGATACCTTATTATAAAATCAGCTTTGTGTT,
- AGCTCAAATTCTTTTACTAATTGTTACATCGAAAGTTCAAAATTAAATTTTAAACGTTTT,
- AATTTAGAGGAACTCTATATAAACAACATCGAAACTTTGCTTCATGCACAAAATTTAAAA,
- CAGAAATGCACGCACAGGCACAACAGCATCGAAACCGGTTCTTTGGAGGCTCAGTTTTTG,
- CCTACATATACATGGAATATTCCTGGTATCGAAATATTTTAGGTAATCATTATTTGTCTA.

2. A process according to claim 1 comprising typing at least 7 chromosome interactions represented by (i) the said probes of Marker Set 1 or (ii) the said probes of Marker Set 2.

3. A process according to claim 1 or 2 comprising typing all of the chromosome interactions represented by all of the said probes of Marker Set 1.

4. A process according to any one of the preceding claims comprising typing all of the chromosome interactions represented by all the said probes of Marker Set 2.

5. A process according to any one of the preceding claims in which the chromosome interactions are typed by detecting the presence or absence of a DNA loop at the site of the chromosome interactions.

6. A process according to any one of the preceding claims in which the chromosome interactions are typed by detecting the presence or absence of distal regions of a chromosome being brought together in a chromosome conformation.

7. A process according to any one of the preceding claims in which the chromosome interactions are typed by detecting the presence of a ligated nucleic acid which is generated during said typing and whose sequence comprises two regions each corresponding to the regions of the chromosome which come together in the chromosome interaction.

8. A process according to claim 7 wherein detection of the ligated nucleic acid is performed by PCR.

9. A process according to claim 8 wherein said PCR uses at least one of the following primers:

- TGTAGTAGTTACCCTGTTGTTG;
- GCCTCACAGTGCTCTTATG;
- GTGCTTTGTAAACCATGAAGTG;
- TCGTGGGCATATGACTGAG;
- GGCATTGCTTTGCCTTATC;
- CAACTTCCTTGGGTGTAGAG;
- CGCTATATGTGGTTCTGTACG;
- CTTCTCTAAAGGGAGATTTGGG;
- TGTTGAGCAAGATGGATAGC;
- ATATTCAGGATGGAACCCAAG;
- TCCAGAGGTTATGGAATTTGAG;
- AAGAAACAGACTGGGCTTG;
- ACTCAAATACTGCTCTACACTG;
- AAGGAAGTTAAGCCCTATGC;
- ACCCTCCTTCACTCACATAG;
- GCACCTAATCTACCTAACATCAC.

10. A process according to any one of the preceding claims, wherein the process is carried out to diagnose breast cancer.

11. A process according to any one of the preceding claims, wherein the process is carried out to select an individual for a medical treatment.

12. A process according to any one of the preceding claims, wherein the process is carried out on an individual who has been selected based on gender or another physical characteristic.

13. A process according to any one of the preceding claims, wherein the process is carried out to detect malignant breast cancer.

14. A process according to any of the preceding claims wherein the typing or detecting comprises specific detection of the ligated product by quantitative PCR (qPCR) which uses primers capable of amplifying the ligated product and a probe which binds the ligation site during the PCR reaction, wherein said probe comprises sequence which is complementary to sequence from each of the chromosome regions that have come together in the chromosome interaction.

15. A process according to claim 14 wherein said probe comprises an oligonucleotide which specifically binds to said ligated product.

16. A process according to claim 14 or 15 wherein said probe comprises a quencher covalently attached to the 3' end of the oligonucleotide.

17. A process according to any one of claims 14 to 16 wherein said probe comprises a fluorophore covalently attached to the 5' end of the oligonucleotide.

18. A process according to claim 17 wherein said fluorophore is selected from HEX, Texas Red and FAM.

19. A process according to any one of claims 14 to 18 wherein said probe comprises a nucleic acid sequence of length 10 to 40 nucleotide bases.

20. A process according to claim 19 wherein said probe comprises a nucleic acid sequence of length 20 to 30 nucleotide bases.