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(54) Title: DESIGNING PADLOCK PROBES FOR TARGETED GENOMIC SEQUENCING

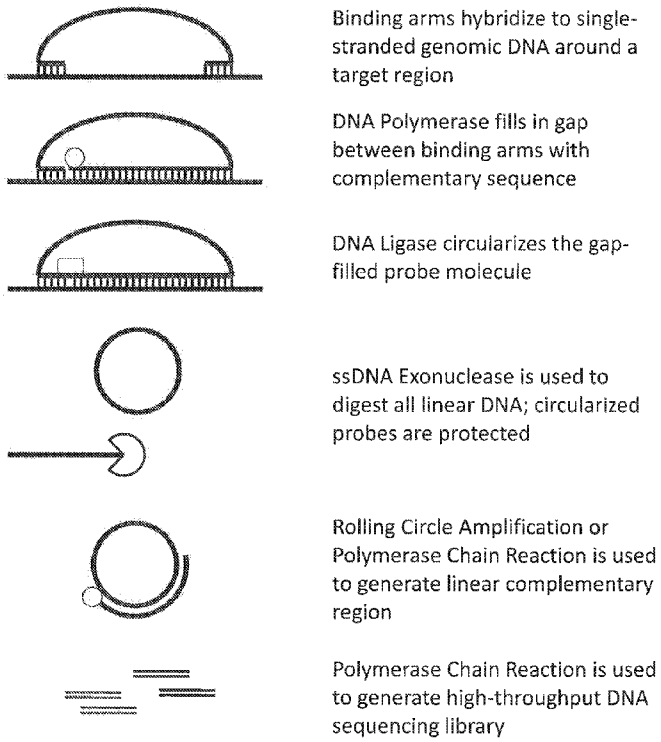

FIGURE 1

(57) Abstract: Methods, systems, and computer programs for designing probes or primers for nucleic acid sequencing, generating libraries of nucleic acid sequences, and mapping genomic sequences are provided herein,

WO 2012/149171 A1

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DESIGNING PADLOCK PROBES FOR TARGETED GENOMIC SEQUENCING

SEQUENCE LISTING

[0001] The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on April 26, 2012, is named 378665WO-1.txt and is 99,190 bytes in size.

TECHNICAL FIELD

[0002] The subject matter described herein relates generally to the fields of molecular biology and bioinformatics. More specifically, the subject matter described herein relates to systems, methods, and computer programs for designing probes for use in nucleic acid sequencing, particularly padlock probes useful in carrying out targeted genomic and methylation sequencing.

BACKGROUND

[0003] Padlock Probe (PP) technology is a multiplex genomic enrichment method allowing for accurate targeted high-throughput sequencing. PP technology has been used to perform highly multiplexed genotyping, digital allele quantification, targeted bisulfite sequencing, and exome sequencing. *See* Hardenbol, P. et al., (2005) *Genome Res.* 15: 269-275; Wang, Y. et al., (2005) *Nucleic Acids Res.*, 2005, 33(21) e183: 1-14; Porreca, G.J. et al., (2007) *Nat. Methods* 4(11): 931-936; Zhang, K. et al., (2009) *Nat. Methods* 6: 613-618; Turner, E.H. et al., (2009) *Nat. Methods* 6: 315-316; Deng, J. et al., (2009) *Nat. Biotechnol.* 27: 353-360; Shoemaker, R. et al., (2010) *Genome Res.* 20: 883-889, and Gore, A. et al., (2011) *Nature* 471(7336): 63-67.

[0004] Padlock probe technology may utilize a linear oligonucleotide molecule with two binding sequences at each end joined by a common linker sequence. The probe's binding arms may be hybridized several base pairs apart surrounding a target single-stranded genomic DNA region. A DNA polymerase (with each of the four standard dNTP molecules) may be used to fill in the gap between the two binding arms, and a DNA ligase may be used to circularize the resulting molecule. A mixture of exonucleases may then used to digest all

arm; the resulting circular molecules can be amplified using rolling circle amplification or polymerase chain reaction to generate a DNA library compatible with modern high-throughput sequencers.

[0005] Previous padlock probe work has relied on relatively arbitrary methods for padlock probe design. Padlock probes are generally designed to have binding arms with complementary sequence around a chosen target region of approximately 100-200 base pairs; each binding arm is generally designed to have a specific DNA melting temperature. However, padlock probe efficacy from molecule to molecule has been found to vary greatly, ranging across several orders of magnitude. Previous work identified a complex nonlinear relationship between padlock probe efficiency and many probe characteristics. (Deng, J. et al., (2009) *Nat. Biotechnol.* 27: 353-360; Li, J.B. et al., (2009) *Genome Res.* 19(9): 1606-1615). The bias inherent in padlock probe design has been demonstrated to show a complex nonlinear relationship with many probe characteristics, confounding attempts to generate efficient probes. Because of this, padlock probe results are highly biased towards certain genomic regions. Certain genomic regions are therefore extremely difficult to target using padlock probes due to the lack of knowledge of probe capturing efficiency.

[0006] There remains a need in the art for methods and algorithms that are useful for designing probes from a large set of probe characteristics to ensure optimal capture of DNA sequences, particularly genomic DNA.

SUMMARY

[0007] The subject matter described herein relates to methods, systems, and computer program products that can be used to design oligonucleotide primers and probes, particularly padlock probes for high-density multiplex genome sequencing. The subject matter disclosed herein is particularly useful for detecting methylation status and/or single nucleotide variants in a target nucleic acid sequence of interest.

[0008] Accordingly, in some aspects, the subject matter described herein provides a method of designing probes or primers for sequencing a target nucleic acid molecule, comprising the steps of selecting one or more inputs associated with efficiency of the probe or primer; selecting a target nucleic acid sequence; generating a first library of probe or primer sequences that comprise a target capturing sequence that is complementary to the target nucleic acid sequence; determining the efficiency of each probe or primer sequence in the

first library by using an algorithm comprising the one or more selected inputs defined herein; ranking the probe or primer sequences in the first library by efficiency; extracting the probe or primer sequences having the highest efficiency to generate a second library; and adding a linker sequence to each of the probe or primer sequences in the second library. In some embodiments, the method further comprises synthesizing the probe or primer.

[0009] In some embodiments, the probe is a padlock probe. The one or more inputs may comprise target length, target folding energy, target GC content, extension arm A%, extension arm G%, target A%, target T%, target G%, number of "GG" dinucleotides in ligation arm, number of "AT" dinucleotides in extension arm, number of "GG" dinucleotides in extension arm, number of "AA" dinucleotides in target, number of "AT" dinucleotides in target, number of "TA" dinucleotides in target, number of "GT" dinucleotides in target, number of "GA" dinucleotides in target, ligation arm terminal dinucleotide, extension arm terminal dinucleotide, target 5' terminal dinucleotide, ligation arm melting temperature, extension arm melting temperature, ligation arm length, extension arm length, local single-stranded folding energy of the target, and dinucleotides present at the extension site and ligation site during probe capture.

[0010] In some embodiments, the target nucleic acid sequence is derived from a human.

[0011] The target-capturing sequence may include a ligation arm and an extension arm. In some embodiments, the target-capturing sequence contains one or more CpG dinucleotides. The target-capturing sequences in the first library may also contain all possible methylation state combinations of the one or more CpG dinucleotides. In some embodiments, the extension arm comprises one or more priming sites for amplification of the target nucleic acid sequence and may be universal priming sites. The target capturing sequence may also include one or more restriction sites.

[0012] The methods disclosed herein involve an algorithm that may comprise one or more neural networks. The one or more neural networks may comprise the one or more inputs, *e.g.*, seven or more inputs.

[0013] In certain embodiments, the method further comprises, after the extracting step, pooling the non-extracted probe or primer sequences and repeating certain steps defined herein.

[0014] In some embodiments, the linker sequence is a sequence that is common to each probe or primer sequence in the second library.

[0015] In another aspect, an apparatus is provided, comprising at least one processor and at least one memory including code which when executed by the at least one processor provides operations comprising: selecting one or more inputs associated with efficiency of the probe or primer; selecting a target nucleic acid sequence; generating a first library of probe or primer sequences that comprise a target capturing sequence that is complementary to the target nucleic acid sequence; determining the efficiency of each probe or primer sequence in the first library by using an algorithm comprising the one or more selected inputs; ranking the probe or primer sequences in the first library by efficiency; extracting the probe or primer sequences having the highest efficiency to generate a second library; and adding a linker sequence to each of the probe or primer sequences in the second library.

[0016] In another aspect of the subject matter described herein, a computer-readable storage medium including code is provided, which when executed by at least one processor provides operations comprising: selecting one or more inputs associated with efficiency of the probe or primer; selecting a target nucleic acid sequence; generating a first library of probe or primer sequences that comprise a target capturing sequence that is complementary to the target nucleic acid sequence; determining the efficiency of each probe or primer sequence in the first library by using an algorithm comprising the one or more selected inputs; ranking the probe or primer sequences in the first library by efficiency; extracting the probe or primer sequences having the highest efficiency to generate a second library; and adding a linker sequence to each of the probe or primer sequences in the second library.

[0017] Other features and advantages of the subject matter described herein will be apparent from the following detailed description and claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0018] The following Detailed Description, given by way of example, but not intended to limit the subject matter described herein to specific embodiments described, may be understood in conjunction with the accompanying figures, incorporated herein by reference, in which:

[0019] Figure 1 is a block diagram of a single padlock probe capture experiment, demonstrating an example of a workflow for targeted genomic resequencing consistent with some of the exemplary embodiments described herein;

[0020] Figure 2 is a flowchart of a process for designing padlock probes from input files consistent with some of the exemplary embodiments described herein;

[0021] Figure 3 is a block diagram of a back propagation neural network used to derive the probe efficiency scoring equation consistent with some of the exemplary embodiments described herein;

[0022] Figure 4 is a depiction of two examples of customized linker sequences and the function provided thereby, each of which is consistent with some of the exemplary embodiments described herein. Figure 4 discloses SEQ ID NOS 420-424, respectively, in order of appearance;

[0023] Figures 5A-5E depicts the design of padlock probes for targeted bisulfite sequencing. (A) is a diagram showing that each padlock probe has a common linker sequence flanked by two target-specific capturing arms (H1 and H2). H1 and H2 are melting temperature normalized, and a spacer sequence is included to normalize probe lengths. The linker sequence contains priming sites (AP1 and AP2) for universal primers, two *Mme*I sites and a central *Alu*I recognition site. (B) is a diagram depicting a CpG island (or other target region) covered by multiple padlock probes targeting partially overlapped regions on alternating strands. (C) is a diagram showing a library of padlock probes annealed to bisulfite-converted genomic DNA. (D) is a flow chart showing the generation of a shotgun sequencing library. (E) is a picture showing gel electrophoresis analysis of the padlock-captured products from two independent capturing reactions (1 and 2) and a no-template control (NTC).

[0024] Figures 6A-6G are graphs summarizing an analysis of the effect of probe characteristics on capturing efficiency. The statistical package R and its effects module were used for this analysis. A linear model was used, and each individual factor was assumed to be independent. The dashed lines represent a 95% confidence interval. (A) High GC content in the ligation arm was found to increase probe capturing efficiency. (B) Longer ligation arms were found to capture probes with higher efficiency than short ligation arms. (C) The frequency of 12-mers within the ligation arm in the human genome did not have a statistically significant effect on probe efficiency. (D) GC content of the extension arm did not have a statistically significant effect on probe efficiency. (E) Extension arm length did not have a statistically significant effect on probe efficiency; the trend was that a longer extension arm would be better. (F) The frequency of 12-mers within the extension arm in the human

genome did not have a statistically significant effect on probe efficiency. (G) Shorter target sequences were captured with higher efficiency than long target sequences.

[0025] Figures 7A-7D depict experimental normalization of padlock-capturing efficiency. (A) shows the "subsetting" strategy. (B) depicts the 'suppressor oligo' strategy. (C) shows the distribution of normalized abundance for all captured targets with one 30,000-probe set and with four probe sets. The x-axis is the normalized abundance of each captured target, which is calculated by dividing the counts of the target by the average counts of all targets. The y-axis is the fraction of probes with the coverage equal to or greater than the normalized coverage. (D) Comparison of relative abundance for each target before and after normalization. The vertical dash lines indicate the clear separation of four subsets of targets, as well as the fifth set normalized with the suppressor oligos.

[0026] Figures 8A-8C show the results of a validation experiment demonstrating the digital methylation assay. (A) Comparison of methylation measurements from both strands for the same CpG sites. The methylation levels of the forward strand were plotted against the levels of the reverse strand on 2697 CpG sites that were covered by on both strands by different probes. (B) Methylation levels of 182 randomly selected CpG sites in the BJ fibroblast lines were measured by the conventional bisulfite Sanger sequencing. (C) Comparison of the methylation levels of 25,665 CpG sites (at least 50x sequencing reads per site) between two biological replicates on the IMR90 fibroblasts.

[0027] Figure 9 is a schematic for the probe design software (ppDesigner).

[0028] Figure 10 are graphs comparing probe capture efficiencies between the DMR220K, LC4K probe sets and the CGI30K set. The first three plots were generated from data without subsetting or suppressor oligos to allow for a direct comparison of probe design.

[0029] Figure 11 is a scatter plot of number of characterized CpG sites versus mappable sequencing data for the DMR330K probe set. Variability in sequencing quality of individual sequencing runs is responsible for the different number of CpG sites characterized with similar sequencing effort.

[0030] Figure 12 is a graph showing the number of CpG sites called per sample as a function of sequencing effort. The horizontal dash line represents 4Gbps of sequences per library.

[0031] Figures 13A-13B are graphs showing captured CpG sites that were tested for potential regulatory interactions with genes by GREAT. (A) Most CpG sites were interacting with 1-2

genes. (B) Distance of CpG sites to the transcriptional start sites (TSS) of the predicted regulating genes.

[0032] Figures 14A-14E are graphs showing the accuracy of digital quantification by BSPP. (A, B) show a comparison of the methylation levels obtained at 10x depth from multiple capture reactions of the same sample (PGP1iPS) within batches and between batches. (C, D, E) Within sample comparison of methylation levels obtained from different probes capturing the same CpG site on different strands at 10x depth within one capture reaction.

[0033] Figure 15 is a graph showing the comparison between BSPP and whole genome bisulfite sequencing (WGBS). Two H1 ESC datasets were compared, using sites with at least 10x read depth in each.

[0034] Figure 16 is a graph depicting the variation in amount of sequencing data obtained per sample in a multiplexed BSPP capture experiment. Forty-eight whole blood samples were captured and sequenced in one batch using the library-free BSPP method.

[0035] Figure 17 depicts exemplary padlock probes ordered from (A) Agilent's oligonucleotide synthesis service (SEQ ID NOS 425-427, respectively, in order of appearance) and (B) LC Sciences' oligonucleotide synthesis service (SEQ ID NOS 428-430, respectively, in order of appearance).

[0036] Figure 18 is a diagram depicting the addition of a second neural network specifically for bisulfite-converted DNA. This network contains two hidden layers with 10 and 12 nodes, respectively, and accepts 25 pieces of information as input.

DETAILED DESCRIPTION

[0037] The features, structures, or characteristics described throughout this specification may be combined in any suitable manner in one or more embodiments. For example, the usage of the phrases "exemplary embodiments," "example embodiments," "some embodiments," or other similar language, throughout this specification refers to the fact that a particular feature, structure, or characteristic described in connection with an embodiment may be included in at least one embodiment described herein. Thus, appearances of the phrases "exemplary embodiments," "example embodiments," "in some embodiments," "in other embodiments," or other similar language, throughout this specification do not necessarily all refer to the same group of embodiments, and the described features, structures, or characteristics can be combined in any suitable manner in one or more embodiments.

[0038] To facilitate the understanding of this disclosure, a number of terms are defined below. Terms defined herein have meanings as commonly understood by a person of ordinary skill in the areas relevant to the subject matter described herein. Terms such as "a", "an" and "the" are not intended to refer to only a singular entity, but include the general class of which a specific example may be used for illustration. The terminology herein is used to describe specific embodiments of the subject matter described herein, but their usage does not delimit the subject matter, except as outlined in the claims.

[0039] The term "read" refers to a nucleic acid sequence of sufficient length (*e.g.*, at least about 30 bp) that can be used to identify a larger sequence or region, *e.g.* that can be aligned and specifically assigned to a chromosome or genomic region or gene.

[0040] As used herein, the terms "aligned", "alignment", or "aligning" refer to one or more sequences that are identified as a match in terms of the order of their nucleic acid molecules to a known sequence from a reference genome. Such alignment can be done manually or by a computer algorithm. Examples include, without limitation, the Efficient Local Alignment of Nucleotide Data (ELAND) computer program distributed as part of the Illumina Genomics Analysis, Bowtie, BWA, and SOAP2Align. The matching of a sequence read in aligning can be a 100% sequence match or less than 100% (non-perfect match).

[0041] "Amplification" or "amplifying" methods include but are not limited to the polymerase chain reaction (PCR), the ligase chain reaction (LCR) (*e.g.*, Wu, D.Y. and Wallace, R.B. (1989) *Genomics* 4: 560-569; Landegren, U. et al., (1998) *Science* 241(4869): 1077-1080; and Barringer, K.J. et al. (1990) *Gene* 89(1): 117-122), transcription amplification (Kwoh, D.Y. et al., (1989) *Proc. Natl. Acad. Sci.* 86(4): 1173-1177 and WO88/10315), self-sustained sequence replication (Guatelli, J.C. et al., (1990) *Proc. Nat. Acad. Sci.* 87(5): 1874-1878 and WO90/06995), selective amplification of target polynucleotide sequences (U.S. Patent No. 6,410,276), consensus sequence primed polymerase chain reaction (CP-PCR) (U.S. Patent No. 4,437,975), arbitrarily primed polymerase chain reaction (AP-PCR) (U.S. Patent Nos. 5,413,909, 5,861,245) and nucleic acid based sequence amplification (NABSA; U.S. Patent Nos. 5,409,818, 5,554,517, and 6,063,603 each of which is incorporated herein by reference). Other amplification methods that may be used are described in U.S. Patent Nos. 5,242,794, 5,494,810, 4,988,617. Additional methods of sample preparation and techniques for reducing the complexity of a nucleic sample are described in Dong, S. et al., (2001) *Genome Res.* 11(8): 1418-1424, in

U.S. Patent Nos. 6,361,947, 6,391,592 and U.S. Patent Application Ser. Nos. 09/916,135, 09/920,491, 09/910,292, and 10/013,598.

[0042] A "nucleotide" is a monomer that includes a base, such as a pyrimidine, purine, or synthetic analogs thereof, linked to a sugar and one or more phosphate groups. Nucleotides include adenine (A) residues, guanine (G) residues, cytosine (C) residues, thymine (T) residues, and uracil (U) residues. The major nucleotides of DNA are deoxyadenosine 5'-triphosphate (dATP or A), deoxyguanosine 5'-triphosphate (dGTP or G), deoxycytidine 5'-triphosphate (dCTP or C) and deoxythymidine 5'-triphosphate (dTTP or T). The major nucleotides of RNA are adenosine 5'-triphosphate (ATP or A), guanosine 5'-triphosphate (GTP or G), cytidine 5'-triphosphate (CTP or C) and uridine 5'-triphosphate (UTP or U). Nucleotides also include chemical entities containing modified bases, modified sugar moieties and modified phosphate backbones, for example as described in U.S. Patent No. 5,866,336. Such modifications however, can allow for incorporation of the nucleotide into a growing nucleic acid chain or for binding of the nucleotide to the complementary nucleic acid chain.

[0043] Nucleotides can be modified at any position on their structures. Examples include, but are not limited to, the modified nucleotides 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N-6-sopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, methoxyarninomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-S-oxyacetic acid, 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, and 2,6-diaminopurine.

[0044] Examples of modified sugar moieties which can be used to modify nucleotides at any position on their structures include, but are not limited to: arabinose, 2-fluoroarabinose, xylose, and hexose, or a modified component of the phosphate backbone, such as phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a

phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, or a formacetal or analog thereof.

[0045] As used herein the terms "nucleic acid", "nucleic acid molecule", "polynucleotide" and "oligonucleotide" are used interchangeably and refers to the polymeric form of nucleotides, either ribonucleotides and/or deoxyribonucleotides or a modified form of either type of nucleotide. Nucleic acids include, without limitation, cDNA, mRNA, genomic DNA, and synthetic (such as chemically synthesized) DNA or RNA, plasmids, amplicons, cosmids, and fragments thereof. The nucleic acid can be double stranded (ds) or single stranded (ss). Where single stranded, the nucleic acid molecule can be the sense strand or the antisense strand. Nucleic acids can include natural nucleotides (such as adenine, thymine/uracil, cytosine, and guanine) and can also include analogs of natural nucleotides. A set of bases linked to a peptide backbone, as in a peptide nucleic acid (PNA), can be used as a substitute for a nucleic acid molecule. Nucleic acids can be modified by means of a fluorophore that is directly or indirectly excitable. "Fluorescent DNA dye" as used herein may refer to a composition, for example SYBR Green I or SYBR Gold that becomes fluorescently excitable when it associates with double-stranded DNA. Other examples of fluorescent DNA dyes are known in the art and include, *e.g.*, 5-carboxyfluorescein, 2'7'-dimethoxy-4'5'-dichloro-6-carboxyfluorescein, fluorescein (FL); N,N,N',N'-tetramethyl-6-carboxyrhodamine; 6-carboxy-X-rhodamine; CY3; CY5; tetrachloro-fluorescein; and hexachloro-fluorescein; NED; 6-FAM; VIC; PET; LIZ, SID, TED, and TAZ.

[0046] A "target" nucleic acid molecule is a nucleic acid to be sequenced, identified, or detected, and can be obtained or isolated in purified form, by any method known to those skilled in the art (for example, as described in U.S. Patent No. 5,674,743), but need not be in purified form. Various other biomolecules can also be present with the target nucleic acid molecule. For example, the target nucleic acid molecule can be present in a cell or a biological sample (which can include other nucleic acid molecules and proteins). The target nucleic acid molecule may be a whole genome or a portion of a genome, such as chromosomal sequences, or may be extrachromosomal sequences such as plasmids. The target nucleic acid molecule can be derived from any species, including but not limited to, vertebrates such as humans, cows, dogs, cats, mice, rats, sheep, horse, goat, invertebrates, bacteria, viruses, fungi, and the like. The target nucleic acid may be derived from any source, including tissues, primary cells, cultured cells, cell lines, tumor specimens, bodily fluids, and

the like. The target nucleic acid may also be wholly or partly synthetic. A "complementary" nucleic acid molecule is complementary to the target nucleic acid molecule and is the nucleic acid strand that is elongated when sequencing the target nucleic acid molecule.

[0047] An "oligonucleotide" refers to a linear nucleic acid molecule (such as DNA or RNA) sequence of at least 6 nucleotides, for example at least 9, at least 15, at least 18, at least 24, at least 30, at least 50, at least 100, at least 200 or even at least 500 nucleotides long. However shorter or longer oligonucleotides may be used. Oligonucleotides may be designed to have different length. In some embodiments, the sequence of the nucleic acid molecule may be divided up into a plurality of shorter sequences that can be synthesized in parallel and assembled into a single or a plurality of desired nucleic acid molecules using the methods described herein. In certain embodiments, the oligonucleotides are designed to provide the full sense and antisense strands of the nucleic acid molecule. After hybridization of the plus and minus strand oligonucleotides, two double stranded oligonucleotides are subjected to ligation or polymerization in order to form a first subassembly product. Subassembly products are then subjected to ligation or polymerization to form a larger DNA or the full DNA sequence.

[0048] A "primer" is a short nucleic acid molecule, for example sequences of at least 9 nucleotides, which can be annealed to a complementary target nucleic acid molecule by nucleic acid hybridization to form a hybrid between the primer and the target nucleic acid strand. A primer can be extended along the target nucleic acid molecule by a polymerase enzyme. Therefore, individual primers can be used for nucleic acid sequencing, wherein the sequence of the primer is specific for the target nucleic acid molecule, for example so that the primer will hybridize to the target nucleic acid molecule under stringent hybridization conditions. In particular examples, a primer is at least 10 nucleotides in length, such as at least 10 contiguous nucleotides complementary to a target nucleic acid molecule to be sequenced. In order to enhance specificity, longer primers can be employed, such as primers having at least 12, at least 15, at least 20, or at least 30 contiguous nucleotides complementary to a target nucleic acid molecule to be sequenced. Methods for preparing and using primers are described in, for example, Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY; Ausubel et al. (1987) Current Protocols in Molecular Biology, Greene Publ. Assoc. & Wiley-Intersciences.

[0049] As used herein a "probe" refers to an oligonucleotide sequence that may or may not be extended in the amplification reaction by a DNA polymerase. Probes that are very specific for a perfectly complementary target sequence and strongly reject closely related sequences having one or a few mismatched bases are known in the art as "allele discriminating". Probes that hybridize under at least one applicable detection condition not only to perfectly complementary sequences, but also to partially complementary sequences having one or more mismatched bases, are "mismatch tolerant" probes.

[0050] As used herein an "oligonucleotide set", "primer set" or "probe set" refers to a collection of primers or primers and probes for performing amplification or sequencing reactions. Another analogous term is "oligonucleotide library", "primer library", or "probe library". In some embodiments, methods of assembling libraries containing nucleic acids, primers, and probes having predetermined sequence variations are provided herein. Assembly strategies provided herein can be used to generate very large libraries representative of many different nucleic acid probe sequences of interest. In some embodiments, libraries of nucleic acids are libraries of sequence variants. Sequence variants may be variants of a single naturally-occurring sequence. However, in some embodiments, sequence variants may be variants of a plurality of different sequences. A high-density nucleic acid library may include more than 100 different sequence variants (*e.g.*, about 10^2 to 10^3; about 10^3 to 10^4; about 10^4 to 10^5; about 10^5 to 10^6; about 10^6 to 10^7; about 10^7 to 10^8; about 10^8 to 10^9; about 10^9 to 10^{10}; about 10^{10} to 10^{11}; about 10^{11} to 10^{12}; about 10^{12} to 10^{13}; about 10^{13} to 10^{14}; about 10^{14} to 10^{15}; or more different sequences) wherein a percentage of the different sequences are specified sequences as opposed to random sequences (*e.g.*, more than about 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99% of the sequences are predetermined sequences of interest). In some embodiments, the libraries may contain information obtained from sequencing reactions, such as target nucleic acid sequences, sequence reads, sequence alignments, bisulfite-converted sequences, methylation frequencies, allele frequencies, single nucleotide variants or polymorphisms, among others. Libraries may be stored or kept on high-density arrays, microarrays, microchips, computer-readable media, or by any method or apparatus known in the art.

[0051] In some embodiments, the oligonucleotides, primers, and probes may comprise universal (common to all oligonucleotides), semi-universal (common to at least of portion of

the oligonucleotides) or individual or unique primer (specific to each oligonucleotide) binding sites (also referred to herein as "priming sites") on either the 5' end or the 3' end or both. As used herein, the term "universal" primer or primer binding site means that a sequence used to amplify the oligonucleotide is common to all oligonucleotides such that all such oligonucleotides can be amplified using a single set of universal primers. In other circumstances, an oligonucleotide contains a unique primer binding site. As used herein, the term "unique primer binding site" refers to a set of primer recognition sequences that selectively amplifies a subset of oligonucleotides. In yet other circumstances, an oligonucleotide contains both universal and unique amplification sequences, which can optionally be used sequentially.

[0052] A "linker" or "linker sequence" is a structure, which can be a unique nucleic acid sequence, that joins one molecule to another, such as attachment of a probe as described herein to another molecule or a substrate, wherein one portion of the linker is operably linked to a substrate, and wherein another portion of the linker is operably linked to the probe. As used herein, a linker or linker sequence may refer to a common nucleic acid sequence that is preferably non-complementary to a target nucleic acid sequence. A library of oligonucleotides, probes or primers as described herein may each contain a single common linker sequence.

[0053] A "barcode" or "barcode sequence" as used herein refers to a short stretch of nucleotides in a particular order, and different barcodes are different combinations of nucleotides. A barcode may be of any length, but is preferably between 4 to 15, more preferably between 4 to 10, and most preferably between 4 and 8, such as 4, 5, 6, 7 or 8 nucleotides long. Ideally, the barcodes are designed such that they can be unambiguously called post-sequencing or post-amplification. The sequence of the barcode may be identical or different for each nucleic acid molecule in a particular sequencing run, or according to a number of different parameters, such as target nucleic acid origin, sequence reads derived from the 5' strand or the 3' strand, sequence length or molecular weight of a sequence read. Barcode sequences may be derived computationally or by hand. In some aspects, barcode sequences can be randomly generated using an iterative script program, such as Perl.

[0054] Oligonucleotides, primers, and probes may also contain one or more sites ("restriction sites") for cleavage by restriction endonucleases (also known as "restriction enzymes"). Type I enzymes cut DNA at random far from their recognition sequences. Type II enzymes

cut DNA at defined positions close to or within their recognition sequences. Type III enzymes are also large combination restriction-and-modification enzymes. They cleave outside of their recognition sequences and require two such sequences in opposite orientations within the same DNA molecule to accomplish cleavage. Type IV enzymes recognize modified, typically methylated DNA. Restriction endonucleases are commercially available and restriction site sequences are well known in the art (New England Biolabs, Beverly, MA). Any restriction site sequence may be included in oligonucleotides, primers, and probes as described herein.

[0055] Oligonucleotides, primers, and probes may be isolated from natural sources or purchased from commercial sources. Oligonucleotides, primers, and probes described herein may also be synthesized by any suitable method, *e.g.*, standard phosphoramidite methods such as those described by Beaucage, S.L. and Caruthers, M.H. (1981) *Tetrahedron Lett.* 22: 1859-1862 or the triester method according to Matteucci, M.D. and Caruthers, M.H. (1981) *J. Am. Chem. Soc.* 103(11): 3185-3191, or by other chemical methods using either a commercial automated oligonucleotide synthesizer or high-throughput, high-density array methods known in the art (see U.S. Patent Nos. 5,602,244, 5,574,146, 5,554,744, 5,428,148, 5,264,566, 5,141,813, 5,959,463, 4,861,571 and 4,659,774, incorporated herein by reference in its entirety for all purposes). Pre-synthesized oligonucleotides may also be obtained commercially from a variety of vendors. Oligonucleotides, primers, and probes may be prepared using a variety of microarray technologies known in the art. Pre-synthesized oligonucleotide and/or polynucleotide sequences may be attached to a support or synthesized in situ using light-directed methods, flow channel and spotting methods, inkjet methods, pin-based methods and bead-based methods set forth in the following references: McGall et al. (1996) *Proc. Natl. Acad. Sci. U.S.A.* 93(24): 13555-13560; Synthetic DNA Arrays In Genetic Engineering, Vol. 20:111, Plenum Press (1998); Duggan, D.J. et al., (1999) *Nat. Genet.* 21 (Suppl 1): 10-14; Microarrays: Making Them and Using Them In Microarray Bioinformatics, Cambridge University Press, 2003; U.S. Patent Application Publication Nos. 20030068633 and 20020081582; U.S. Patent Nos. 6,833,450, 6,830,890, 6,824,866, 6,800,439, 6,375,903 and 5,700,637; and PCT Application Nos. WO 04/031399, WO 04/031351, WO 04/029586, WO 03/100012, WO 03/066212, WO 03/065038, WO 03/064699, WO 03/064027, WO 03/064026, WO 03/046223, WO 03/040410 and WO 02/24597.

[0056] In the context of the subject matter described herein, references are made to melting temperatures (T_m) of oligonucleotides, primers, and probes. T_m means the temperature at which half of the subject material exists in double-stranded form and the remainder is single stranded. Generally, the T_m of a primer is a calculated value using any method knows in the art, particularly the "% GC" method (Wetmar, J.G. (1991) *Crit. Rev. Biochem. Mol. Biol.* 26: 227-259), the "2(A+T) plus 4(G+C)" method at a standard condition of primer and salt concentration, or methods described in Santa Lucia, J. (1998) *Proc. Natl. Acad. Sci.* 95(4): 1460-1465 and von Ahsen, N. et al., (2001) *Clin. Chem.* 47: 1956-1961.

[0057] The term "methylation" as used herein, denotes the type of chemical modification of nucleic acids that involves the addition of a methyl group, for example to the C5 carbon atom of the cytosine pyrimidine ring or to the N6 nitrogen atom of the adenosine purine ring, with the first option being particularly preferred. This modification can be inherited and subsequently removed without changing the original nucleic acid sequence. As such, it is part of the epigenetic code and the most well characterized epigenetic mechanism. Methylation is reversible: methyl-transferases catalyze the transfer of a methyl group from S-adenosyl-L-methionine to cytosine or adenosine residues. Polymerases such as DNA polymerases do not copy the methylated status during replication (reviewed, *e.g.*, in Robertson, K.D. and Wolffe, A.P. (2000) *Nat. Rev. Genet.* 1(1): 11-19; Li, E. (2002) *Nat. Rev. Genet.* 3: 662-673; Bird, A.P. (2002) *Genes Dev.* 16: 6-21).

[0058] The term "CpG dinucleotide sites" (or "CpG sites"), as used herein, refers to regions of DNA where a cytosine nucleotide is located immediately adjacent to a guanine nucleotide in the linear sequence. "CpG" refers to cytosine and guanine separated by a phosphate (i.e., --C--phosphate--G--). The "CpG" notation is used to distinguish a cytosine followed by guanine from a cytosine base paired to a guanine. Regions of the DNA that have a higher frequency or concentration of CpG sites are known as "CpG islands". "CpG islands" may also define a contiguous region of genomic DNA that satisfies the criteria of (1) having a frequency of CpG dinucleotides corresponding to an "observed/expected ratio" greater than 0.6; (2) having a "GC content" greater than 0.5; and (3) having a length of at least 0.2 kb (as described in Gardiner-Garden et al., (1987) *J. Mol. Biol.*, 196: 262-282), with the exception that repeat regions matching these criteria are excluded (or masked). "CpG island fragment" or "CGI fragment" are used interchangeably to refer to a nucleic acid molecule fragment mapping to and containing at least part of a CpG island. The term "CpG target sequence"

refers to a stretch of bases targeted to selectively enrich for CpG island fragments and/or other methylation informative GC-rich fragments. Many genes in mammalian genomes have CpG islands associated with the transcriptional start site (including the promoter) of the gene, which play a pivotal role in controlling gene expression.

[0059] Methylation at the C5 of cytosine has been found in bacteria, fungi, plant and mammalian genomes. Approximately 60-90% of CpG dinucleotides are methylated in most mammalian cell types. The CpG dinucleotides are not uniformly distributed in mammalian genomes. For example, sequence analysis of the human genome has estimated nearly 30,000 CpG islands, which accounts for about 0.7% of the genome. CpG dinucleotides in the remaining 99.3% of the genome are sparsely distributed. Because of the high cytosine-guanine frequency of CpG islands, it is possible to identify them without knowledge of the methylation pattern of the DNA.

[0060] In normal tissue, CpG islands are often unmethylated but a subset of islands becomes methylated during oncogenesis, cellular development, and various disease states. Hypermethylation (i.e. an increased level of methylation) of CpG sites within the promoters of genes can lead to their silencing, a feature found, *e.g.*, in a number of human cancers (for example the silencing of tumor suppressor genes). In contrast, the hypomethylation (i.e. a reduced level of methylation) of CpG sites has been associated with the over-expression of oncogenes within cancer cells (reviewed, *e.g.*, in Robertson, K.D. and Wolffe, A.P. (2000) *Nat. Rev. Genet.* 1(1): 11-19; Li, E. (2002) *Nat. Rev. Genet.* 3: 662-673; Bird, A.P. (2002) *Genes Dev.* 16: 6-21; Klose, R.J. and Bird, A.P. (2006) *Trends Biochem. Sci.* 31: 89-97). Accordingly, there is great interest in determining the methylation status or profiles of promoters and CpG islands (CGIs) in various tissues, especially with regard to methylation differences accounting for altered patterns of expression in normal development and in various disease states which would greatly improve understanding of these processes and provide potential diagnostic markers and therapeutic targets for diseases (Berman, B.P. et al., (2009) *Nat. Biotech.*, 27(4): 341-342).

[0061] The term "methylation assay" refers to any assay for determining the methylation status of one or more CpG dinucleotide sequences within one or more nucleic acid sequences. "Methylation frequency", "methylation state" or "methylation status" refers to a determination of the presence or absence of 5-methylcytosine ("5-mCyt") or any other methylation modification at one or a plurality of CpG dinucleotides within a target nucleic

acid sequence by a methylation assay. The methylation status of a particular nucleic acid fragment or sequence can indicate the methylation state of every base in the sequence or can indicate the methylation state of a subset of the base pairs (*e.g.*, whether the base is cytosine or 5-methylcytosine) within the sequence. Methylation states at one or more particular CpG methylation sites (each having two CpG dinucleotide sequences) within a nucleic acid sequence may include "unmethylated," "fully-methylated" and "hemi-methylated" sites. Methylation status can also indicate information regarding regional methylation density within the sequence without specifying the exact location at the single nucleotide position level.

[0062] A "methylation profile" refers to a set of data representing the methylation states or methylation frequencies of one or more loci within a molecule of DNA from *e.g.*, the genome of an individual or cells or tissues from an individual. The profile can indicate the methylation state of every base in an individual, can have information regarding a subset of the base pairs (*e.g.*, the methylation state of specific promoters or quantity of promoters) in a genome, or can have information regarding regional methylation density or methylation frequency of one or more loci with or without specifying the exact location at the single nucleotide position level.

[0063] "Differential methylation" denotes a condition in which a particular candidate genomic locus is (at one or more nucleic acid sites comprised in its sequence) methylated in at least one target sample but unmethylated in at least one reference sample, or vice versa, in which a particular candidate genomic locus is (at one or more nucleic acid sites comprised in its sequence) unmethylated in the reference sample but methylated in the target sample. The determination of the differential methylation pattern or frequency of the one or more candidate genes/loci already includes the identification of the exact nucleic acid sites (i.e. sequence elements, genetic loci) comprised in the one or more candidate genes. Preferably, the nucleic acid sites comprised in the one or more candidate genes/loci that are differentially methylated are CpG dinucleotide sites.

[0064] Generally, the determination of the methylation pattern, methylation frequency, or methylation status of the one or more candidate genes/loci may be accomplished by any means known in the art. Preferably, methylation is determined by means of one or more methods selected from reverse-phase HPLC, thin-layer chromatography, *Sss*I methyltransferases with incorporation of labeled methyl groups, the chloracetaldehyde

reaction, differentially sensitive restriction enzymes, hydrazine or permanganate treatment (m5C is cleaved by permanganate treatment but not by hydrazine treatment), bisulfite sequencing, combined bisulfite-restriction analysis, pyrosequencing, methylation-sensitive single-strand conformation analysis (MS-SSCA), high resolution melting analysis (HRM), methylation-sensitive single nucleotide primer extension (MS-SnuPE), base-specific cleavage/MALDI-TOF, methylation-specific PCR (MSP), microarray-based methods, and *Msp*I cleavage (reviewed, *e.g.*, in Rein, T. et al. (1998) *Nucl. Acids Res.* 26: 2255-2264). Methods for detecting methylation status have been described in, for example U.S. Patent Nos. 6,214,556, 5,786,146, 6,017,704, 6,265,171, 6,200,756, 6,251,594, 5,912,147, 6,331,393, 6,605,432, 5,786,146, 6,143,504, 6,596,493, 6,884,586, 6,300,071, and 7,195,870 and U.S. Patent Application Publication Nos. 20030148327, 20030148326, 20030143606, 20050009059, and 20060292564, each of which are incorporated herein by reference. Other array based methods of methylation analysis are disclosed in U.S. Patent Application Publication No. 20050196792. See also, Oakeley, E.J., (1999) *Pharmacol. Ther.* 84: 389-400; Fraga et al., (2002) *BioTechniques* 33: 632-649; and Dahl et al., (2003) *Biogerontology* 4: 233-250.

[0065] Methods for identifying methylation may be based on differential cleavage by restriction enzymes are used. Methylation-sensitive restriction analysis followed by PCR amplification or Southern analysis have been disclosed, for example, in Huang, T.H. et al. (1997) *Cancer Res.* 57: 1030-1034; Zuccotti, M. et al, (1993) *Meth. Enzymol.* 225: 557-567; Carrel, L. et al. (1996) *Am. J. Med. Genet.* 64: 27-30; and Chang et al. (1992) *Plant Mol. Biol. Rep.* 10: 362-366.

[0066] In some aspects, enzymes that include at least one CpG dinucleotide in the recognition site may be used. Enzymes with a recognition site that includes the sequence CCGG include, for example, *Msp*I, *Hpa*II, *Age*I, *Xma*I, *Sma*I, *Ngo*MIV, *Nae*I, and *Bsp*EI. Enzymes with a recognition site that includes the sequence CGCG include, for example, *Bst*UI (CGCG, MSRE), *Mlu*I (ACGCGT, MSRE), *Sac*II (CCGCGG, MSRE), *Bss*HII (GCGCGC, MSRE) and *Nru*I (TCGCGA, MSRE). *Not*I, *Bst*ZI, *Csp*I and *Eag*I have two CpGs in their recognition sites and cleavage is blocked by CpG methylation. Enzymes with a recognition site that includes the sequence GCGC include, for example, *Hin*P1I, *Hha*I, *Afe*I, *Kas*I, *Nar*I, *Sfo*I, *Bbe*I, and *Fsp*I. Enzymes with a recognition site that includes the sequence TCGA include, for example, *Taq*I, *Cla*I (MSRE), *Bsp*DI (MSRE), *Pae*R7I, *Tli*I, *Xho*I, *Sal*I,

and *Bst*BI. For additional enzymes that contain CpG in the recognition sequence and for information about the enzyme's sensitivity to methylation, *see*, for example, the New England Biolabs catalog and web site. In some aspects two restriction enzymes may have a different recognition sequence but generate identical overhangs or compatible cohesive ends. For example, the overhangs generated by cleavage with *Hpa*II or *Msp*I can be ligated to the overhang generated by cleavage with *Taq*I. Some restriction enzymes that include CpG in the recognition site are unable to cleave if the site is methylated; these are methylation sensitive restriction enzymes (MSRE). Other enzymes that contain CpG in their recognition site can cleave regardless of the presence of methylation; these are methylation insensitive restriction enzymes (MIRE). A third type of enzyme cleaves only when the recognition site is methylated, and are referred to herein as methylation dependent restriction enzymes (MDRE). Examples of MIREs that have a CpG in the recognition sequence include, for example, *Bsa*WI (WCCGGW), *Bso*BI, *Bss*SI, *Msp*I, and *Taq*I. Examples of MSREs, that include a CpG in the recognition site, include *Aat*II, *Aci*I, *Acl*I, *Afe*I, *Age*I, *Asc*I, *Ava*I, *Bmg*BI, *Bsa*AI, *Bsa*HI, *Bsp*DI, *Cla*I, *Eag*I, *Fse*I, *Fau*I, *Hae*III, *Hpa*II, *Hin*P1I, *Mlu*I, *Nar*I, *Not*I, *Nru*I, *Pvu*I, *Sac*II, *Sal*I, *Sma*I and *Sna*BI. In preferred aspects a pair of enzymes that have differential sensitivity to methylation and cleave at the same recognition sequence with one member of the pair being a MSRE and the other member being MIRE is used. Still other enzymes include *Bth*CI, *Gla*I, *Hpa*I, *Hin*P1I, *Dpn*I, *Mbo*I, *Cha*I and *Bst*KTI.

[0067] Bisulfite sequencing is a commonly used method in the art for generating methylation data at single-base resolution. The term "bisulfite conversion" refers to a biochemical process for converting unmethylated cytosine residue to uracil or thymine residues, whereby methylated cytosine residues are preserved. "Bisulfite conversion" may be carried out computationally from a nucleic acid sequence contained in a computer file (such as those in FASTA, FASTQ or any file format known in the art), wherein all cytosine residues in a sequence of interest are changed to thymine or uracil residues. Exemplary reagents for bisulfite conversion include sodium bisulfite and magnesium bisulfite. "Bisulfite reagent" refers to a reagent comprising bisulfite, disulfite, hydrogen sulfite or combinations thereof, useful as disclosed herein to distinguish between methylated and unmethylated CpG dinucleotide sequences. One way to obtain such methylation data for the CGIs is to sequence the entire epigenome directly. Due to the difficulty in mapping bisulfite converted sequence reads and the methylation heterogeneity in a cell population, approximately 100 gigabases

(Gb) of sequence data would be needed to generate a high-resolution human DNA methylation map (Lister, R. et al., (2009) *Nature*, 462(7271): 315-322). Other methylation profiling approaches include array capture (Hodges, E. et al., (2009) *Genome Res.* 19(9): 1593-1605), padlock probe capture (Deng, J. et al., (2009) *Nat. Biotech.* 27: 353-360; Ball, M.P. et al., (2009) *Nat. Biotech.*, 27(4): 361-368) and reduced representation bisulfite sequencing (Gu et al., (2010) *Nat. Methods* 7(2): 133-136).

[0068] In particular, bisulfite sequencing involves conversion of unmethylated cytosine to uracil or thymine through a three-step process during sodium bisulfite modification. The steps are sulfonation to convert cytosine to cytosine sulfonate, deamination to convert cytosine sulfonate to uracil sulfonate or thymine sulfonate and alkali desulfonation to convert uracil sulfonate to uracil or thymine sulfonate to thymine. Conversion of methylated cytosine is much slower and is not observed at significant levels in a 4-16 hour reaction (Clark, S.J. et al, (1994) *Nucleic Acids Res.*, 22(15): 2990-7). If the cytosine is methylated it will remain a cytosine. If the cytosine is unmethylated, it will be converted to uracil or thymine. When the modified strand is copied, through, for example, extension of a locus specific primer, a random or degenerate primer or a primer to an adaptor, a G will be incorporated in the interrogation position (opposite the C being interrogated) if the C was methylated and an A will be incorporated in the interrogation position if the C was unmethylated. When the double stranded extension product is amplified, those Cs that were converted to Us or Ts and resulted in incorporation of A in the extended primer will be replaced by Ts during amplification. Those Cs that were not modified and resulted in the incorporation of G will remain as C. Bisulfite treatment can degrade the DNA making it difficult to amplify. The sequence degeneracy resulting from the treatment also complicates primer design. The treatment may also result in incomplete desulfonation, depurination and other as yet uncharacterized DNA damage, making downstream processing more challenging. The treatment can also result in preferential amplification of unmethylated DNA relative to methylated DNA. This may be mitigated by increasing the PCR extension time.

[0069] Kits for DNA bisulfite modification are commercially available from, for example, Human Genetic Signatures' Methyleasy and Chemicon's CpGenome Modification Kit. *See* also, WO04096825, which describes bisulfite modification methods and Olek, A. et al. (1994) *Nucl. Acids Res.* 24(24): 5064-6, which discloses methods of performing bisulfite treatment and subsequent amplification on material embedded in agarose beads. In some

aspects a catalyst such as diethylenetriamine may be used in conjunction with bisulfite treatment, see Komiyama, M. and Oshima, S., (1994) *Tetrahedron Lett.* 35(44): 8185-8188. Diethylenetriamine has been shown to catalyze bisulfite ion-induced deamination of 2'-deoxycytidine to 2'-deoxyuridine at pH 5 efficiently. Other catalysts include ammonia, ethylene-diamine, 3,3'-diaminodipropylamine, and spermine. In some aspects, deamination is performed using sodium bisulfite solutions of 3-5 M with an incubation period of 12-16 hours at about 50°C. A faster procedure has also been reported using 9-10 M bisulfite pH 5.4 for about 10 minutes at 90°C., see Hayatsu, H. et al., (2004) *Proc. Jpn. Acad. Ser. B* 80(4): 189-194.

[0070] Bisulfite treatment allows the methylation status of cytosines to be detected by a variety of methods. For example, any method that may be used to detect a single nucleotide polymorphism (SNP) may be used, for examples, see Syvanen, A.C. (2001) *Nature Rev. Gen.* 2(12): 930-942. In a preferred aspect, bisulfite sequencing methods, systems, and computer program products described herein may provide information regarding not only methylation frequencies or methylation status of a sequence of interest at single base resolution, but also information regarding SNPs, preferably in the same sequencing run. Other methods such as single base extension (SBE) may be used or hybridization of sequence specific probes similar to allele specific hybridization methods. "Variants" or "alleles" generally refer to one of a plurality of species each encoding a similar sequence composition, but with a degree of distinction from each other. The distinction may include any type of variation known to those of ordinary skill in the related art, that include, but are not limited to, polymorphisms such as SNPs, insertions or deletions (the combination of insertion/deletion events are also referred to as "indels"), differences in the number of repeated sequences (also referred to as tandem repeats), and structural variations. Detection of such variants or alleles is also within the ambit of the subject matter described herein.

[0071] In a preferred aspect, molecular inversion probes (MIP), described in Hardenbol, P. et al. *Genome Res.* 15:269-275 (2005) and in U.S. Patent No. 6,858,412, may be used to determine methylation status after methylation dependent modification. A MIP may be designed for each cytosine to be interrogated. In a preferred aspect the MIP includes a locus specific region that hybridizes upstream and one that hybridizes downstream of an interrogation site and can be extended through the interrogation site, incorporating a base that is complementary to the interrogation position. The interrogation position may be the

cytosine of interest after bisulfite modification and amplification of the region and the detection can be similar to detection of a polymorphism. Separate reactions may be performed for each NTP so extension only takes place in the reaction containing the base corresponding to the interrogation base or the different products may be differentially labeled.

[0072] The term "padlock probe" (PLP) refers to circularized nucleic acid molecules which may combine specific molecular recognition and universal amplification (or specific amplification and general recognition), thereby increasing sensitivity and multiplexing capabilities without limiting the range of potential target organisms. PLPs are long oligonucleotides of approximately 100 bases (but can be of any length), containing target complementary regions (referred to herein as "target-capturing sequences") at both their 5' and 3' ends (See, for example, Figure 5). These regions recognize adjacent sequences on the target nucleic acid sequence (Nilsson, M., et al. (1994) *Science* 265: 2085-2088) and may also contain "binding arms" which comprise "extension arms" having priming sites (*e.g.*, universal priming sites"), sites recognized by ligase enzymes, and unique sequence identifiers, sometimes referred to as a "ZipCode" or "barcode". Upon hybridization, the ends of the probes are situated into adjacent position, and can be joined by enzymatic ligation at the ligation sites (also referred to herein as "ligation arms") converting the probe into a circular molecule (also known in the art and referred to herein as an "amplicon") that is threaded on the target strand. This ligation and the resulting circular molecule can only take place when both ligation arm and extension arm segments recognize their target sequences correctly. Non-circularized probes may be removed by exonuclease treatment, while the circularized entities may be amplified with universal primers, which may or may not contain barcode or ZipCode sequences. This mechanism ensures reaction specificity, even in a complex nucleotide extract with a large number of padlock probes. Subsequently, the target-specific products are detected by a universal cZipCode microarray (Shoemaker, D.D., et al., (1996) *Nat. Genet.* 14: 450-456). PLPs have high specificity and multiplexing capabilities in genotyping assays (Hardenbol, P., et al., (2003) *Nat. Biotechnol.*, 21: 673-678.).

[0073] A "formula," "algorithm," or "model" is any mathematical equation, algorithmic, analytical or programmed process, or statistical technique that takes one or more continuous or categorical inputs (herein called "parameters") and calculates an output value, sometimes referred to as an "index" or "index value." Non-limiting examples of "algorithms" include

sums, ratios, and regression operators, such as coefficients or exponents, value transformations and normalizations, rules and guidelines, statistical classification models, pattern recognition, linear and quadratic discrimination, support vector machines, principal component analysis, nearest neighbor search, naïve Bayesian classifiers, and neural networks.

[0074] A "neural network" can be an Artificial Neural Network (ANN) and are information processing systems composed of varying numbers of simple elements called neurons distributed into layers. Neurons are organized in an input layer, one or more hidden layers, and an output layer. The connections between elements determine network function just as in natural biological nervous systems. ANN is an intelligent technique that mimics the functioning of a human brain, and emulates human intuition of making decisions and drawing conclusions even when presented with complex, noisy, irrelevant and partial information. ANNs may have any number of hidden layers. The neurons are connected to each other by weighted links over which signals can pass. Each neuron receives multiple inputs from other neurons, except the neurons in the input layer, in proportion to their connection weights and then generates a single output in accordance with an activation function. An activation function can be linear or nonlinear depending on the application. Sigmoid or Hyperbolic Tangent activation function can be used to improve the performance of ANNs in power system applications.

[0075] An ANN can be trained to perform a particular function by adjusting values of the interconnections called weights, and neuron thresholds. The process of adjusting interconnection weights and neuron thresholds to achieve output of the ANN the same as the target value or desired output for a given input is referred to as "training" of ANN. Training an ANN consists of adjusting interconnection weights of neurons using a learning algorithm. Back propagation with momentum is the commonly used learning algorithm. Multilayer Feed Forward ANNs with Error Back Propagation learning algorithm are also commonly used. Feed Forward calculations, and propagating error from output layer to input layer and weight updating in hidden and output layers are major steps of training algorithm.

[0076] By way of example, the neural networks described herein comprise one or more inputs that are associated with efficiency of the probe or primer described herein. By "efficiency" is meant the amount of target nucleic acid sequence represented by a particular probe or primer in a sequencing library. Standard methods can be used to calculate the efficiency by measuring or counting the amount of target nucleic acid(s) and the amount of

unbound target nucleic acid(s) via sequencing. The efficiency of a probe or primer described herein is typically compared to the capture efficiency of a control probe or primer under the same incubation conditions (*e.g.*, using same buffer and temperature).

[0077] Such inputs comprise, without limitation, target length, target folding energy, target GC content, extension arm A%, extension arm G%, target A%, target T%, target G%, number of "GG" dinucleotides in ligation arm, number of "AT" dinucleotides in extension arm, number of "GG" dinucleotides in extension arm, number of "AA" dinucleotides in target, number of "AT" dinucleotides in target, number of "TA" dinucleotides in target, number of "GT" dinucleotides in target, number of "GA" dinucleotides in target, ligation arm terminal dinucleotide, extension arm terminal dinucleotide, target 5' terminal dinucleotide, ligation arm melting temperature, extension arm melting temperature, ligation arm length, extension arm length, local single-stranded folding energy of the target, and the dinucleotides present at the extension site and ligation site during probe capture. The neural networks described herein may comprise one, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen, sixteen, seventeen, eighteen, nineteen, twenty, twenty-one, twenty-two, twenty-three, twenty-four, twenty-five or more inputs.

[0078] The methods, computer program products, and systems described herein may comprise one, two, three, four, five or more neural networks. In a preferred embodiment, the probe designing algorithm used to predict efficiency of probes or primers as described herein utilizes two neural networks.

[0079] In some exemplary embodiments, there is provided methods, systems, and computer program products for designing large numbers of efficient, low-bias padlock probe molecules using a computational algorithm for probe or primer efficiency prediction. For example, a process may be implemented as a software application or computer program product (also known as software, programs, applications, components, or code) stored in memory and executed by one or more processors contained in a system. The software application or computer program product may accept a complete genome and a tabular list of specific regions of interest, along with several customizable probe properties (such as the inputs described herein, including, *e.g.*, a desired target length, a desired binding arm length, and a desired DNA melting temperature). The software application or computer program product may use this information in an intelligent decision mechanism, such as for example a back propagation neural network-derived equation, to predict probe efficiency based on many

probe characteristics. The software application or computer program product may output the optimal set of probes to obtain maximum coverage of a desired set of genomic regions in a high-throughput sequencing experiment. The software application or computer program product may also add a customized linker sequence to each probe molecule, allowing easy usage in modern high-throughput sequencers. Alternatively, the probe design software application may also perform an *in silico* bisulfite conversion prior to probe design in order to generate an optimal set of probes for targeted bisulfite sequencing. An additional feature may be implemented to allow the probe design software application or computer program product to output the most efficient padlock probe molecules for specific unique regions of a genome, allowing for efficient multiplex organism detection.

[0080] In some implementations, the methods, systems, and computer program products described herein may exhibit features, such as an increased number of targetable genomic regions for padlock probe sequencing, more efficient and unbiased capture of genomic regions, and reduced cost of sequencing to obtain target coverage. Moreover, the method, system, and computer program product described herein may extend to designing efficient padlock probes for SNP genotyping, mutation discovery, targeted genomic sequencing, quantification of allele specific gene expression, analysis of DNA methylation profiles or frequencies, and organism presence detection.

[0081] In some exemplary embodiments, the methods, systems, and computer program products described herein may be applied, in some implementations, with particular advantage to easily generate optimal, high-efficiency sets of padlock probes for targeted genomic sequencing experiments. Figure 2 depicts a process 200 which may be implemented by a system comprising one or more processors and at least one memory including code which when executed by a processor provide the one or more of the operations depicted at process 200. For example, the process 200 may comprise a software application or computer program product implemented on at least one processor and at least one memory. In this example, the software application or computer program product may read three files presented by the user, such as for example, a job file, a target file, and/or a genome or chromosome file. Parameters or inputs provided in these files are associated with probe or primer efficiency and can be used to design padlock probes. The system may optionally comprise one or more databases containing libraries of information related to sequencing data, such as raw sequence reads, sequence alignments, methylation status or frequencies of a

target nucleic acid sequence, and information regarding SNPs, allele frequencies, or other variations in nucleic acid sequences of interest. The system may also comprise one or more communication links connecting the one or more processors to the one or more databases.

[0082] The job file may include several customizable parameters or inputs, such as for example a range for desired binding arm size, a range for target region size, and/or a flag for whether single stranded folding energy of each target should be calculated using an external module. The job file may also include links to one or more software modules to be used and the location on disk of the software, target file, and/or genome/chromosome file.

[0083] The genome/chromosome file may include the genome or chromosome on which targeted sequencing is to be performed. The file may be configured in a FASTA or FASTQ format. In FASTA, an organism or chromosome name may be presented, beginning with the ">" character; example: ">chr1" may be the first line of a FASTA or FASTQ file. The subsequent lines may contain a string of DNA base characters (A, T, G, and C) or DNA ambiguity characters as determined by IUPAC (M, R, W, S, Y, K, V, H, D, B, or N). Line break characters may be allowed to enable easier reading. Multi-chromosome organisms (such as, for example, humans) may still require one ">" character per file; multiple chromosomes may be split into separate files (for example "human_chr1.fa" or "human_chr2.fa").

[0084] The target file may include a tab-delimited list of specific genomic regions or target nucleic acid sequences to be sequenced with padlock probes. The first column in the file may include a specific identifier chosen (*e.g.*, by an experimenter) to describe the target nucleic acid molecule, for example the name of a specific gene or specific regulatory region of interest. The second column may list the chromosome or genome for the specific targeted region. The third and fourth columns may list the beginning and end of the specific target region, in linear bases; the distance spanned can be as short as a single base pair (for genotyping) or thousands of base pairs (for larger-scale sequencing). The fifth column (which may not be included in the file) may accept a strand designation; if one is provided, only probes targeting one specifically chosen strand of DNA (either forward or reverse) may be designed; otherwise, probes may be designed utilizing both strands in combination to provide optimal capture.

[0085] The software application or computer program product implemented on one or more processors and at least one memory may read in the specified job file to obtain desired probe

parameters or inputs. The software application or computer program product may then proceed sequentially through the target file, designing optimal probe sets for each target nucleic acid molecule. The software application or computer program product may begin by opening the first specified genome/chromosome and loading, for example, the entire sequence into random access memory for rapid access. The software application or computer program product may then extract the specific target region of interest or target capture sequence, along with flanking sequence both linearly upstream and downstream of the target, from the genome.

[0086] The software application or computer program may design most, if not all, possible binding arms (which include extension arms and ligation arms as defined herein) for a given target nucleic acid molecule or region using the extracted sequence. The software application or computer program product may take into account the desired binding arm size from the job file, and remove from consideration any very low-complexity binding arms (i.e., more than six nucleotides of the same kind in a row). The software application or computer program product may next associate each binding arm with every possible partner binding arm, taking into account the desired target region length provided in the job file. These arm pairs would represent the two sequences surrounding the common linker. The software application or computer program product may designs arm pairs using both the forward and reverse strand of the provided genome unless the target file specifies a chosen strand; in this case, arm pairs will only be generated for the designated strand.

[0087] The software application or computer program product may then obtains a portion of information or inputs (*e.g.*, six to seven key pieces of information) about each binding arm pair, such as for example the target length, the target GC content, the melting temperature of each binding arm, the length of each binding arm, and/or optionally the local single-stranded folding energy of the target. The software application or computer program product may then use a previously developed equation to predict the probe efficiency. This equation may be developed by an intelligent decision mechanism, such as a neural network, pattern recognizer, and/or other numerical techniques. For example, the equation to predict the probe efficiency may be developed by performing a multiplex genome sequencing reaction using hundreds of thousands of separate padlock probe molecules; the capturing efficiency of each molecule is measured in this sequencing experiment and modeled using one or more neural networks, including for example a back propagation neural network (with three layers

containing 13, 8, and 5 nodes respectively, see Figure 3) to the aforementioned 6-7 pieces of information as input. Additional neural networks may be added (such as, *e.g.*, a network with two hidden layers having 10 and 12 nodes, see Figure 18) with at least 25 pieces of information as input. The software application or computer program product may then divide each probe into separate categories based on which regions of the target sequence are captured, and ranks each available probe by probe efficiency. The probes having the highest efficiency are extracted and included in the generation of a library or probe set. The non-extracted probes of lower efficiency can be pooled and resubmitted for additional rounds of probe design as defined by the methods described herein.

[0088] The software application or computer program product may then generate candidate "probe sets" or "libraries" from the list of valid probes. The software application or computer program product may consider all possible sets of probe sets, and choose the set that provides maximum coverage of the sample and the highest aggregate probe scores, under the constraint that no binding arms in a probe set can bind to the same sequence (in order to prevent probe competition for hybridization). The software application or computer program product may also penalize sets that require many probes in order to control the cost of sequencing; this parameter may be adjustable.

[0089] The software application or computer program product may then report all binding arm pairs for the optimal probe set to the user. If the user is satisfied, the software application may then attach a common linker sequence between the two binding arms and generate a single molecular sequence for each probe that can be generated using commercial DNA synthesis services (such as that provided by Integrated DNA Technologies) or via other high-throughput methods (*see, e.g.*, U.S. Patent Application Ser. No. 60/765,978). Each linker sequence may be customizable to include specific adaptor sequences for current high-throughput sequencers, allowing designed padlock probes to be converted into linear sequencing libraries in just one experimental step. Linker sequences can also be customized to include barcode sequences for even greater multiplex capability or restriction enzyme sites for easy linearization of the circular padlock probe modules (*see, e.g.*, Figure 4). The user may thus integrate the generated probes into many custom experimental environments.

[0090] Once an optimal probe set is designed, the software application or computer program product may output it and proceed to the next target listed in the target file. The software application or computer program product may then repeat the genomic loading and probe

design process. In some embodiments, the software application or computer program product may exclude probes having the highest efficiency and pool the remaining non-extracted probes and repeat the steps of generating a library or set of probe or primer sequences, determining efficiency of the probes in the generated library or set, and ranking the probe or primer sequences in the library or set by efficiency.

[0091] In some exemplary embodiments, the methods, systems, and computer program products described herein are related to characterizing the DNA methylation profile of a sample using bisulfite sequencing and include mapping a genomic sequence of interest to determine methylation status, methylation frequency, and detection of single nucleotide polymorphisms. During bisulfite sequencing, all cytosines present in the DNA molecule except those that are methylated are converted to thymines. Almost every methylated cytosine is present as a cytosine-guanine dinucleotide (CpG), though not all CpGs are methylated. In such embodiments, after the genome/chromosome is loaded into memory, the software application or computer program product performs an *in silico* bisulfite conversion, computationally converting all cytosines except those present in a CpG to thymines. The application or program then designs probes as previously, but penalizes the inclusion of CpG sites in each binding arm. During linker sequence insertion, the software application or computer program product generates multiple probes for those arm pairs containing a CpG; one probe assumes a methylated state (and contains a CpG dinucleotide) while the other assumes an unmethylated state (and contains a TpG instead). This procedure allows for efficient targeted bisulfite sequencing of hundreds of thousands of CpG sites in parallel.

[0092] In some exemplary embodiments, the methods, systems, and computer program products described herein use the probes or primers to obtain sequence reads of a target genome or sequence of interest by bisulfite sequencing and loading it into memory. A software application or computer program product encodes the sequence reads by predicting the forward and reverse orientation of each of the sequence reads to generate at least one forward sequence read and at least one reverse sequence read. The forward and reverse sequence reads are then converted by the software application or computer program product by computationally changing all cytosine residues in the forward sequence reads to thymine residues *in silico*, and changing all guanine residues to adenine residues in the reverse sequence reads. The bisulfite-converted genome sequence and all forward and reverse sequence reads are then aligned computationally by an alignment software application or

computer program (*e.g.*, ELAND, SOAP2Align, Bowtie, BWA, BLAST or any other alignment program known in the art). The alignment application or program can be a stand-alone application or integrated into the system, software application or computer program product described herein. The aligned sequences are then combined to create a map of the target genomic sequence. The software application or computer program product then analyzes and computes methylation frequencies or methylation status of the mapped sequences in entirety. In preferred embodiments, the mapped sequences may also be analyzed by the software application or computer program product for the presence of single nucleotide polymorphisms. Because bisulfite sequencing provides sequence read information at single-base resolution, this technique (and modifications thereof described in the methods, systems, and computer program products described herein) is particularly advantageous for calculating methylation frequencies and detecting SNPs in a single sequencing reaction.

[0093] In some exemplary embodiments, the methods, systems and computer program products described herein are related to organism detection in a mixed sample. Many cellular samples are heterogeneous, and contain mixtures of organisms in unknown quantities; padlock probes can be used to detect which and how many organisms of each of a given type are present. In this example, the software application or computer program product may accept a fourth input file, known as a "homer file," which contains lists of preferred arm sequences in FASTA format (generally those found via genome annotation to be unique to a given genome or chromosome). The job file also contains at least one additional parameter: the number of probes to generate per genome or chromosome. The software application or computer program product then designs binding arm pairs as previously described, but favors binding arms containing a user-provided homer sequence. Instead of creating a "probe set" to maximize coverage of a target region, the software application or computer program instead returns the user-specified number of probes (in order of decreasing capturing efficiency) per genome. Probes designed for many separate genomes can be combined into a single padlock probe reaction, allowing detection of multiple organisms present at low frequency in a mixed population.

[0094] The subject matter described herein may be embodied in systems, computer program products, and methods, depending on the desired configuration. For example, the control module may be realized in digital electronic circuitry, integrated circuitry, specially designed ASICs (application specific integrated circuits), computer hardware, firmware, software,

and/or combinations thereof. These various implementations may include implementation in one or more computer programs that are executable and/or interpretable on a programmable system including at least one programmable processor, which may be special or general purpose, coupled to receive data and instructions from, and to transmit data and instructions to, a storage system, at least one input device, and at least one output device.

[0095] These software applications or computer program products include machine instructions for a programmable processor, and may be implemented in a high-level procedural and/or object-oriented programming language, and/or in assembly/machine language. As used herein, the term "computer-readable medium" refers to any computer program product, apparatus and/or device (*e.g.*, magnetic discs, optical disks, memory, Programmable Logic Devices (PLDs)) used to provide machine instructions and/or data to a programmable processor, including a machine-readable medium.

[0096] A computer may include any type of computer platform such as a workstation, a personal computer, a server, or any other present or future computer. Computers typically include known components such as a processor, an operating system, system memory, memory storage devices, input-output controllers, input-output devices, and display devices. Many possible configurations and components of a computer exist in the art and may also include cache memory, a data backup unit, and other additional devices.

[0097] Display devices may include display devices that provide visual information, this information typically may be logically and/or physically organized as an array of pixels. An interface controller may also be included that may comprise any of a variety of known or future software programs for providing input and output interfaces. For example, interfaces may include "Graphical User Interfaces" (often referred to as GUI's) that provide one or more graphical representations to a user. Interfaces are typically enabled to accept user inputs using means of selection or input known to those of ordinary skill in the related art.

[0098] In the same or alternative embodiments, applications on a computer may employ an interface that includes what are referred to as "command line interfaces" (often referred to as CLI's). CLI's typically provide a text based interaction between an application and a user. Typically, command line interfaces present output and receive input as lines of text through display devices. For example, some implementations may include a "shell" such as Unix Shells known to those of ordinary skill in the related art, or Microsoft Windows Powershell

that employs object-oriented type programming architectures such as the Microsoft .NET framework. Interfaces may include one or more GUI's, CLI's or a combination thereof.

[0099] A processor may include a commercially available processor such as an Itanium® or Pentium® processor made by Intel Corporation, a SPARC® processor made by Sun Microsystems, an Athlon™ or Opteron™ processor made by AMD corporation, or it may be one of other processors that are or will become available. Some embodiments of a processor may also include Multi-core processors and/or employ parallel processing technology in a single or multi-core configuration. For example, a multi-core architecture typically comprises two or more processor "execution cores". Each execution core may perform as an independent processor that enables parallel execution of multiple threads. In addition, a processor may be configured in what is generally referred to as 32 or 64 bit architectures, or other architectural configurations now known or that may be developed in the future.

[00100] A processor typically executes an operating system, which may be, for example, a Windows®-type operating system (such as Windows® XP, Windows Vista®, Windows 7) from the Microsoft Corporation; the Mac OS X operating system from Apple Computer Corp. (such as 7.5 Mac OS X v10.4 "Tiger" or 7.6 Mac OS X v10.5 "Leopard" operating systems); a Unix® or Linux-type operating system available from many vendors or an open source; another or a future operating system; or some combination thereof. An operating system interfaces with firmware and hardware in a well-known manner, and facilitates the processor in coordinating and executing the functions of various computer programs that may be written in a variety of programming languages. An operating system, typically in cooperation with a processor, coordinates and executes functions of the other components of a computer. An operating system also provides scheduling, input-output control, file and data management, memory management, and communication control and related services, all in accordance with known techniques.

[00101] System memory may include any of a variety of known or future memory storage devices. Examples include any commonly available random access memory (RAM), magnetic medium such as a resident hard disk or tape, an optical medium such as a read and write compact disc, or other memory storage device. Memory storage devices may include any of a variety of known or future devices, including a compact disk drive, a tape drive, a removable hard disk drive, USB or flash drive, or a diskette drive. Such types of memory storage devices typically read from, and/or write to, a program storage medium such as,

respectively, a compact disk, magnetic tape, removable hard disk, USB or flash drive, or floppy diskette. Any of these program storage media, or others now in use or that may later be developed, may be considered a computer program product. As will be appreciated, these program storage media typically store a computer software program and/or data. Computer software programs, also called computer control logic, typically are stored in system memory and/or the program storage device used in conjunction with memory storage device.

[00102] In some embodiments, a computer program product is described comprising a computer usable medium having control logic (computer software program, including program code) stored therein. The control logic, when executed by a processor, causes the processor to perform functions described herein. In other embodiments, some functions are implemented primarily in hardware using, for example, a hardware state machine. Implementation of the hardware state machine so as to perform the functions described herein will be apparent to those skilled in the relevant arts.

[00103] Input-output controllers could include any of a variety of known devices for accepting and processing information from a user, whether a human or a machine, whether local or remote. Such devices include, for example, modem cards, wireless cards, network interface cards, sound cards, or other types of controllers for any of a variety of known input devices. Output controllers could include controllers for any of a variety of known display devices for presenting information to a user, whether a human or a machine, whether local or remote. As presently described herein, the functional elements of a computer may communicate with each other via a system bus. Some embodiments of a computer may communicate with some functional elements using network or other types of remote communications.

[00104] As will be evident to those skilled in the relevant art, an instrument control and/or a data processing application, if implemented in software, may be loaded into and executed from system memory and/or a memory storage device. All or portions of the instrument control and/or data processing applications may also reside in a read-only memory or similar device of the memory storage device, such devices not requiring that the instrument control and/or data processing applications first be loaded through input-output controllers. It will be understood by those skilled in the relevant art that the instrument control and/or data processing applications, or portions of it, may be loaded by a processor in

a known manner into system memory, or cache memory, or both, as advantageous for execution.

[00105] Also a computer may include one or more library files, experiment data files, and an internet client stored in system memory. For example, experiment data could include data related to one or more experiments or assays such as detected signal values, or other values associated with one or more sequencing experiments or processes. Additionally, an internet client may include an application enabled to accesses a remote service on another computer using a network and may for instance comprise what are generally referred to as "Web Browsers". In the present example some commonly employed web browsers include Microsoft® Internet Explorer available from Microsoft Corporation, Mozilla Firefox® from the Mozilla Corporation, Safari from Apple Computer Corp., Google Chrome available from Google, Inc., or other type of web browser currently known in the art or to be developed in the future. An internet client may include, or could be an element of, specialized software applications enabled to access remote information via a network such as a data processing application for sequencing applications.

[00106] A network may include one or more of the many various types of networks well known to those of ordinary skill in the art. For example, a network may include a local or wide area network that employs what is commonly referred to as a TCP/IP protocol suite to communicate. A network may include a network comprising a worldwide system of interconnected computer networks that is commonly referred to as the internet, or could also include various intranet architectures. Some users in networked environments may prefer to employ what are generally referred to as "firewalls" (also sometimes referred to as Packet Filters, or Border Protection Devices) to control information traffic to and from hardware and/or software systems. For example, firewalls may comprise hardware or software elements or some combination thereof and are typically designed to enforce security policies put in place by users, such as for instance network administrators, etc.

[00107] The subject matter described herein may also make use of additional computer program products and software for a variety of purposes, such as probe design, management of data, analysis, and instrument operation. See, U.S. Patent Nos. 5,593,839, 5,795,716, 5,733,729, 5,974,164, 6,066,454, 6,090,555, 6,185,561, 6,188,783, 6,223,127, 6,229,911 and 6,308,170. Additionally, the subject matter described herein may also make use of methods for providing genetic information over networks such as the Internet as shown in U.S. Patent

Application Ser. Nos. 10/197,621, 10/063,559 (United States Publication No. 20020183936), 10/065,856, 10/065,868, 10/328,818, 10/328,872, 10/423,403, and 60/482,389.

[00108] The subject matter described herein also provides for design of oligonucleotides, primers, and probes useful for general sequencing reactions and assays. Commercial sequencing by synthesis platforms are available, such as the Genome Sequencer from Roche/454 Life Sciences, the Genome Analyzer from Illumina/Solexa, the SOLiD system from Applied BioSystems, Pacific Biosystems and the Heliscope system from Helicos Biosciences. Exemplary sequencing platforms may have one or more of the following features: 1) four differently optically labeled nucleotides are utilized (*e.g.*, Genome Analyzer); 2) sequencing-by-ligation is utilized (*e.g.*, SOLiD); 3) pyrosequencing is utilized (*e.g.*, Roche/454); and 4) four identically optically labeled nucleotides are utilized (*e.g.*, Helicos).

[00109] Such sequencing reactions and assays include sequencing by ligation methods commercialized by Applied Biosystems (*e.g.*, SOLiD sequencing). In general, double stranded fragment nucleic acid molecules can be prepared by the methods described herein, and then incorporated into a water-in-oil emulsion along with polystyrene beads and amplified, for example by PCR. In some cases, alternative amplification methods can be employed in the water-in-oil emulsion such as any of the methods provided herein. The amplified product in each water microdroplet formed by the emulsion can interact, bind, or hybridize with the one or more beads present in that microdroplet leading to beads with a plurality of amplified products of substantially one sequence. When the emulsion is broken, the beads float to the top of the sample and are placed onto an array. The methods can include a step of rendering the nucleic acid bound to the beads single-stranded or partially single stranded. Sequencing primers are then added along with a mixture of four different fluorescently labeled oligonucleotide probes. The probes bind specifically to the two bases in the nucleic acid molecule to be sequenced immediately adjacent and 3' of the sequencing primer to determine which of the four bases are at those positions. After washing and reading the fluorescence signal from the first incorporated probe, a ligase is added. The ligase cleaves the oligonucleotide probe between the fifth and sixth bases, removing the fluorescent dye from the nucleic acid molecule to be sequenced. The whole process is repeated using a different sequence primer until all of the intervening positions in the sequence are imaged. The process allows the simultaneous reading of millions of DNA fragments in a "massively

parallel" manner. This "sequence-by-ligation" technique uses probes that encode for two bases rather than just one, allowing error recognition by signal mismatching and leading to increased base determination accuracy.

[00110] Other sequencing methods include sequencing by synthesis methods commercialized by 454/Roche Life Sciences including but not limited to the methods and apparatus described in Margulies et al., Nature (2005) 437:376-380 (2005); and U.S. Patent Nos. 7,244,559; 7,335,762; 7,211,390; 7,244,567; 7,264,929; and 7,323,305. In general, double stranded fragment nucleic acid molecules can be prepared by the methods described herein, immobilized onto beads, and compartmentalized in a water-in-oil PCR emulsion. In some cases, alternative amplification methods can be employed in the water-in-oil emulsion such as any of the methods provided herein. When the emulsion is broken, amplified fragments remain bound to the beads. The methods can include a step of rendering the nucleic acid bound to the beads single stranded or partially single stranded. The beads can be enriched and loaded into wells of a fiber optic slide so that there is approximately 1 bead in each well. Nucleotides are flowed across and into the wells in a fixed order in the presence of polymerase, sulfhydrolase, and luciferase. Addition of nucleotides complementary to the target strand can result in a chemiluminescent signal that is recorded, such as by a camera. The combination of signal intensity and positional information generated across the plate allows software to determine the DNA sequence.

[00111] Other sequencing methods include those commercialized by Helicos BioSciences Corporation (Cambridge, MA) as described in U.S. Patent Application Ser. No. 11/167,046, and U.S. Patent Nos. 7,501,245; 7,491,498; 7,276,720; and in U.S. Patent Application Publication Nos. 20090061439; 20080087826; 20060286566; 20060024711; 20060024678; 20080213770; and 20080103058. In general, double stranded fragment nucleic acid molecules can be isolated and purified, then immobilized onto a flow-cell surface. The methods can include a step of rendering the nucleic acid bound to the flow-cell surface stranded or partially single stranded. Polymerase and labeled nucleotides are then flowed over the immobilized DNA. After fluorescently labeled nucleotides are incorporated into the DNA strands by a DNA polymerase, the surface is illuminated with a laser, and an image is captured and processed to record single molecule incorporation events to produce sequence data.

[00112] Other methods include sequencing by ligation methods commercialized by Dover Systems. Generally, oligonucleotides, primers, and probes can be prepared by the methods described herein. The nucleic acid molecules can then be amplified in an emulsion in the presence of magnetic beads. Any amplification methods can be employed in the water-in-oil emulsion. The resulting beads with immobilized clonal nucleic acid polonies are then purified by magnetic separation, capped, amine functionalized, and covalently immobilized in a series of flow cells. The methods can include a step of rendering the nucleic acid bound to the flow-cell surface stranded or partially single stranded. A series of anchor primers are flowed through the cell, where they hybridize to the synthetic oligonucleotide sequences at the 3' or 5' end of proximal or distal genomic DNA tags. Once an anchor primer is hybridized, a mixture of fully degenerate nonanucleotides ("nonamers") and T4 DNA ligase is flowed into the cell. Each of the nonamer mixture's four components is labeled with one of four fluorophores, which correspond to the base type at the query position. The fluorophore-tagged nonamers selectively ligate onto the anchor primer, providing a fluorescent signal that identifies the corresponding base on the genomic DNA tag. Once the probes are ligated, fluorescently labeling the beads, the array is imaged in four colors. Each bead on the array will fluoresce in only one of the four images, indicating whether there is an A, C, G, or T at the position being queried. After imaging, the array of annealed primer-fluorescent probe complex, as well as residual enzyme, are chemically striped using guanidine HCl and sodium hydroxide. After each cycle of base reads at a given position have been completed, and the primer-fluorescent probe complex has been stripped, the anchor primer is replaced, and a new mixture of fluorescently tagged nonamers is introduced, for which the query position is shifted one base further into the genomic DNA tag. Seven bases are queried in this fashion, with the sequence performed from the 5' end of the proximal tag, followed by six base reads with a different anchor primer from the 3' end of the proximal tag, for a total of 13 base pair reads for this tag. This sequence is then repeated for the 5' and 3' ends of the distal tag, resulting in another 13 base pair reads. The ultimate result is a read length of 26 bases (thirteen from each of the paired tags). However, it is understood that this method is not limited to 26 base read lengths.

[00113] Other useful methods for sequencing include those commercialized by Illumina as described U.S. Patent Nos. 5,750,341; 6,306,597; and 5,969,119. In general, oligonucleotides, primers, and probes can be prepared by the methods described herein to

produce amplified nucleic acid sequences tagged at one (*e.g.*, (A)/(A') or both ends (*e.g.*, (A)/(A') and (C)/(C')). In some cases, single stranded nucleic acid tagged at one or both ends is amplified by the methods described herein (*e.g.*, by SPIA or linear PCR). The resulting nucleic acid is then denatured and the single stranded amplified nucleic acid molecules are randomly attached to the inside surface of flow-cell channels. Unlabeled nucleotides are added to initiate solid-phase bridge amplification to produce dense clusters of double-stranded DNA. To initiate the first base sequencing cycle, four labeled reversible terminators, primers, and DNA polymerase are added. After laser excitation, fluorescence from each cluster on the flow cell is imaged. The identity of the first base for each cluster is then recorded. Cycles of sequencing are performed to determine the fragment sequence one base at a time. For paired-end sequencing, such as for example, when the nucleic acid molecules are labeled at both ends by the methods described herein, sequencing templates can be regenerated in-situ so that the opposite end of the fragment can also be sequenced.

[00114] Still other sequencing methods include those commercialized by Pacific Biosciences as described in U.S. Patent Nos. 7,462,452; 7,476,504; 7,405,281; 7,170,050; 7,462,468; 7,476,503; 7,315,019; 7,302,146; 7,313,308; and U.S. Patent Application Publication Nos. US20090029385; US20090068655; US20090024331; and US20080206764. In general, oligonucleotides, primers and probes can be prepared by the methods described herein. Target nucleic acid molecules can then be immobilized in zero mode waveguide arrays. The methods may include a step of rendering the nucleic acid bound to the waveguide arrays single stranded or partially single stranded. Polymerase and labeled nucleotides are added in a reaction mixture, and nucleotide incorporations are visualized via fluorescent labels attached to the terminal phosphate groups of the nucleotides. The fluorescent labels are clipped off as part of the nucleotide incorporation. In some cases, circular templates are utilized to enable multiple reads on a single molecule.

[00115] Another example of a sequencing technique that can be used in the methods described herein is nanopore sequencing (see *e.g.* Soni, G.V. and Meller, A. (2007) *Clin. Chem.* 53: 1996-2001). A nanopore can be a small hole of the order of one nanometer in diameter. Immersion of a nanopore in a conducting fluid and application of a potential across it can result in a slight electrical current due to conduction of ions through the nanopore. The amount of current that flows is sensitive to the size of the nanopore. As a DNA molecule passes through a nanopore, each nucleotide on the DNA molecule obstructs the nanopore to a

different degree. Thus, the change in the current passing through the nanopore as the DNA molecule passes through the nanopore can represent a reading of the DNA sequence.

[00116] Another example of a sequencing technique that can be used is semiconductor sequencing provided by Ion Torrent (*e.g.*, using the Ion Personal Genome Machine (PGM)). Ion Torrent technology can use a semiconductor chip with multiple layers, *e.g.*, a layer with micro-machined wells, an ion-sensitive layer, and an ion sensor layer. Nucleic acids can be introduced into the wells, *e.g.*, a clonal population of single nucleic can be attached to a single bead, and the bead can be introduced into a well. To initiate sequencing of the nucleic acids on the beads, one type of deoxyribonucleotide (*e.g.*, dATP, dCTP, dGTP, or dTTP) can be introduced into the wells. When one or more nucleotides are incorporated by DNA polymerase, protons (hydrogen ions) are released in the well, which can be detected by the ion sensor. The semiconductor chip can then be washed and the process can be repeated with a different deoxyribonucleotide. A plurality of nucleic acids can be sequenced in the wells of a semiconductor chip. The semiconductor chip can comprise chemical-sensitive field effect transistor (chemFET) arrays to sequence DNA (for example, as described in U.S. Patent Application Publication No. 20090026082). Incorporation of one or more triphosphates into a new nucleic acid strand at the 3' end of the sequencing primer can be detected by a change in current by a chemFET. An array can have multiple chemFET sensors.

[00117] Although a few variations have been described in detail above, other modifications or additions are possible. In particular, further features and/or variations may be provided in addition to those set forth herein. For example, the implementations described above may be directed to various combinations and subcombinations of the disclosed features and/or combinations and subcombinations of several further features disclosed above. In addition, the logic flow depicted in the accompanying figures and/or described herein does not require the particular order shown, or sequential order, to achieve desirable results. Other embodiments may be within the scope of the following claims.

EXAMPLES

Example 1: Changes in DNA methylation detected by targeted bisulfite sequencing

[00118] A method to specifically capture an arbitrary subset of genomic targets for single-molecule bisulfite sequencing and digital quantification of DNA methylation at single-nucleotide resolution is presented herein. A set of ~30,000 padlock probes was designed to

assess the methylation state of ~66,000 CpG sites within 2,020 CpG islands on human chromosome 12, chromosome 20, and 34 selected regions. To investigate epigenetic differences associated with de-differentiation, methylation in three human fibroblast lines and eight human pluripotent stem cell lines was compared. Chromosome-wide methylation patterns were similar among all lines studied, but cytosine methylation was slightly more prevalent in the pluripotent cells than in the fibroblasts. Induced pluripotent stem (iPS) cells appeared to display more methylation than embryonic stem cells. In fibroblasts and pluripotent cells, 288 regions were methylated differently. This targeted approach is particularly useful for analyzing DNA methylation in large genomes.

[00119] Padlock probes have been previously used for exon capture and resequencing (Porreca, G.J. et al. (2007) *Nat. Methods* 4: 931-936). This approach to targeted bisulfite sequencing involves the *in situ* synthesis of long (~150 nt) oligonucleotides on programmable microarrays, followed by their cleavage and enzymatic conversion into padlock probes. A library of padlock probes was annealed to the template DNA, circularized, and amplified by PCR before shotgun sequencing (Figure 5A-5C). There are, however, two major challenges in performing padlock capture for bisulfite sequencing. First, bisulfite treatment converts all unmethylated cytosines into uracils, resulting in marked reduction of sequence complexity. Achieving specific target capture on bisulfite-converted DNA is more difficult than on native genomic DNA. Second, low capturing sensitivity, high bias and random losses of alleles was initially observed with the "eMIP method" previously disclosed in the art (Porreca, G.J. et al. (2007) *Nat. Methods* 4: 931-936). Obtaining accurate and efficient quantification of DNA methylation was not possible with the existing protocol, especially with the presence of allelic drop-outs.

Materials and Methods

[00120] Padlock probe design

[00121] A probe design algorithm was developed to search for an optimal set of padlock probes covering an arbitrary set of non-repetitive genomic targets. This algorithm weights candidate probes based on several sequence features that were previously not considered in eMIP probe design, including the melting temperature, size and word statistics (distribution of 12-mers in the bisulfite-converted genome) of the capturing arms, and gap sizes. When the capturing arms contain one or more CpG dinucleotides, we used multiple

probes to iterate all possible methylation state combinations of the CpGs contained within the arms. Chromosome positions of CpG islands were retrieved from the UCSC genome browser based on the hg18 annotation.

[00122] Padlock probe production

[00123] Libraries of long oligonucleotides (~150 nt) were synthesized by ink-jet printing on a programmable microarray and released (Agilent Technologies). The estimated total yield is 10 fmol per library. PCR amplification was performed in 32–96 reactions (100 µl each) with 0.1 nM template oligonucleotides, 200 µM dNTPs, 400 nM Ap1V4IU primer, 400 nM Ap2V4 primer, 0.8× SybrGreen I, 36 units JumpStart *Taq* polymerase in 1× JumpStart buffer (Sigma), at 94°C for 2 minutes, 22 cycles of 94°C for 30 seconds, 55°C for 2 minutes, 72°C for 45 seconds and, finally, 72°C for 5 minutes. The amplicons were purified by either column purification (Zymo DNA Concentrator- 100 columns) or ethanol precipitation.

[00124] Approximately 40–60 µg of the purified PCR amplicons were digested with 40 units Lambda Exonuclease (5 U/µl; New England Biolabs (NEB)) in 1× lambda exonuclease buffer (NEB) at 37°C for 2 hours, followed by denaturing at 90°C for 5 minutes, and purified with six Qiagen Qiaquick PCR purification columns. The resulting single-stranded DNA was subsequently digested with 6 units USER enzyme (1 U/µl; NEB) in 1× *Dpn*II buffer (NEB) at 37°C for 4 hours. Ten µl of 100 µM *Dpn*II_V4 guide oligo was added into the reaction and denatured the mixture at 95°C for 5 minutes in a thermocycler, followed by a gradual decrease of temperature (0.1°C/s) to 60°C and a 20-minute incubation at 60°C. The mixture was digested with 100 U *Dpn*II (50 U/µl) at 37°C for 2 hours. The single-stranded 102-nt probes were finally purified from the digestion with 6% denaturing PAGE (6% TB-Urea 2D gel; Invitrogen).

[00125] Multiplex capture on bisulfite-converted DNA

[00126] Genomic DNA was extracted from frozen pellets of fibroblast, iPS or hES cells using Qiagen DNeasy columns and bisulfite converted with the Zymo DNA Methylation Gold Kit (Zymo Research). Padlock probes (60 nM) and 200 ng of bisulfite-converted genomic DNA were mixed in 10 µl 1× Ampligase Buffer (Epicentre), denatured at 95°C for 10 minutes, then hybridized at 55°C for 18 hours, after which 1 µl gap-filling mix (200 µM

dNTPs, 2 U AmpliTaq Stoffel Fragment (ABI) and 0.5 units Ampligase (Epicentre) in 1× Ampligase buffer) was added to the reaction. For circularization, the reactions were incubated at 55°C for 4 hours, followed by five cycles of 95°C for 1 minute and 55°C for 4 hours. To digest linear DNA after circularization, 2 µl exonuclease mix containing 10 U/µl exonuclease I and 100 U/µl exonuclease III (USB) was added to the reaction, and the reactions were incubated at 37°C for 2 hours and then inactivated at 95°C for 5 minutes.

[00127] Capture circles amplification

[00128] Ten microliters of circularization products were amplified by PCR in 100 µl reactions with 200 nM AmpF6.2-SoL primer, 200 nM AmpR6.2-SoL primer, 0.4× SybrGreen I and 50 µl iProof High-Fidelity Master Mix (Bio-Rad) at 98°C for 30 seconds, eight cycles of 98°C for 10 seconds, 58°C for 20 seconds, 72°C for 20 seconds, 14 cycles of 98°C for 10 seconds, 72°C for 20 seconds and 72°C for 3 minutes. The amplicons of the expected size range (344–394 bp) were purified with 6% PAGE (6% TBE gel; Invitrogen).

[00129] Shotgun sequencing library construction

[00130] Purified PCR products with the four probe sets on the same template DNA were pooled in equal molar ratio, and reamplified in 4× 100 µl reactions with 4-µl template (10-15 ng/µl), 200µM dNTPs, 20 µM dUTP, 200 nM AmpF6.3 primer, 200 nM AmpR6.3 primer, 0.4× SybrGreen I and 200 µl 2× Taq Master Mix (NEB) at 94°C for 3 minutes, 8 cycles of 94°C for 45 seconds, 55°C for 45 seconds, 72°C for 45 seconds and 72°C for 3 minutes. PCR amplicons were purified with Qiaquick columns, and digested with *Mme*I: ~3.6 nmole purified PCR amplicons, 16 units of *Mme*I (2 U/µl; NEB), 100 µM SAM in 1× NEB Buffer 4 at 37°C for 1 hour. The digestions were again column purified, and digested with 3 U USER enzyme (1 U/µl) at 37°C for 2 hours, then with 10 units S1 nuclease (10 U/µl; Invitrogen) in 1× S1 nuclease buffer at 37°C for 10 minutes. The fragmented DNA was column purified, and end repaired at 25°C for 45 minutes in 25-µl reactions containing 2.5 µl 10× buffer, 2.5 µl dNTP mix (2.5 mM each), 2.5 µl ATP (10 mM), 1 µl end-repair enzyme mix (Epicentre), and 15 µl DNA. Approximately 100–500 ng of the end-repaired DNA was ligated with 60 µM Solexa sequencing adaptors in 30 µl of 1× QuickLigase Buffer (NEB) with 1 µl QuickLigase for 15 minutes at 25°C. Ligation products of 150-175 bp in size were size selected with 6% PAGE, and amplified by PCR in 100 µl reactions with 15 µl template,

200 nM Solexa PCR primers, 0.8× SybrGreen I and 50 µl iProof High-Fidelity Master Mix (Bio-Rad) at 98°C for 30 seconds, 12 cycles of 98°C for 10 seconds, 65°C for 20 seconds, 72°C for 20 seconds and 72°C for 3 minutes. The PCR amplicons were purified with Qiaquick PCR purification columns, and sequenced on Illumina Genome Analyzer. All primer sequences are listed in Table 1.

[00131] Table 1: Sequences of primers used in padlock capture and sequencing library construction.

Primer Name	Primer Sequence
AP1v4IU	5'-G*T*AGACTGGAAGAGCACTGTU-3' (SEQ ID NO: 1)
AP2v4	5'-/Phos/TAGCCTCATGCGTATCCGAT-3' (SEQ ID NO: 2)
DpnII_v4	5'- ATGCGTATCCGATC-3' (SEQ ID NO: 3)
AmpF6.2Sol	5'-AATGATACGGCGACCACCGACACTCTCTGCAGATGTTATCGAGGT-3' (SEQ ID NO: 4)
AmpR6.2Sol	5'-CAAGCAGAAGACGGCATACGAGCTCTTCACGCAGCTGAATAGGAACGAT-3' (SEQ ID NO: 5)
AmpF6.3	CAGATGTTATCGAGGTCCGAC (SEQ ID NO: 6)
AmpR6.3	GGAACGATGAGCCTCCAAC (SEQ ID NO: 7)

"*" indicates a phosphorothioate bond

[00132] Read mapping and data analysis

[00133] Mapping of bisulfite sequencing reads was performed with SOAP (Li, R. et al. (2008) *Bioinformatics* 24: 713-714) driven by a customized Perl script. An unbiased mapping strategy in which the mapping success rate is independent of the methylation status was developed. Sequences of the captured targets were extracted from the repeat-masked human genome (hg18), and both strands were "bisulfite converted" *in silico* assuming no methylation on all CpG dinucleotides. The raw sequencing reads were also converted to "unmethylated reads". To do this, the C/T and A/G ratios for each read were first compared to determine whether the reads corresponding to the bisulfite-converted strand or the reverse-complementary strand. In the latter case, the raw sequence was reverse complemented. All Cs were replaced by Ts in the resulting sequences. The unmethylated reads were then aligned to the unmethylated template sequences using SOAP. The false mapping rate that was due to the use of captured targets instead of the full human genome sequence was 0.21%. Finally, based on the mapping position, the methylation status of each CpG site was retrieved from the unconverted raw reads. Cluster analyses and statistical analyses were performed

with R, Cluster3 Perl module, and in-house Perl scripts. The UCSC Genome Browser and Multiexperiment Viewer were used for data visualization.

[00134] Discussion

[00135] Approximately 10,580 padlock probes were designed, each capturing a 175- to 225-bp region, including 9,350 probes covering 2,020 CpG islands (Table 2) on human chromosomes 12 and 20, 705 probes covering 237 promoters in eight ENCODE (the Encyclopedia of DNA Elements) regions, and 527 probes targeting 4-kb regions centered on the transcription start sites (TSS) of 26 genes related to development or pluripotency (Table 3).

[00136] Table 2: Summary statistics of padlock captured CGIs

	Total	Chromosome 12	Chromosome 20
CpG islands on the chromosome	2020	1221	799
CpG islands covered	2020	1221	799
CpG islands percentage covered*	82%	82%	83%
CpG islands on promoter regions	857	551	306
CpG islands on gene body	667	350	317
CpG islands outside promoter and gene body region	496	320	176

*Calculated as the fraction of base-pairs within CGIs that were covered by the padlock probes. Some CGIs were not completely covered due to the presence of repetitive sequences, or extreme nucleotide composition such that no probe could be designed.

[00137] Table 3: List of 26 selected genes and 8 ENCODE regions

Target position	Gene
chr1:063559560-63563560	FOXD3
chr1:224193561-224197561	LEFTY2
chr10:134891777-134895777	UTF1
chr11:031787344-31791344	PAX6
chr12:007831291-7835291	NANOG
chr14:056344940-56348940	OTX2
chr14:100360210-100364210	MEG3
chr15:022749222-22753222	SNRPN
chr15:022910785-22914785	IPW
chr15:047500765-47504765	FGF7
chr17:037791988-37795988	STAT3
chr17:042249080-42253080	WNT3
chr19:001601591-1605591	TCF3
chr2:066513974-66517974	MEIS1
chr20:030811877-30815877	DNMT3B
chr3:055488366-55492366	WNT5A
chr3:057207043-57211043	HESX1
chr3:171556146-171560146	SKIL
chr3:182910526-182914526	SOX2
chr4:054788195-54792195	PDGFRA

Target position	Gene
chr4:057466834-57470834	REST
chr4:123965397-123969397	FGF2
chr5:149524548-149528548	CDX
chr5:153836005-153840005	HAND1
chr6:031244421-31248421	OCT4
chrX:136474004-136478004	ZIC3
chr5:153836005-153840005	HAND1
chr22:3033954-31833953	ENm004
chr21:32668237-34364221	ENm005
chrX:152767492-154063081	ENm006
chr19:59023585-60024460	ENm007
chr7:26924046-27424045	ENm010
chr11:1699992-2306039	ENm011
chr12:38626477-39126476	ENr123
chr20:33304929-33804928	ENr333

[00138] The total size of captured fragments was 2.1 Mbp, representing 0.064% of the human genome. Because some probes contain CpG sites within the capturing arms, all possible C/T combinations were iterated on these CpG sites, and a total of 30,000 nondegenerate probes were synthesized. CpG islands were chosen to perform the proof-of-concept study primarily because they represent a relatively well-defined set of genomic features in the human genome annotation. To increase the sensitivity and reduce bias, the probe/target ratio was increased by more than tenfold, the reaction time was extended, and five additional cycles of circularization were added in comparison with the published protocol (Porreca, G.J. et al. (2007) *Nat. Methods* 4: 931-936). To integrate construction of sequencing libraries with padlock capture, a new method that uses a combination of uracil-specific excision reagent (USER) enzymes and S1 nuclease to create fragments with random ends was developed (Figure 5D).

[00139] Validation of the targeted bisulfite sequencing method was achieved by capturing bisulfite-converted Jurkat cell genomic DNA with all 30,000 padlock probes in a single-tube reaction. PCR amplicons from the circularization reactions were template specific, and PAGE analysis showed the expected size distribution (Figure 5E). The specificity of the capturing reaction was estimated by ligating captured DNA fragments to a sequencing vector, cloning these into *Escherichia coli*, and sequencing 96 clones. Of 89 high-quality Sanger sequencing reads obtained, 80 were from the targeted regions, indicating a specificity of 90%. An Illumina Genome Analyzer was then used to sequence the ends of the captured fragments. Approximately 5.5 million reads were mapped to 10,364 of 10,582 targets, which translates to a sensitivity of 98%. These results indicate that padlock probes

can specifically extract a large set of genomic targets for single molecule bisulfite sequencing.

[00140] Although 98% of the targets were observed at least once in the end-sequencing analysis, the abundance of different captured fragments varied across a 10,000-fold range. Analysis of variance revealed that the bias resulted from a combination of factors, including GC content and length of the ligation arms, and the size of the targets to be captured (Figure 6). To normalize the relative abundance among different DNA fragments, a combination of two strategies was used: 'subsetting' and 'suppressor oligos' (Figure 7A-7D). All 30,000 padlock probes were ranked based on the capturing efficiency determined by end sequencing, and divided into four subsets, two containing 5,000, and two containing 10,000, oligos. The three less efficient subsets were resynthesized. For each DNA sample, four capturing reactions were performed separately using probes from the original set of 30,000 and the three resynthesized subsets. The PCR amplicons from the capturing reactions were pooled in equal molar ratios before constructing a shotgun sequencing library (Figure 7A). This subsetting strategy increased the relative abundance of less efficient targets by orders of magnitude. A very small number of probes were extremely efficient. For example, the top 48 (0.016%) most efficient probes account for 13.3% of mappable reads in the end-sequencing analysis.

[00141] Although the subsetting strategy allowed for adjusting the relative abundance among several relatively large subsets of probes, a method was also needed to specifically reduce the efficiency of a small number of probes in a library. For this, a set of 48 suppressor oligos was designed, which contained chimeric sequences: the 5' region was reverse-complementary to the extension arm H2, and the 3' region contained a short sequence unrelated to the ligation arm H1. When these suppressor oligos were mixed with padlock probes in a high molar ratio (100-fold molar excess of suppressor oligos), the 48 most efficient probes tended to anneal to the suppressor oligos, were extended from the 3' ends and yielded linear-extended sequences that were removed in the subsequent exonuclease digestion (Figure 7B). This normalization strategy was tested on the same bisulfite-converted Jurkat cell DNA, end sequencing on the captured DNA fragments was performed and 2.2 million mappable reads were obtained. The effect of normalization resulted in the fraction of probes with at least half of the average abundance increased from 31% to 49%; the average efficiency for the 48 most abundant probes was reduced by fivefold (Figure 7C-7D).

[00142] To validate the measurement accuracy of this method, advantage was taken of the built-in redundancy in our probe design. Each CpG island was covered by multiple probes targeting partially overlapping DNA fragments on alternating strands (Figure 5B). The CpG sites in the overlapping regions were captured independently from two DNA strands with different probes. Because the sequencing reads were mapped in a strand-specific manner and CpG methylation is symmetric on the two DNA strands, the accuracy of the assay can be determined by comparing the methylation level of these CpG sites on the two strands. For 2,697 such CpG sites that were covered by >50 sequencing reads, the Pearson correlation coefficient (R) was 0.987 (Figure 8A). To confirm the measurement accuracy with an independent method, the methylation levels of 182 randomly selected CpG sites were quantified with conventional bisulfite Sanger sequencing. Correlation between the two assays was high (R = 0.975; Figure 8B). Finally, the methylation measurements from two batches of IMR90 fibroblast cultures were compared. Correlation was observed between the biological replicates (R = 0.970; Figure 8C). Taken together, these three validation experiments indicate that this assay is highly robust.

[00143] To demonstrate the utility of targeted bisulfite sequencing, the changes of chromosome-wide methylation status during reprogramming of human fibroblasts to pluripotent cells was characterized. The methylation assay was performed on three sets of fibroblasts and iPS cells from three laboratories: IMR/IMR90-iPS17 reprogrammed with four factors (Oct4, Sox2, Nanog and Lin28); hFib2/hFib2-iPS18 reprogrammed with a different set of factors (Oct4, Sox2, Klf4 and Myc); BJ/BJ-iPS11/BJ-iPS12 (Maherali, N. et al. (2008) *Cell Stem Cell* 3: 340-345) reprogrammed with the five factors (Oct4, Sox2, Nanog, Klf4 and Myc) controlled by an inducible promoter. A line of hybrid stem cells (BJHues6-Hybrid1), which were reprogrammed by fusing the human fibroblasts (BJ) with hES cells (Hues6), as well as three hES cell lines (Hues12, Hues42, Hues63) were also characterized. Bisulfite conversion, padlock capture and construction of shotgun sequencing libraries were performed on each DNA sample. Each library in one lane was sequenced in the flow cell of an Illumina Genome Analyzer, and yielding 2–3 million reads that were mapped to the targeted regions. The bisulfite conversion rates were >98.5%. To avoid stochastic sampling drift, CpG sites that were covered by <10 reads were removed from the following analyses.

[00144] The global methylation patterns in all 12 samples (11 cell lines plus a biological replicate on IMR90 fibroblasts) were visualized using the UCSC Genome

Browser. The chromosome-wide patterns of CpG island methylation were highly similar among all the cell lines. Globally, the methylation level of CpG dinucleotides followed similar bimodal distribution: 67% were weakly methylated (<20% methylation), 22% were highly methylated (>80% methylation) and the remaining 11% had intermediate levels of methylation. To distinguish CpG islands with different methylation patterns, a histogram (bin size = 0.05) for the distribution of methylation on all CpG sites within a CpG island was generated. Treating such histograms as 20-component vectors, hierarchical clustering was performed to partition the CpG islands and divide all CpG islands into three clusters based on the similarity of distribution between pluripotent and fibroblast lines. In cluster 1, the CpG islands (1,451; 77.3%) have similar distributions in the two cell types ($R > 0.5$); in cluster 2, CpG islands (252; 13.4%) have less similar distributions ($0.5 \geq R > 0$); in cluster 3, CpG islands (173; 9.2%) are anti-correlated ($R \leq 0$). Therefore, only a small fraction of CpG islands show cell-type-specific methylation.

[00145] Because CpG islands are not defined in a functional manner, the CpG islands were divided into three categories. The first comprises CpG islands in the regions from 2 kb upstream to 500 bp downstream of TSS. These "upstream regions" often include promoter regions. The second class ("gene body CpG islands") comprises CpG islands in the regions from 500 bp downstream of TSS to the ends of the last exons. The final category comprises CpG islands outside of gene body and promoter regions. CpG islands in each category were further divided into three groups according to CpG density. Consistent with previous findings, most (91.8%) CpG islands in promoter regions were weakly methylated (<20% methylation), 3.4% were highly methylated (>80% methylation) and the remaining 4.8% showed an intermediate level of methylation (20– 80% methylation). The distributions were quite similar among the three groups with different CpG densities. In contrast, only 45.2% of CpG islands in the gene body were weakly methylated, whereas roughly one-third of them (37.7%) were highly methylated. Methylated CpGs tended to locate in islands with low CpG density. In regions outside of gene body and promoter regions, more weakly methylated CpG islands (58.9%) than highly methylated CpG islands (26.6%) were found. Similarly, CpG islands with low CpG density were more methylated. There were 80 genes in the data set that contained both promoter-region CpG islands and gene-body CpG islands. Sixty-two of these genes were weakly methylated in promoter regions. Among these, 48.4% were highly methylated in the gene body and 29.0% displayed weak gene-body methylation.

[00146] In summary, these experiments demonstrated that padlock probes can specifically extract a large number of genomic regions in single-tube reactions for bisulfite sequencing analysis. The degree of multiplexity is at least four orders of magnitude greater than that possible with conventional PCR-based bisulfite sequencing. The high capturing specificity is contributed by the cooperative annealing of the two capturing arms on the target molecules in proper orientation and distance, the selectivity of DNA polymerase and ligase, and the removal of linear DNA with exonuclease.

[00147] Although padlock probes have been successfully applied to exon capturing (Porreca, G.J. et al. (2007) *Nat. Methods* 4: 931-936) and SNP genotyping (Hardenbol, P. et al. (2003) *Nat. Biotechnol.* 21: 673-678), these experiments demonstrate their use with bisulfite-converted DNA with highly skewed nucleotide composition and low sequence complexity. Recent studies showed that most methylation changes are restricted to a very small fraction of the genome outside of CpG islands (Meissner, A. et al. (2008) *Nature* 454: 766-770; Ball, M.P. et al. (2009) *Nat. Biotechnol.* 27(4): 361-8; Irizarry, R.A. et al. (2009) *Nat. Genet.* 41: 178-186). Padlock capture is more efficient than full-genome bisulfite sequencing (Cokus, S.J. et al. (2008) *Nature* 452: 215-219; Lister, R. et al. (2008) *Cell* 133: 523-536) for quantifying DNA methylation, as it allows for focused sequencing on the most informative genomic regions. It also provides a much greater flexibility than reduced representation bisulfite sequencing (Meissner, A. et al. (2008) *Nature* 454: 766-770; Ball, M.P. et al. (2009) *Nat. Biotechnol.* 27(4): 361-8) in the selection of genomic targets, because the latter method is limited to genomic regions closely adjacent to the recognition sites of restriction enzymes.

Example 2: Library-free methylation sequencing with bisulfite padlock probes

[00148] The program ppDesigner was developed to aid in the design of efficient padlock probes for bisulfite analysis. It accepts as input the genome of any organism, a list of user-specified arbitrary targets and user-desired probe constraints matching requirements of the experimental protocol. It 'bisulfite-converts' the genome *in silico* (that is, it changes all cytosines to thymines) and outputs padlock probes to cover the chosen targets while avoiding CpGs on the capturing arms that could be methylated and not converted to be recognized as thymine. ppDesigner uses a back-propagation neural network to predict probe efficiency (Figure 9). This network was previously trained using data from probes for exomic targets

(Gore, A. et al. (2011) *Nature* 471: 63-67) based on seven properties. Using bisulfite capture data from the first BSPPs (Deng, J. et al. (2009) *Nat. Biotechnol.* 27: 353-360), the network was refined with two additional factors. ppDesigner can explain ~50% of the variance in capturing efficiency for genomic DNA and ~20% of the variance in capturing efficiency for bisulfite-converted DNA; additional variation could be due to factors such as variability in oligonucleotide synthesis and sample DNA quality. ppDesigner is extremely flexible and has been used to design a variety of genomic and bisulfite probes for Homo sapiens (Liu, G.H. et al. (2011) *Nature* 472: 221-225; Liu, G.H. et al. (2011) *Cell Stem Cell* 8: 688-694), *Mus musculus* (Xu, Y. et al. (2011) *Mol. Cell* 42: 451-464) and *Drosophila melanogaster* (Wang, H. et al. (2010) *Genome Res.* 29: 981-988).

[00149] Key requirements for methylation analysis of large sample sizes include low cost, simple workflow and automation compatibility. As the cost of DNA sequencing has rapidly decreased, sample processing has become a bottleneck in terms of cost and throughput. A complicated workflow increases variability between samples and reduces power in large-scale studies. To address these issues, we extended a "library-free" protocol (Turner, E.H. et al. (2009) *Nat. Methods* 6: 315-316) to multiplexed BSPP capture. This method eliminates five steps from Illumina's library-construction protocol such that multiplexed libraries can be generated from DNA in only four steps (Table 4). Table 4 herein shows the number of enzymatic reactions, number of purifications, cost per sample, and mapping rates for first-generation padlock probes, second-generation library-free padlock probes, reduced representation bisulfite sequencing (RRBS), and whole genome bisulfite sequencing (WGBS).

[00150] Table 9: Comparison of bisulfite sequencing methods

	Published BSPP	**Library-Free BSPP**	**RRBS**	**WGBS**
Enzymatic reactions	10	3	4	3
Purification	6	1	3	3
Size-selection	2	1[1]	1	1
Sample preparation cost per sample	$71.15[1]	$37.86[2]	$28.15	$31.10
Mapping rate	44%	87%	27%[3]	N.D.
Genome coverage obtained at 10x depth	<0.1%	0.6%-1%	~1%[3]	76-96%[4]
Sequencing (Gbps)	0.5	4.0	1.4	70.0
Sequencing cost per sample[5]	$24.38	$195.00	$68.25	$3412.50

[1]Unlike other methods, in the library-free BSPP protocol, size selection is typically performed on 48-96 pooled libraries.
[2]Includes the cost of ordering 400,000 synthesized probes from LC Sciences and reagents for preparing probes, bisulfite conversion, capture, and sequencing library preparation. Estimates assume that 10,000 samples will be processed.
[3]Estimated from: Gu et al., (2010) *Nat. Methods* 7(2):133-136.
[4]Adapted from: Beck et al., (2010) *Nat. Biotechnol.* 28:1026-1028.
[5]Assumes sequencing using an Illumina HiSeq to generate 300 Gbps of sequencing data, with cost of $4920 for a flowcell, $6815 for sequencing reagents, and $2890 for service fee. ($48.75 per Gbps)

[00151] Using multiplexed primers with 6–base pair (bp) barcodes, libraries for 96 samples in 96-well plates were routinely generated and sequenced all at once in a single Illumina HiSeq flowcell. Additionally, barcodes to process 384 samples per batch were designed. As sample-specific barcodes were added, barcoded libraries can be pooled for size selection, which is the most time consuming, contamination-prone and error-prone step if performed individually. The protocol is compatible with the use of multichannel pipettes or liquid-handling devices. It dramatically reduced experimental cost and time, and improved reproducibility and read mapping rates (Tables 4 and 5).

[00152] Table 5: Representative cost per sample for oligonucleotide synthesis, sequencing library construction, and Illumina sequencing

Expected number of samples to be processed	Probe Set Sizes		
	4,000	40,000	400,000
10	$134.57	$872.04	$9,298.78
100	$35.57	$129.54	$1,131.28
1000	$25.67	$55.29	$314.53
10,000	$24.68	$47.86	$232.86

[00153] For large sample sizes, the library preparation cost (including probes) with our protocol was comparable to that of the restricted-representation bisulfite sequencing and whole-genome bisulfite sequencing protocols, and the sequencing cost was much lower than that of whole-genome bisulfite sequencing owing to targeting of CpG sites of interest. Restricted-representation bisulfite sequencing is more cost-effective than BSPPs, but the former lacks BSPPs' flexibility in selecting specific sites or regions.

[00154] Another bottleneck in sequencing of bisulfite-converted DNA is a lack of computational tools to efficiently analyze sequencing data generated from hundreds of samples. To overcome this issue, an analysis pipeline for read mapping and methylation quantification was developed, called bisReadMapper. In previous padlock probe studies, reads had been mapped only against target regions owing to the computational requirements

of sequence alignment (Deng, J. et al. (2009) *Nat. Biotechnol.* 27: 353-360). In contrast, bisReadMapper was designed to map to the full genome sequence, allowing processing of data from both targeted and whole-genome sequencing of bisulfite-converted DNA. bisReadMapper also determines the origin strand of the read based on base composition and maps reads as if they were fully bisulfite-converted to a fully bisulfite-converted genome sequence, allowing mapping of both bi- and unidirectional bisulfite libraries in an unbiased manner. Another feature is the capability to call single-nucleotide polymorphisms (SNPs) from sequences of bisulfite-converted DNA; this feature not only allows for analysis of allele-specific methylation (Shoemaker, R. et al. (2010) *Genome Res.* 20: 883-889) but also allows accurate sample tracking in large-scale experiments. Finally, bisReadMapper can call methylation levels at both CpG and non-CpG sites.

[00155] To test this assay, a genome-scale probe set based on our previous results was generated, as well as new information about differential methylation (Deng, J. et al. (2009) *Nat. Biotechnol.* 27: 353-360). The new design was targeted for evaluation of methylation at genomic locations known to contain differentially methylated regions or differentially methylated sites (DMSs; Irizarry, R.A. et al. (2009) *Nat. Genet.* 41: 178-186; Doi, A. et al. (2009) *Nat. Genet.* 41: 1350-1353; Lister, R. et al. (2009) *Nature* 462: 315-322; Figueroa, M.E. et al. (2010) *Cancer Cell* 17: 13-27), transcriptional repressor CTCF binding sites and DNase I hypersensitive regions. All microRNA genes and all promoters for human U.S. National Center for Biotechnology Information reference sequence (RefSeq) genes were targeted. Using ppDesigner, ~330,000 padlock probes that covered 140,749 non-overlapping regions with a total size of 34 megabases were designed. Capturing experiments and end-sequencing were performed, and found that these probes were slightly more specific (~96% on-target) and uniform than previous probes (Deng, J. et al. (2009) *Nat. Biotechnol.* 27: 353-360) (Figure 10). To improve uniformity, the experimental capturing performance of these probes was normalized using subsetting and suppressor oligonucleotides as described previously (Deng, J. et al. (2009) *Nat. Biotechnol.* 27: 353-360). Roughly 500,000 CpG sites with ~4 gigabases of sequencing reads could be characterized, and additional sites became callable with deeper sequencing (Figures 11 and 12). A schematic for the padlock probes is illustrated in Figure 17.

Materials and Methods

[00156] Bisulfite padlock probe production (oligonucleotides from Agilent)

[00157] Libraries of oligonucleotides (~150 nt) were synthesized by ink-jet printing on programmable microarrays (Agilent Technologies) and released to form a combined library of 330,000 oligonucleotides. The oligonucleotides were amplified by PCR in 96 reactions (100 µl each) with 0.02 nM template oligonucleotide, 400 nM each of pAP1V61U primer and AP2V6 primer (Table 6), and 50 µl of KAPA SYBG fast Universal 2× qPCR Master Mix (Kapabiosystems) at 95°C for 30 seconds, 15–16 cycles of 95°C for 3 seconds; 55°C for 30 seconds; and 60°C for 20 seconds and 60°C for 2 minutes.

[00158] Table 6: Primer sequences used for padlock probe production, padlock capture, sequencing library construction, and Illumina sequencing

Primer Name	Primer Sequences
Primers used with Agilent Probes	
pAP1V61U	5'-G*G*G*TCATATCGGTCACTGTU-3' (SEQ ID NO: 8)
AP2V6	5'-/5Phos/CACGGGTAGTGTGTATCCTG-3' (SEQ ID NO: 9)
RE-DpnII-V6	5'-GTGTATCCTGATC-3' (SEQ ID NO: 10)
AmpF6.4Sol	5'-AATGATACGGCGACCACCGAGATCTACACCACTCTCAGATGTTA TCGAGGTCCGAC-3' (SEQ ID NO: 11)
AmpF6.3NH2	5'-/5AmMC6/CAGATGTTATCGAGGTCCGAC-3' (SEQ ID NO: 12)
AmpR6.3NH2	5'-/5AmMC6/GGAACGATGAGCCTCCAAC-3' (SEQ ID NO: 13)
PCR_F	5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTC-3' (SEQ ID NO: 14)
PE_t_N2	5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCTN*N-3' (SEQ ID NO: 15)
PE_b_A	5'-/5Phos/AGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG-3' (SEQ ID NO: 16)
SolSeq6.3.3 (Read1)	5'-TACACCACTCTCAGATGTTATCGAGGTCCGAC-3' (SEQ ID NO: 17)
SolSeqV6.3.2r(Read2)	5'-GCTAGGAACGATGAGCCTCCAAC-3' (SEQ ID NO: 18)
AmpR6.3IndSeq(IndexRead)	5'-GTTGGAGGCTCATCGTTCCTAGC-3' (SEQ ID NO: 19)
Primers used with LC Sciences Probes	
eMIP_CA1_F	5'- TGCCTAGGACCGGATCAACT-3' (SEQ ID NO: 20)
eMIP_CA1_R	5'- GAGCTTCGGTTCACGCAATG-3' (SEQ ID NO: 21)
CP-2-FA	5'-GCACGATCCGACGGTAGTGT-3' (SEQ ID NO: 22)
CP-2-RA	5'-CCGTAATCGGGAAGCTGAAG-3' (SEQ ID NO: 23)
CA-2-FA.Indx7Sol	5'-CAAGCAGAAGACGGCATACGAGATGATCTGCGGTCTGCCATCCG ACGGTAGTGT-3' (SEQ ID NO: 24)
CA-2-FA.Indx45Sol	5'-CAAGCAGAAGACGGCATACGAGATCGTAGTCGGTCTGCCATCCG ACGGTAGTGT-3' (SEQ ID NO: 25)
CA-2-FA.Indx76Sol	5'-CAAGCAGAAGACGGCATACGAGATAATAGGCGGTCTGCCATCCG ACGGTAGTGT-3' (SEQ ID NO: 26)
CA-2-RA.Sol	5'- AATGATACGGCGACCACCGAGATCTACACGCCTATCGGGAAGCT GAAG-3' (SEQ ID NO: 27)
Switch.CA-2-FA.Sol	5'- AATGATACGGCGACCACCGAGATCTACACGCCTATCCGACGGTA GTGT-3' (SEQ ID NO: 28)
Switch.CA-2-RA.Ind7Sol	5'-CAAGCAGAAGACGGCATACGAGATGATCTGCGGTCTGCCATCGG GAAGCTGAAG-3' (SEQ ID NO: 29)
Switch.CA-2-RA.Ind45Sol	5'-CAAGCAGAAGACGGCATACGAGATCGTAGTCGGTCTGCCATCGG GAAGCTGAAG-3' (SEQ ID NO: 30)

Switch.CA-2-RA.Ind76Sol	5'-CAAGCAGAAGACGGCATACGAGATAATAGGCGGTCTGCCATCG GGAAGCTGAAG-3' (SEQ ID NO: 31)
CP-2-SeqRead1.x (Read1)	5'-TACACGCCTATCGGGAAGCTGAAG-3' (SEQ ID NO: 32)
CP-2-IndSeq.x (IndexRead)	5'-ACACTACCGTCGGATGGCAGACCG-3' (SEQ ID NO: 33)
CP-2-SeqRead1.y (Read1)	5'-TACACGCCTATCCGACGGTAGTGT-3' (SEQ ID NO: 34)
CP-2-IndSeq.y (IndexRead)	5'-CTTCAGCTTCCCGATGGCAGACCG-3' (SEQ ID NO: 35)

* Indicates a phosphorothioate bond

[00159] The amplicons were purified by ethanol precipitation and repurified with Qiaquick PCR purification columns (Qiagen). Approximately 20 μg of the purified amplicons were digested with 50 units of lambda exonuclease (5 U/μl; New England Biolabs (NEB)) at 37°C for 1 hour in lambda exonuclease reaction buffer. The resulting single-strand amplicons were purified with Qiaquick PCR purification column. Approximately 5–8 μg of single strand amplicons were subsequently digested with 5 units USER (1 U μl−1, NEB) at 37°C for 1 hour. The digested DNA was annealed to 5.88 μM RE-*Dpn*II-V6 guide oligo (Table 6) and denatured at 94°C for 2 minutes decreased the temperature to 37°C and incubated at 37 °C for 3 minutes. The mixture was digested with 50 U of *Dpn*II (10 U μl−1, NEB) in NEBuffer *Dpn*II at 37°C for 2 hours. The mixture was further digested with 5 U of USER at 37°C for 2 hours followed by enzyme inactivation at 75°C for 20 minutes. The USER and *Dpn*II–digested DNA was purified with Qiaquick PCR purification column. The single-strand 102-nt probes were purified with 6% denaturing PAGE (6% TB-urea two dimensional (2D) gel; Invitrogen).

[00160] Bisulfite padlock probe production (oligonucleotides from LC Sciences)

[00161] The oligonucleotides (100 nt) were synthesized using a programmable microfluidic microarray platform (LC Sciences) and released to form a mix of 3,918 oligonucleotides. The oligonucleotides were amplified by two-step PCR in a 200 μl reaction with 1 nM template oligonucleotides, 400 nM each of eMIP_CA1_F primer and eMIP_CA1_R primer (Table 6), and 100 μl of KAPA SYBR fast Universal qPCR Master Mix at 95°C for 30 seconds, 5 cycles of 95°C for 5 seconds; 52°C for 1 minute; and 72°C for 30 seconds, 10–12 cycles of 95°C for 5 seconds; 60°C for 30 seconds; and 72°C for 30 seconds, and 72°C for 2 minutes. The resultant amplicons were purified with Qiaquick PCR purification columns and re-amplified in 32 PCRs (100 μl each) with 0.02 nM first round amplicons, 400 nM each of eMIP_CA1_F primer and eMIP_CA1_R primer and 50 μl of KAPA SYBR fast Universal qPCR master mix at 95°C for 30 seconds, 13–15 cycles of 95°C

for 5 seconds; 60°C for 30 seconds; and 72°C for 30 seconds and 72°C for 2 minutes. The resultant amplicons were purified by ethanol precipitation and repurified with Qiaquick PCR purification columns as described above. Approximately 4 μg of the purified amplicons were digested with 100 U of Nt.*Alw*I (100 U/μl, NEB) at 37°C for 1 hour in NEBuffer 2. The enzyme was heat-inactivated at 80°C for 20 minutes. The digested amplicons were then incubated with 100 U of Nb.*Brs*DI (10 U μl−1, NEB) at 65°C for 1 hour. The nicked DNA was purified by Qiaquick PCR purification column. The probe molecules (~70 bases) were purified by 6% denaturing PAGE (6% TB-urea 2D gel).

[00162] Sample preparation and capture

[00163] Genomic DNA was extracted using the AllPrep DNA/RNA Mini kit (Qiagen) and bisulfite-converted with the EZ-96 DNA methylation Gold kit (Zymoresearch) in a 96-well plate. Normalized amount of padlock probes, 200 ng of bisulfite converted gDNA and 4.2 nM oligo suppressor were mixed in 25 μl 1× Ampligase buffer (Epicentre) in 96-well plate, denatured at 95°C for 10 minutes, gradually lowered the temperature at 0.02°C/s to 55°C in a thermocycler and hybridized at 55°C for 20 hours. 2.5 μl of SLN (Stoffel fragment, ligase, nucleotides) mix (100 μM dNTP, 2 U μl−1AmpliTaq Stoffel Fragment (ABI) and 0.5 U/μl Ampligase (Epicentre) in 1× Ampligase buffer) was added to the reaction for gap-filling. For circularization, the reactions were incubated at 55°C for 20 hours, followed by enzyme inactivation at 94°C for 2 minutes. To digest linear DNA after circularization, 2 μl of exonuclease mix (10 U/μl exonuclease I and 100 U/μl exonuclease III, USB) was added to the reactions, and the reactions were incubated at 37°C for 2 hours and then inactivated at 94°C for 2 minutes.

[00164] Capture-circles amplification (library-free BSPP protocol, Agilent oligonucleotides)

[00165] Ten microliters of circularized DNA was amplified and barcoded in 100-μl reactions with 400 nM each of AmpF6.3Sol primer (Table 6) and AmpR6.3 indexing primer (Table 6), 0.4× SYBR Green I (Invitrogen) and 50 μl Phusion High-Fidelity 2× master mix (NEB) at 98°C for 30 seconds, 5 cycles of 98°C for 10 seconds; 58°C for 20 seconds; and 72°C for 20 seconds, 9-12 cycles of 98°C for 10 seconds; and 72°C for 20 seconds and 72°C for 3 minutes.

[00166] Capture-circles amplification (library-free BSPP protocol, LC Sciences oligonucleotides)

[00167] Ten microliters of circularized DNA was amplified in a 100-µl reaction with 200 nM each of CP-2-FA primer and CP-2-RA primer (Table 6) and 50 µl KAPA SYBR fast Universal qPCR Master Mix at 98°C for 30 seconds, 5 cycles of 98°C for 10 seconds; 52°C for 30 seconds; and 72°C for 30 seconds, 15 cycles of 98°C for 10 seconds; 60°C for 30 seconds; and 72°C for 30 seconds and 72°C for 3 minutes. The resultant amplicons with the corresponding expected size of ~260 bp were purified by 6% PAGE (6% 5-well gel, Invitrogen) and resuspended in 12 µl of TE buffer. Thirty percent of the gel-purified amplicons were reamplified and barcoded in a 100-µl reaction with 200 nM each of two different sets of primers to enable single-end sequencing of both ends of the amplicons (CP-2-FA.IndSol primer and CP-2-RA.Sol primer or Switch. CP-2-FA and Switch.CP-2-RA.IndSol) and 50 µl KAPA SYBR fast Universal qPCR Master Mix at 98°C for 30 seconds, 4 cycles of 98°C for 10 seconds; 54°C for 30 seconds; and 72°C for 30 seconds and 72°C for 3 minutes.

[00168] Primer barcode design for multiplexing

[00169] An in-house Perl script was written to randomly generate 6-nt-long sequences. A sequence was kept if it did not have more than two matching positions with another accepted barcode and if it had 2–4 guanines or cytosines. The script reiterated until the desired number of barcodes have been obtained. A total of 384 primers were designed (Table 7).

[00170] Table 7: Sequences of multiplexed barcoded primers used in the library-free protocol.

Barcode ID	Barcode	Primer	SEQ ID NO:
Ind1	CGTGAT	CAAGCAGAAGACGGCATACGAGATCGTGATGCTAGGAACGATGAGCCT CCAAC	36
Ind2	ACATCG	CAAGCAGAAGACGGCATACGAGATACATCGGCTAGGAACGATGAGCCT CCAAC	37
Ind3	GCCTAA	CAAGCAGAAGACGGCATACGAGATGCCTAAGCTAGGAACGATGAGCCT CCAAC	38
Ind4	TGGTCA	CAAGCAGAAGACGGCATACGAGATTGGTCAGCTAGGAACGATGAGCCT CCAAC	39

Barcode ID	Barcode	Primer	SEQ ID NO:
Ind5	CACTGT	CAAGCAGAAGACGGCATACGAGATCACTGTGCTAGGAACGATGAGCCT CCAAC	40
Ind6	ATTGGC	CAAGCAGAAGACGGCATACGAGATATTGGCGCTAGGAACGATGAGCCT CCAAC	41
Ind7	GATCTG	CAAGCAGAAGACGGCATACGAGATGATCTGGCTAGGAACGATGAGCCT CCAAC	42
Ind8	TCAAGT	CAAGCAGAAGACGGCATACGAGATTCAAGTGCTAGGAACGATGAGCCT CCAAC	43
Ind9	CTGATC	CAAGCAGAAGACGGCATACGAGATCTGATCGCTAGGAACGATGAGCCT CCAAC	44
Ind10	AAGCTA	CAAGCAGAAGACGGCATACGAGATAAGCTAGCTAGGAACGATGAGCCT CCAAC	45
Ind11	GTAGCC	CAAGCAGAAGACGGCATACGAGATGTAGCCGCTAGGAACGATGAGCCT CCAAC	46
Ind12	TACAAG	CAAGCAGAAGACGGCATACGAGATTACAAGGCTAGGAACGATGAGCCT CCAAC	47
Ind13	TCATGG	CAAGCAGAAGACGGCATACGAGATTCATGGGCTAGGAACGATGAGCCT CCAAC	48
Ind14	TGTCTT	CAAGCAGAAGACGGCATACGAGATTGTCTTGCTAGGAACGATGAGCCT CCAAC	49
Ind15	AGGAA G	CAAGCAGAAGACGGCATACGAGATAGGAAGGCTAGGAACGATGAGCC TCCAAC	50
Ind16	AACCCC	CAAGCAGAAGACGGCATACGAGATAACCCCGCTAGGAACGATGAGCCT CCAAC	51
Ind17	GATGAG	CAAGCAGAAGACGGCATACGAGATGATGAGGCTAGGAACGATGAGCCT CCAAC	52
Ind18	TGAACT	CAAGCAGAAGACGGCATACGAGATTGAACTGCTAGGAACGATGAGCCT CCAAC	53
Ind19	TGCGTC	CAAGCAGAAGACGGCATACGAGATTGCGTCGCTAGGAACGATGAGCCT CCAAC	54
Ind20	GACAGG	CAAGCAGAAGACGGCATACGAGATGACAGGGCTAGGAACGATGAGCCT CCAAC	55
Ind21	GGGTTG	CAAGCAGAAGACGGCATACGAGATGGGTTGGCTAGGAACGATGAGCCT CCAAC	56
Ind22	TCCGAG	CAAGCAGAAGACGGCATACGAGATTCCGAGGCTAGGAACGATGAGCCT CCAAC	57
Ind23	TTTCGA	CAAGCAGAAGACGGCATACGAGATTTTCGAGCTAGGAACGATGAGCCT CCAAC	58
Ind24	GCGAAT	CAAGCAGAAGACGGCATACGAGATGCGAATGCTAGGAACGATGAGCCT CCAAC	59
Ind25	GCAGTA	CAAGCAGAAGACGGCATACGAGATGCAGTAGCTAGGAACGATGAGCCT CCAAC	60
Ind26	TCACGA	CAAGCAGAAGACGGCATACGAGATTCACGAGCTAGGAACGATGAGCCT CCAAC	61
Ind27	CGCGTA	CAAGCAGAAGACGGCATACGAGATCGCGTAGCTAGGAACGATGAGCCT CCAAC	62
Ind28	GCACCT	CAAGCAGAAGACGGCATACGAGATGCACCTGCTAGGAACGATGAGCCT CCAAC	63
Ind29	GTTCGT	CAAGCAGAAGACGGCATACGAGATGTTCGTGCTAGGAACGATGAGCCT CCAAC	64
Ind30	CACTAA	CAAGCAGAAGACGGCATACGAGATCACTAAGCTAGGAACGATGAGCCT CCAAC	65
Ind31	GTGGTG	CAAGCAGAAGACGGCATACGAGATGTGGTGGCTAGGAACGATGAGCCT CCAAC	66

Barcode ID	Barcode	Primer	SEQ ID NO:
Ind32	CCTTGC	CAAGCAGAAGACGGCATACGAGATCCTTGCGCTAGGAACGATGAGCCT CCAAC	67
Ind33	GTTGCG	CAAGCAGAAGACGGCATACGAGATGTTGCGGCTAGGAACGATGAGCCT CCAAC	68
Ind34	TCAGTT	CAAGCAGAAGACGGCATACGAGATTCAGTTGCTAGGAACGATGAGCCT CCAAC	69
Ind35	CCCGAT	CAAGCAGAAGACGGCATACGAGATCCCGATGCTAGGAACGATGAGCCT CCAAC	70
Ind36	TGCTTG	CAAGCAGAAGACGGCATACGAGATTGCTTGGCTAGGAACGATGAGCCT CCAAC	71
Ind37	TGTAGC	CAAGCAGAAGACGGCATACGAGATTGTAGCGCTAGGAACGATGAGCCT CCAAC	72
Ind38	GCGTGA	CAAGCAGAAGACGGCATACGAGATGCGTGAGCTAGGAACGATGAGCCT CCAAC	73
Ind39	CTCTGG	CAAGCAGAAGACGGCATACGAGATCTCTGGGCTAGGAACGATGAGCCT CCAAC	74
Ind40	CCGTCA	CAAGCAGAAGACGGCATACGAGATCCGTCAGCTAGGAACGATGAGCCT CCAAC	75
Ind41	GTTCCC	CAAGCAGAAGACGGCATACGAGATGTTCCCGCTAGGAACGATGAGCCT CCAAC	76
Ind42	CTTTTC	CAAGCAGAAGACGGCATACGAGATCTTTTCGCTAGGAACGATGAGCCT CCAAC	77
Ind43	GGCACT	CAAGCAGAAGACGGCATACGAGATGGCACTGCTAGGAACGATGAGCCT CCAAC	78
Ind44	GGATGC	CAAGCAGAAGACGGCATACGAGATGGATGCGCTAGGAACGATGAGCCT CCAAC	79
Ind45	CGTAGT	CAAGCAGAAGACGGCATACGAGATCGTAGTGCTAGGAACGATGAGCCT CCAAC	80
Ind46	GAAATG	CAAGCAGAAGACGGCATACGAGATGAAATGGCTAGGAACGATGAGCCT CCAAC	81
Ind47	GGAGA G	CAAGCAGAAGACGGCATACGAGATGGAGAGGCTAGGAACGATGAGCC TCCAAC	82
Ind48	TAACGT	CAAGCAGAAGACGGCATACGAGATTAACGTGCTAGGAACGATGAGCCT CCAAC	83
Ind49	ACACAG	CAAGCAGAAGACGGCATACGAGATACACAGGCTAGGAACGATGAGCCT CCAAC	84
Ind50	AAAGGT	CAAGCAGAAGACGGCATACGAGATAAAGGTGCTAGGAACGATGAGCCT CCAAC	85
Ind51	GCGATA	CAAGCAGAAGACGGCATACGAGATGCGATAGCTAGGAACGATGAGCCT CCAAC	86
Ind52	CGTGTC	CAAGCAGAAGACGGCATACGAGATCGTGTCGCTAGGAACGATGAGCCT CCAAC	87
Ind53	GTAGAA	CAAGCAGAAGACGGCATACGAGATGTAGAAGCTAGGAACGATGAGCCT CCAAC	88
Ind54	GGACGT	CAAGCAGAAGACGGCATACGAGATGGACGTGCTAGGAACGATGAGCCT CCAAC	89
Ind55	AGTCGA	CAAGCAGAAGACGGCATACGAGATAGTCGAGCTAGGAACGATGAGCCT CCAAC	90
Ind56	GTCTGA	CAAGCAGAAGACGGCATACGAGATGTCTGAGCTAGGAACGATGAGCCT CCAAC	91
Ind57	GAAGG A	CAAGCAGAAGACGGCATACGAGATGAAGGAGCTAGGAACGATGAGCC TCCAAC	92
Ind58	ATGCTG	CAAGCAGAAGACGGCATACGAGATATGCTGGCTAGGAACGATGAGCCT CCAAC	93

Barcode ID	Barcode	Primer	SEQ ID NO:
Ind59	TCTATC	CAAGCAGAAGACGGCATACGAGATTCTATCGCTAGGAACGATGAGCCT CCAAC	94
Ind60	ATCTGT	CAAGCAGAAGACGGCATACGAGATATCTGTGCTAGGAACGATGAGCCT CCAAC	95
Ind61	ATAGAG	CAAGCAGAAGACGGCATACGAGATATAGAGGCTAGGAACGATGAGCCT CCAAC	96
Ind62	GCTAAA	CAAGCAGAAGACGGCATACGAGATGCTAAAGCTAGGAACGATGAGCCT CCAAC	97
Ind63	ACCAGG	CAAGCAGAAGACGGCATACGAGATACCAGGGCTAGGAACGATGAGCCT CCAAC	98
Ind64	CCAACT	CAAGCAGAAGACGGCATACGAGATCCAACTGCTAGGAACGATGAGCCT CCAAC	99
Ind65	AAGGA A	CAAGCAGAAGACGGCATACGAGATAAGGAAGCTAGGAACGATGAGCC TCCAAC	100
Ind66	CCTCCA	CAAGCAGAAGACGGCATACGAGATCCTCCAGCTAGGAACGATGAGCCT CCAAC	101
Ind67	CACGTC	CAAGCAGAAGACGGCATACGAGATCACGTCGCTAGGAACGATGAGCCT CCAAC	102
Ind68	CATAAC	CAAGCAGAAGACGGCATACGAGATCATAACGCTAGGAACGATGAGCCT CCAAC	103
Ind69	CCATAT	CAAGCAGAAGACGGCATACGAGATCCATATGCTAGGAACGATGAGCCT CCAAC	104
Ind70	GAAGTC	CAAGCAGAAGACGGCATACGAGATGAAGTCGCTAGGAACGATGAGCCT CCAAC	105
Ind71	CAAAGA	CAAGCAGAAGACGGCATACGAGATCAAAGAGCTAGGAACGATGAGCCT CCAAC	106
Ind72	TGGCAG	CAAGCAGAAGACGGCATACGAGATTGGCAGGCTAGGAACGATGAGCCT CCAAC	107
Ind73	GAGTCC	CAAGCAGAAGACGGCATACGAGATGAGTCCGCTAGGAACGATGAGCCT CCAAC	108
Ind74	TCGCCA	CAAGCAGAAGACGGCATACGAGATTCGCCAGCTAGGAACGATGAGCCT CCAAC	109
Ind75	AAGTCG	CAAGCAGAAGACGGCATACGAGATAAGTCGGCTAGGAACGATGAGCCT CCAAC	110
Ind76	AATAGG	CAAGCAGAAGACGGCATACGAGATAATAGGGCTAGGAACGATGAGCCT CCAAC	111
Ind77	ACCCGT	CAAGCAGAAGACGGCATACGAGATACCCGTGCTAGGAACGATGAGCCT CCAAC	112
Ind78	AACACG	CAAGCAGAAGACGGCATACGAGATAACACGGCTAGGAACGATGAGCCT CCAAC	113
Ind79	GCTTGG	CAAGCAGAAGACGGCATACGAGATGCTTGGGCTAGGAACGATGAGCCT CCAAC	114
Ind80	TTACCA	CAAGCAGAAGACGGCATACGAGATTTACCAGCTAGGAACGATGAGCCT CCAAC	115
Ind81	CCAGGT	CAAGCAGAAGACGGCATACGAGATCCAGGTGCTAGGAACGATGAGCCT CCAAC	116
Ind82	CGTTTG	CAAGCAGAAGACGGCATACGAGATCGTTTGGCTAGGAACGATGAGCCT CCAAC	117
Ind83	GACCAC	CAAGCAGAAGACGGCATACGAGATGACCACGCTAGGAACGATGAGCCT CCAAC	118
Ind84	ACAAGA	CAAGCAGAAGACGGCATACGAGATACAAGAGCTAGGAACGATGAGCCT CCAAC	119
Ind85	ACCGCA	CAAGCAGAAGACGGCATACGAGATACCGCAGCTAGGAACGATGAGCCT CCAAC	120

Barcode ID	Barcode	Primer	SEQ ID NO:
Ind86	TGGGTA	CAAGCAGAAGACGGCATACGAGATTGGGTAGCTAGGAACGATGAGCCT CCAAC	121
Ind87	ATTCCG	CAAGCAGAAGACGGCATACGAGATATTCCGGCTAGGAACGATGAGCCT CCAAC	122
Ind88	GAATGT	CAAGCAGAAGACGGCATACGAGATGAATGTGCTAGGAACGATGAGCCT CCAAC	123
Ind89	GCTGAT	CAAGCAGAAGACGGCATACGAGATGCTGATGCTAGGAACGATGAGCCT CCAAC	124
Ind90	AGTGCT	CAAGCAGAAGACGGCATACGAGATAGTGCTGCTAGGAACGATGAGCCT CCAAC	125
Ind91	CAGGGA	CAAGCAGAAGACGGCATACGAGATCAGGGAGCTAGGAACGATGAGCCT CCAAC	126
Ind92	CATGCG	CAAGCAGAAGACGGCATACGAGATCATGCGGCTAGGAACGATGAGCCT CCAAC	127
Ind93	TGCCTA	CAAGCAGAAGACGGCATACGAGATTGCCTAGCTAGGAACGATGAGCCT CCAAC	128
Ind94	CTATAC	CAAGCAGAAGACGGCATACGAGATCTATACGCTAGGAACGATGAGCCT CCAAC	129
Ind95	CCGAGT	CAAGCAGAAGACGGCATACGAGATCCGAGTGCTAGGAACGATGAGCCT CCAAC	130
Ind96	ACCTGC	CAAGCAGAAGACGGCATACGAGATACCTGCGCTAGGAACGATGAGCCT CCAAC	131
Ind97	CAGGAC	CAAGCAGAAGACGGCATACGAGATCAGGACGCTAGGAACGATGAGCCT CCAAC	132
Ind98	AAGATG	CAAGCAGAAGACGGCATACGAGATAAGATGGCTAGGAACGATGAGCCT CCAAC	133
Ind99	GGCTTC	CAAGCAGAAGACGGCATACGAGATGGCTTCGCTAGGAACGATGAGCCT CCAAC	134
Ind100	GTGCTT	CAAGCAGAAGACGGCATACGAGATGTGCTTGCTAGGAACGATGAGCCT CCAAC	135
Ind101	TGCGCA	CAAGCAGAAGACGGCATACGAGATTGCGCAGCTAGGAACGATGAGCCT CCAAC	136
Ind102	ACTAGC	CAAGCAGAAGACGGCATACGAGATACTAGCGCTAGGAACGATGAGCCT CCAAC	137
Ind103	TCGAAG	CAAGCAGAAGACGGCATACGAGATTCGAAGGCTAGGAACGATGAGCCT CCAAC	138
Ind104	AGACTA	CAAGCAGAAGACGGCATACGAGATAGACTAGCTAGGAACGATGAGCCT CCAAC	139
Ind105	CGGGTT	CAAGCAGAAGACGGCATACGAGATCGGGTTGCTAGGAACGATGAGCCT CCAAC	140
Ind106	TGACTG	CAAGCAGAAGACGGCATACGAGATTGACTGGCTAGGAACGATGAGCCT CCAAC	141
Ind107	TTGTGT	CAAGCAGAAGACGGCATACGAGATTTGTGTGCTAGGAACGATGAGCCT CCAAC	142
Ind108	TCGCTG	CAAGCAGAAGACGGCATACGAGATTCGCTGGCTAGGAACGATGAGCCT CCAAC	143
Ind109	GATACA	CAAGCAGAAGACGGCATACGAGATGATACAGCTAGGAACGATGAGCCT CCAAC	144
Ind110	TCCTTA	CAAGCAGAAGACGGCATACGAGATTCCTTAGCTAGGAACGATGAGCCT CCAAC	145
Ind111	CGATTT	CAAGCAGAAGACGGCATACGAGATCGATTTGCTAGGAACGATGAGCCT CCAAC	146
Ind112	TTACGG	CAAGCAGAAGACGGCATACGAGATTTACGGGCTAGGAACGATGAGCCT CCAAC	147

Barcode ID	Barcode	Primer	SEQ ID NO:
Ind113	TGTGAC	CAAGCAGAAGACGGCATACGAGATTGTGACGCTAGGAACGATGAGCCT CCAAC	148
Ind114	TTGGAA	CAAGCAGAAGACGGCATACGAGATTTGGAAGCTAGGAACGATGAGCCT CCAAC	149
Ind115	ACCATA	CAAGCAGAAGACGGCATACGAGATACCATAGCTAGGAACGATGAGCCT CCAAC	150
Ind116	GTCGAG	CAAGCAGAAGACGGCATACGAGATGTCGAGGCTAGGAACGATGAGCCT CCAAC	151
Ind117	ACTGCC	CAAGCAGAAGACGGCATACGAGATACTGCCGCTAGGAACGATGAGCCT CCAAC	152
Ind118	TCGGGT	CAAGCAGAAGACGGCATACGAGATTCGGGTGCTAGGAACGATGAGCCT CCAAC	153
Ind119	GGCATG	CAAGCAGAAGACGGCATACGAGATGGCATGGCTAGGAACGATGAGCCT CCAAC	154
Ind120	GTTTCT	CAAGCAGAAGACGGCATACGAGATGTTTCTGCTAGGAACGATGAGCCT CCAAC	155
Ind121	ACCAAT	CAAGCAGAAGACGGCATACGAGATACCAATGCTAGGAACGATGAGCCT CCAAC	156
Ind122	ATGCAT	CAAGCAGAAGACGGCATACGAGATATGCATGCTAGGAACGATGAGCCT CCAAC	157
Ind123	TACGGC	CAAGCAGAAGACGGCATACGAGATTACGGCGCTAGGAACGATGAGCCT CCAAC	158
Ind124	AGTCCC	CAAGCAGAAGACGGCATACGAGATAGTCCCGCTAGGAACGATGAGCCT CCAAC	159
Ind125	CTGCAG	CAAGCAGAAGACGGCATACGAGATCTGCAGGCTAGGAACGATGAGCCT CCAAC	160
Ind126	CTGTTG	CAAGCAGAAGACGGCATACGAGATCTGTTGGCTAGGAACGATGAGCCT CCAAC	161
Ind127	CGGACA	CAAGCAGAAGACGGCATACGAGATCGGACAGCTAGGAACGATGAGCCT CCAAC	162
Ind128	TAAGCG	CAAGCAGAAGACGGCATACGAGATTAAGCGGCTAGGAACGATGAGCCT CCAAC	163
Ind129	GAGAGT	CAAGCAGAAGACGGCATACGAGATGAGAGTGCTAGGAACGATGAGCCT CCAAC	164
Ind130	TACCCG	CAAGCAGAAGACGGCATACGAGATTACCCGGCTAGGAACGATGAGCCT CCAAC	165
Ind131	TTCGTA	CAAGCAGAAGACGGCATACGAGATTTCGTAGCTAGGAACGATGAGCCT CCAAC	166
Ind132	AAAGTG	CAAGCAGAAGACGGCATACGAGATAAAGTGGCTAGGAACGATGAGCCT CCAAC	167
Ind133	TTTGGT	CAAGCAGAAGACGGCATACGAGATTTTGGTGCTAGGAACGATGAGCCT CCAAC	168
Ind134	GTCCCT	CAAGCAGAAGACGGCATACGAGATGTCCCTGCTAGGAACGATGAGCCT CCAAC	169
Ind135	TAGCTT	CAAGCAGAAGACGGCATACGAGATTAGCTTGCTAGGAACGATGAGCCT CCAAC	170
Ind136	GCACTG	CAAGCAGAAGACGGCATACGAGATGCACTGGCTAGGAACGATGAGCCT CCAAC	171
Ind137	ACTATG	CAAGCAGAAGACGGCATACGAGATACTATGGCTAGGAACGATGAGCCT CCAAC	172
Ind138	GAACTT	CAAGCAGAAGACGGCATACGAGATGAACTTGCTAGGAACGATGAGCCT CCAAC	173
Ind139	TTTGAG	CAAGCAGAAGACGGCATACGAGATTTTGAGGCTAGGAACGATGAGCCT CCAAC	174

Barcode ID	Barcode	Primer	SEQ ID NO:
Ind140	AGGCCT	CAAGCAGAAGACGGCATACGAGATAGGCCTGCTAGGAACGATGAGCCT CCAAC	175
Ind141	ACACGC	CAAGCAGAAGACGGCATACGAGATACACGCGCTAGGAACGATGAGCCT CCAAC	176
Ind142	TTGTCG	CAAGCAGAAGACGGCATACGAGATTTGTCGGCTAGGAACGATGAGCCT CCAAC	177
Ind143	TCTCAC	CAAGCAGAAGACGGCATACGAGATTCTCACGCTAGGAACGATGAGCCT CCAAC	178
Ind144	TAGACC	CAAGCAGAAGACGGCATACGAGATTAGACCGCTAGGAACGATGAGCCT CCAAC	179
Ind145	CCTAAG	CAAGCAGAAGACGGCATACGAGATCCTAAGGCTAGGAACGATGAGCCT CCAAC	180
Ind146	GTATCG	CAAGCAGAAGACGGCATACGAGATGTATCGGCTAGGAACGATGAGCCT CCAAC	181
Ind147	TCCAGA	CAAGCAGAAGACGGCATACGAGATTCCAGAGCTAGGAACGATGAGCCT CCAAC	182
Ind148	AGGTGA	CAAGCAGAAGACGGCATACGAGATAGGTGAGCTAGGAACGATGAGCCT CCAAC	183
Ind149	CCCATC	CAAGCAGAAGACGGCATACGAGATCCCATCGCTAGGAACGATGAGCCT CCAAC	184
Ind150	TTGCAC	CAAGCAGAAGACGGCATACGAGATTTGCACGCTAGGAACGATGAGCCT CCAAC	185
Ind151	AACTCA	CAAGCAGAAGACGGCATACGAGATAACTCAGCTAGGAACGATGAGCCT CCAAC	186
Ind152	CGTATA	CAAGCAGAAGACGGCATACGAGATCGTATAGCTAGGAACGATGAGCCT CCAAC	187
Ind153	AGCGAA	CAAGCAGAAGACGGCATACGAGATAGCGAAGCTAGGAACGATGAGCCT CCAAC	188
Ind154	ACGGCT	CAAGCAGAAGACGGCATACGAGATACGGCTGCTAGGAACGATGAGCCT CCAAC	189
Ind155	AGTGAG	CAAGCAGAAGACGGCATACGAGATAGTGAGGCTAGGAACGATGAGCCT CCAAC	190
Ind156	TTTCTC	CAAGCAGAAGACGGCATACGAGATTTTCTCGCTAGGAACGATGAGCCT CCAAC	191
Ind157	GCTCTA	CAAGCAGAAGACGGCATACGAGATGCTCTAGCTAGGAACGATGAGCCT CCAAC	192
Ind158	ACTTGA	CAAGCAGAAGACGGCATACGAGATACTTGAGCTAGGAACGATGAGCCT CCAAC	193
Ind159	CGGTTC	CAAGCAGAAGACGGCATACGAGATCGGTTCGCTAGGAACGATGAGCCT CCAAC	194
Ind160	CATCAT	CAAGCAGAAGACGGCATACGAGATCATCATGCTAGGAACGATGAGCCT CCAAC	195
Ind161	CAAACG	CAAGCAGAAGACGGCATACGAGATCAAACGGCTAGGAACGATGAGCCT CCAAC	196
Ind162	CTATGT	CAAGCAGAAGACGGCATACGAGATCTATGTGCTAGGAACGATGAGCCT CCAAC	197
Ind163	AGCGTT	CAAGCAGAAGACGGCATACGAGATAGCGTTGCTAGGAACGATGAGCCT CCAAC	198
Ind164	AAGACT	CAAGCAGAAGACGGCATACGAGATAAGACTGCTAGGAACGATGAGCCT CCAAC	199
Ind165	CGATAA	CAAGCAGAAGACGGCATACGAGATCGATAAGCTAGGAACGATGAGCCT CCAAC	200
Ind166	CGGCTA	CAAGCAGAAGACGGCATACGAGATCGGCTAGCTAGGAACGATGAGCCT CCAAC	201

Barcode ID	Barcode	Primer	SEQ ID NO:
Ind167	TATACG	CAAGCAGAAGACGGCATACGAGATTATACGGCTAGGAACGATGAGCCT CCAAC	202
Ind168	GAAACC	CAAGCAGAAGACGGCATACGAGATGAAACCGCTAGGAACGATGAGCCT CCAAC	203
Ind169	GAACCG	CAAGCAGAAGACGGCATACGAGATGAACCGGCTAGGAACGATGAGCCT CCAAC	204
Ind170	TTAGGC	CAAGCAGAAGACGGCATACGAGATTTAGGCGCTAGGAACGATGAGCCT CCAAC	205
Ind171	GCCTTT	CAAGCAGAAGACGGCATACGAGATGCCTTTGCTAGGAACGATGAGCCT CCAAC	206
Ind172	GTTGGA	CAAGCAGAAGACGGCATACGAGATGTTGGAGCTAGGAACGATGAGCCT CCAAC	207
Ind173	GTACGC	CAAGCAGAAGACGGCATACGAGATGTACGCGCTAGGAACGATGAGCCT CCAAC	208
Ind174	TGGAAC	CAAGCAGAAGACGGCATACGAGATTGGAACGCTAGGAACGATGAGCCT CCAAC	209
Ind175	AACAGA	CAAGCAGAAGACGGCATACGAGATAACAGAGCTAGGAACGATGAGCCT CCAAC	210
Ind176	AAGCAC	CAAGCAGAAGACGGCATACGAGATAAGCACGCTAGGAACGATGAGCCT CCAAC	211
Ind177	ATGGTA	CAAGCAGAAGACGGCATACGAGATATGGTAGCTAGGAACGATGAGCCT CCAAC	212
Ind178	TCGTTC	CAAGCAGAAGACGGCATACGAGATTCGTTCGCTAGGAACGATGAGCCT CCAAC	213
Ind179	CTTCTA	CAAGCAGAAGACGGCATACGAGATCTTCTAGCTAGGAACGATGAGCCT CCAAC	214
Ind180	TGGGAT	CAAGCAGAAGACGGCATACGAGATTGGGATGCTAGGAACGATGAGCCT CCAAC	215
Ind181	ATCGCC	CAAGCAGAAGACGGCATACGAGATATCGCCGCTAGGAACGATGAGCCT CCAAC	216
Ind182	AGTTGG	CAAGCAGAAGACGGCATACGAGATAGTTGGGCTAGGAACGATGAGCCT CCAAC	217
Ind183	AGCTCT	CAAGCAGAAGACGGCATACGAGATAGCTCTGCTAGGAACGATGAGCCT CCAAC	218
Ind184	GACGGT	CAAGCAGAAGACGGCATACGAGATGACGGTGCTAGGAACGATGAGCCT CCAAC	219
Ind185	CTCAGC	CAAGCAGAAGACGGCATACGAGATCTCAGCGCTAGGAACGATGAGCCT CCAAC	220
Ind186	CTTAGG	CAAGCAGAAGACGGCATACGAGATCTTAGGGCTAGGAACGATGAGCCT CCAAC	221
Ind187	CGACAG	CAAGCAGAAGACGGCATACGAGATCGACAGGCTAGGAACGATGAGCCT CCAAC	222
Ind188	ACATGT	CAAGCAGAAGACGGCATACGAGATACATGTGCTAGGAACGATGAGCCT CCAAC	223
Ind189	ATACGA	CAAGCAGAAGACGGCATACGAGATATACGAGCTAGGAACGATGAGCCT CCAAC	224
Ind190	GAGCAT	CAAGCAGAAGACGGCATACGAGATGAGCATGCTAGGAACGATGAGCCT CCAAC	225
Ind191	ATCCTA	CAAGCAGAAGACGGCATACGAGATATCCTAGCTAGGAACGATGAGCCT CCAAC	226
Ind192	ACGTAA	CAAGCAGAAGACGGCATACGAGATACGTAAGCTAGGAACGATGAGCCT CCAAC	227
Ind193	GGAAAC	CAAGCAGAAGACGGCATACGAGATGGAAACGCTAGGAACGATGAGCCT CCAAC	228

Barcode ID	Barcode	Primer	SEQ ID NO:
Ind194	AGCAAC	CAAGCAGAAGACGGCATACGAGATAGCAACGCTAGGAACGATGAGCCT CCAAC	229
Ind195	CTAGTG	CAAGCAGAAGACGGCATACGAGATCTAGTGGCTAGGAACGATGAGCCT CCAAC	230
Ind196	CCGCTT	CAAGCAGAAGACGGCATACGAGATCCGCTTGCTAGGAACGATGAGCCT CCAAC	231
Ind197	CCAGCA	CAAGCAGAAGACGGCATACGAGATCCAGCAGCTAGGAACGATGAGCCT CCAAC	232
Ind198	ATACTC	CAAGCAGAAGACGGCATACGAGATATACTCGCTAGGAACGATGAGCCT CCAAC	233
Ind199	GGTGAA	CAAGCAGAAGACGGCATACGAGATGGTGAAGCTAGGAACGATGAGCCT CCAAC	234
Ind200	CTCTAT	CAAGCAGAAGACGGCATACGAGATCTCTATGCTAGGAACGATGAGCCT CCAAC	235
Ind201	GACATA	CAAGCAGAAGACGGCATACGAGATGACATAGCTAGGAACGATGAGCCT CCAAC	236
Ind202	GGATCT	CAAGCAGAAGACGGCATACGAGATGGATCTGCTAGGAACGATGAGCCT CCAAC	237
Ind203	AACGAG	CAAGCAGAAGACGGCATACGAGATAACGAGGCTAGGAACGATGAGCCT CCAAC	238
Ind204	CACCTG	CAAGCAGAAGACGGCATACGAGATCACCTGGCTAGGAACGATGAGCCT CCAAC	239
Ind205	CATGAA	CAAGCAGAAGACGGCATACGAGATCATGAAGCTAGGAACGATGAGCCT CCAAC	240
Ind206	CGTGGA	CAAGCAGAAGACGGCATACGAGATCGTGGAGCTAGGAACGATGAGCCT CCAAC	241
Ind207	AGAAG G	CAAGCAGAAGACGGCATACGAGATAGAAGGGCTAGGAACGATGAGCC TCCAAC	242
Ind208	ATCCAG	CAAGCAGAAGACGGCATACGAGATATCCAGGCTAGGAACGATGAGCCT CCAAC	243
Ind209	TTCCTG	CAAGCAGAAGACGGCATACGAGATTTCCTGGCTAGGAACGATGAGCCT CCAAC	244
Ind210	CAACAA	CAAGCAGAAGACGGCATACGAGATCAACAAGCTAGGAACGATGAGCCT CCAAC	245
Ind211	CCTGTT	CAAGCAGAAGACGGCATACGAGATCCTGTTGCTAGGAACGATGAGCCT CCAAC	246
Ind212	CTCGTT	CAAGCAGAAGACGGCATACGAGATCTCGTTGCTAGGAACGATGAGCCT CCAAC	247
Ind213	CTGAGA	CAAGCAGAAGACGGCATACGAGATCTGAGAGCTAGGAACGATGAGCCT CCAAC	248
Ind214	CGCTCA	CAAGCAGAAGACGGCATACGAGATCGCTCAGCTAGGAACGATGAGCCT CCAAC	249
Ind215	TACCAA	CAAGCAGAAGACGGCATACGAGATTACCAAGCTAGGAACGATGAGCCT CCAAC	250
Ind216	CGCAAG	CAAGCAGAAGACGGCATACGAGATCGCAAGGCTAGGAACGATGAGCCT CCAAC	251
Ind217	TACTGA	CAAGCAGAAGACGGCATACGAGATTACTGAGCTAGGAACGATGAGCCT CCAAC	252
Ind218	TCCACT	CAAGCAGAAGACGGCATACGAGATTCCACTGCTAGGAACGATGAGCCT CCAAC	253
Ind219	ATCGTG	CAAGCAGAAGACGGCATACGAGATATCGTGGCTAGGAACGATGAGCCT CCAAC	254
Ind220	TAGGCA	CAAGCAGAAGACGGCATACGAGATTAGGCAGCTAGGAACGATGAGCCT CCAAC	255

Barcode ID	Barcode	Primer	SEQ ID NO:
Ind221	CAAGAG	CAAGCAGAAGACGGCATACGAGATCAAGAGGCTAGGAACGATGAGCCT CCAAC	256
Ind222	ACGACA	CAAGCAGAAGACGGCATACGAGATACGACAGCTAGGAACGATGAGCCT CCAAC	257
Ind223	GGTTCC	CAAGCAGAAGACGGCATACGAGATGGTTCCGCTAGGAACGATGAGCCT CCAAC	258
Ind224	TCTTAG	CAAGCAGAAGACGGCATACGAGATTCTTAGGCTAGGAACGATGAGCCT CCAAC	259
Ind225	AGAGG A	CAAGCAGAAGACGGCATACGAGATAGAGGAGCTAGGAACGATGAGCC TCCAAC	260
Ind226	AAAGCA	CAAGCAGAAGACGGCATACGAGATAAAGCAGCTAGGAACGATGAGCCT CCAAC	261
Ind227	AGTCAT	CAAGCAGAAGACGGCATACGAGATAGTCATGCTAGGAACGATGAGCCT CCAAC	262
Ind228	TGCAGT	CAAGCAGAAGACGGCATACGAGATTGCAGTGCTAGGAACGATGAGCCT CCAAC	263
Ind229	TGTTCT	CAAGCAGAAGACGGCATACGAGATTGTTCTGCTAGGAACGATGAGCCT CCAAC	264
Ind230	TATCGC	CAAGCAGAAGACGGCATACGAGATTATCGCGCTAGGAACGATGAGCCT CCAAC	265
Ind231	GCATCA	CAAGCAGAAGACGGCATACGAGATGCATCAGCTAGGAACGATGAGCCT CCAAC	266
Ind232	CTCCGT	CAAGCAGAAGACGGCATACGAGATCTCCGTGCTAGGAACGATGAGCCT CCAAC	267
Ind233	TAAGAC	CAAGCAGAAGACGGCATACGAGATTAAGACGCTAGGAACGATGAGCCT CCAAC	268
Ind234	GAGGCT	CAAGCAGAAGACGGCATACGAGATGAGGCTGCTAGGAACGATGAGCCT CCAAC	269
Ind235	TGATTC	CAAGCAGAAGACGGCATACGAGATTGATTCGCTAGGAACGATGAGCCT CCAAC	270
Ind236	GTCCAA	CAAGCAGAAGACGGCATACGAGATGTCCAAGCTAGGAACGATGAGCCT CCAAC	271
Ind237	ACTCTC	CAAGCAGAAGACGGCATACGAGATACTCTCGCTAGGAACGATGAGCCT CCAAC	272
Ind238	TCAACG	CAAGCAGAAGACGGCATACGAGATTCAACGGCTAGGAACGATGAGCCT CCAAC	273
Ind239	GGGTAC	CAAGCAGAAGACGGCATACGAGATGGGTACGCTAGGAACGATGAGCCT CCAAC	274
Ind240	ACGATT	CAAGCAGAAGACGGCATACGAGATACGATTGCTAGGAACGATGAGCCT CCAAC	275
Ind241	AACTTG	CAAGCAGAAGACGGCATACGAGATAACTTGGCTAGGAACGATGAGCCT CCAAC	276
Ind242	AACGTA	CAAGCAGAAGACGGCATACGAGATAACGTAGCTAGGAACGATGAGCCT CCAAC	277
Ind243	ACCCAC	CAAGCAGAAGACGGCATACGAGATACCCACGCTAGGAACGATGAGCCT CCAAC	278
Ind244	CGGTCT	CAAGCAGAAGACGGCATACGAGATCGGTCTGCTAGGAACGATGAGCCT CCAAC	279
Ind245	TTCTAG	CAAGCAGAAGACGGCATACGAGATTTCTAGGCTAGGAACGATGAGCCT CCAAC	280
Ind246	GGTGTG	CAAGCAGAAGACGGCATACGAGATGGTGTGGCTAGGAACGATGAGCCT CCAAC	281
Ind247	ACCACC	CAAGCAGAAGACGGCATACGAGATACCACCGCTAGGAACGATGAGCCT CCAAC	282

Barcode ID	Barcode	Primer	SEQ ID NO:
Ind248	GACTGC	CAAGCAGAAGACGGCATACGAGATGACTGCGCTAGGAACGATGAGCCT CCAAC	283
Ind249	CTACTT	CAAGCAGAAGACGGCATACGAGATCTACTTGCTAGGAACGATGAGCCT CCAAC	284
Ind250	TAGCGG	CAAGCAGAAGACGGCATACGAGATTAGCGGGCTAGGAACGATGAGCCT CCAAC	285
Ind251	TGTGCG	CAAGCAGAAGACGGCATACGAGATTGTGCGGCTAGGAACGATGAGCCT CCAAC	286
Ind252	CTGGCT	CAAGCAGAAGACGGCATACGAGATCTGGCTGCTAGGAACGATGAGCCT CCAAC	287
Ind253	CGAGAC	CAAGCAGAAGACGGCATACGAGATCGAGACGCTAGGAACGATGAGCCT CCAAC	288
Ind254	GGGATT	CAAGCAGAAGACGGCATACGAGATGGGATTGCTAGGAACGATGAGCCT CCAAC	289
Ind255	TACTCC	CAAGCAGAAGACGGCATACGAGATTACTCCGCTAGGAACGATGAGCCT CCAAC	290
Ind256	GTGGCA	CAAGCAGAAGACGGCATACGAGATGTGGCAGCTAGGAACGATGAGCCT CCAAC	291
Ind257	GTCGTC	CAAGCAGAAGACGGCATACGAGATGTCGTCGCTAGGAACGATGAGCCT CCAAC	292
Ind258	GCATTC	CAAGCAGAAGACGGCATACGAGATGCATTCGCTAGGAACGATGAGCCT CCAAC	293
Ind259	GAGCGA	CAAGCAGAAGACGGCATACGAGATGAGCGAGCTAGGAACGATGAGCCT CCAAC	294
Ind260	AGACAC	CAAGCAGAAGACGGCATACGAGATAGACACGCTAGGAACGATGAGCCT CCAAC	295
Ind261	TCTGGG	CAAGCAGAAGACGGCATACGAGATTCTGGGGCTAGGAACGATGAGCCT CCAAC	296
Ind262	GCCAGT	CAAGCAGAAGACGGCATACGAGATGCCAGTGCTAGGAACGATGAGCCT CCAAC	297
Ind263	CCAGTC	CAAGCAGAAGACGGCATACGAGATCCAGTCGCTAGGAACGATGAGCCT CCAAC	298
Ind264	TTGGCC	CAAGCAGAAGACGGCATACGAGATTTGGCCGCTAGGAACGATGAGCCT CCAAC	299
Ind265	GTAACA	CAAGCAGAAGACGGCATACGAGATGTAACAGCTAGGAACGATGAGCCT CCAAC	300
Ind266	ATTACC	CAAGCAGAAGACGGCATACGAGATATTACCGCTAGGAACGATGAGCCT CCAAC	301
Ind267	TCTCCT	CAAGCAGAAGACGGCATACGAGATTCTCCTGCTAGGAACGATGAGCCT CCAAC	302
Ind268	AGATCA	CAAGCAGAAGACGGCATACGAGATAGATCAGCTAGGAACGATGAGCCT CCAAC	303
Ind269	TCCTAT	CAAGCAGAAGACGGCATACGAGATTCCTATGCTAGGAACGATGAGCCT CCAAC	304
Ind270	ACTCGG	CAAGCAGAAGACGGCATACGAGATACTCGGGCTAGGAACGATGAGCCT CCAAC	305
Ind271	TGCCAT	CAAGCAGAAGACGGCATACGAGATTGCCATGCTAGGAACGATGAGCCT CCAAC	306
Ind272	CATCTC	CAAGCAGAAGACGGCATACGAGATCATCTCGCTAGGAACGATGAGCCT CCAAC	307
Ind273	CTTTGA	CAAGCAGAAGACGGCATACGAGATCTTTGAGCTAGGAACGATGAGCCT CCAAC	308
Ind274	TCGCAT	CAAGCAGAAGACGGCATACGAGATTCGCATGCTAGGAACGATGAGCCT CCAAC	309

Barcode ID	Barcode	Primer	SEQ ID NO:
Ind275	CACACC	CAAGCAGAAGACGGCATACGAGATCACACCGCTAGGAACGATGAGCCT CCAAC	310
Ind276	ACAGAA	CAAGCAGAAGACGGCATACGAGATACAGAAGCTAGGAACGATGAGCCT CCAAC	311
Ind277	ATGTCA	CAAGCAGAAGACGGCATACGAGATATGTCAGCTAGGAACGATGAGCCT CCAAC	312
Ind278	CTGTCC	CAAGCAGAAGACGGCATACGAGATCTGTCCGCTAGGAACGATGAGCCT CCAAC	313
Ind279	CGATCC	CAAGCAGAAGACGGCATACGAGATCGATCCGCTAGGAACGATGAGCCT CCAAC	314
Ind280	TGGAGG	CAAGCAGAAGACGGCATACGAGATTGGAGGGCTAGGAACGATGAGCCT CCAAC	315
Ind281	CGCCAA	CAAGCAGAAGACGGCATACGAGATCGCCAAGCTAGGAACGATGAGCCT CCAAC	316
Ind282	GAATAG	CAAGCAGAAGACGGCATACGAGATGAATAGGCTAGGAACGATGAGCCT CCAAC	317
Ind283	CAACGG	CAAGCAGAAGACGGCATACGAGATCAACGGGCTAGGAACGATGAGCCT CCAAC	318
Ind284	GCGGAA	CAAGCAGAAGACGGCATACGAGATGCGGAAGCTAGGAACGATGAGCCT CCAAC	319
Ind285	TCTGCA	CAAGCAGAAGACGGCATACGAGATTCTGCAGCTAGGAACGATGAGCCT CCAAC	320
Ind286	GATGGC	CAAGCAGAAGACGGCATACGAGATGATGGCGCTAGGAACGATGAGCCT CCAAC	321
Ind287	CCGATG	CAAGCAGAAGACGGCATACGAGATCCGATGGCTAGGAACGATGAGCCT CCAAC	322
Ind288	GATTTC	CAAGCAGAAGACGGCATACGAGATGATTTCGCTAGGAACGATGAGCCT CCAAC	323
Ind289	CCAAAC	CAAGCAGAAGACGGCATACGAGATCCAAACGCTAGGAACGATGAGCCT CCAAC	324
Ind290	AGGATC	CAAGCAGAAGACGGCATACGAGATAGGATCGCTAGGAACGATGAGCCT CCAAC	325
Ind291	CATTCA	CAAGCAGAAGACGGCATACGAGATCATTCAGCTAGGAACGATGAGCCT CCAAC	326
Ind292	AGATTG	CAAGCAGAAGACGGCATACGAGATAGATTGGCTAGGAACGATGAGCCT CCAAC	327
Ind293	CGAAGC	CAAGCAGAAGACGGCATACGAGATCGAAGCGCTAGGAACGATGAGCCT CCAAC	328
Ind294	GGAACG	CAAGCAGAAGACGGCATACGAGATGGAACGGCTAGGAACGATGAGCCT CCAAC	329
Ind295	CGACCA	CAAGCAGAAGACGGCATACGAGATCGACCAGCTAGGAACGATGAGCCT CCAAC	330
Ind296	AGCTTA	CAAGCAGAAGACGGCATACGAGATAGCTTAGCTAGGAACGATGAGCCT CCAAC	331
Ind297	TTCACG	CAAGCAGAAGACGGCATACGAGATTTCACGGCTAGGAACGATGAGCCT CCAAC	332
Ind298	CATTAG	CAAGCAGAAGACGGCATACGAGATCATTAGGCTAGGAACGATGAGCCT CCAAC	333
Ind299	TAGGAG	CAAGCAGAAGACGGCATACGAGATTAGGAGGCTAGGAACGATGAGCCT CCAAC	334
Ind300	CTACCG	CAAGCAGAAGACGGCATACGAGATCTACCGGCTAGGAACGATGAGCCT CCAAC	335
Ind301	ATATCC	CAAGCAGAAGACGGCATACGAGATATATCCGCTAGGAACGATGAGCCT CCAAC	336

Barcode ID	Barcode	Primer	SEQ ID NO:
Ind302	AATGGA	CAAGCAGAAGACGGCATACGAGATAATGGAGCTAGGAACGATGAGCCT CCAAC	337
Ind303	TTCGAC	CAAGCAGAAGACGGCATACGAGATTTCGACGCTAGGAACGATGAGCCT CCAAC	338
Ind304	AACCGG	CAAGCAGAAGACGGCATACGAGATAACCGGGCTAGGAACGATGAGCCT CCAAC	339
Ind305	AAGGCC	CAAGCAGAAGACGGCATACGAGATAAGGCCGCTAGGAACGATGAGCCT CCAAC	340
Ind306	CGTCCT	CAAGCAGAAGACGGCATACGAGATCGTCCTGCTAGGAACGATGAGCCT CCAAC	341
Ind307	AAGAGC	CAAGCAGAAGACGGCATACGAGATAAGAGCGCTAGGAACGATGAGCCT CCAAC	342
Ind308	GTGAAA	CAAGCAGAAGACGGCATACGAGATGTGAAAGCTAGGAACGATGAGCCT CCAAC	343
Ind309	ACGAAC	CAAGCAGAAGACGGCATACGAGATACGAACGCTAGGAACGATGAGCCT CCAAC	344
Ind310	CCGAAA	CAAGCAGAAGACGGCATACGAGATCCGAAAGCTAGGAACGATGAGCCT CCAAC	345
Ind311	CAAGGC	CAAGCAGAAGACGGCATACGAGATCAAGGCGCTAGGAACGATGAGCCT CCAAC	346
Ind312	ATGTGC	CAAGCAGAAGACGGCATACGAGATATGTGCGCTAGGAACGATGAGCCT CCAAC	347
Ind313	CCCTTG	CAAGCAGAAGACGGCATACGAGATCCCTTGGCTAGGAACGATGAGCCT CCAAC	348
Ind314	GAGAA G	CAAGCAGAAGACGGCATACGAGATGAGAAGGCTAGGAACGATGAGCC TCCAAC	349
Ind315	GTAGTT	CAAGCAGAAGACGGCATACGAGATGTAGTTGCTAGGAACGATGAGCCT CCAAC	350
Ind316	TGGTGC	CAAGCAGAAGACGGCATACGAGATTGGTGCGCTAGGAACGATGAGCCT CCAAC	351
Ind317	GACTAT	CAAGCAGAAGACGGCATACGAGATGACTATGCTAGGAACGATGAGCCT CCAAC	352
Ind318	CTCGCA	CAAGCAGAAGACGGCATACGAGATCTCGCAGCTAGGAACGATGAGCCT CCAAC	353
Ind319	TCTTGT	CAAGCAGAAGACGGCATACGAGATTCTTGTGCTAGGAACGATGAGCCT CCAAC	354
Ind320	GCACAA	CAAGCAGAAGACGGCATACGAGATGCACAAGCTAGGAACGATGAGCCT CCAAC	355
Ind321	CCTTTA	CAAGCAGAAGACGGCATACGAGATCCTTTAGCTAGGAACGATGAGCCT CCAAC	356
Ind322	ACGTTG	CAAGCAGAAGACGGCATACGAGATACGTTGGCTAGGAACGATGAGCCT CCAAC	357
Ind323	AAGCGT	CAAGCAGAAGACGGCATACGAGATAAGCGTGCTAGGAACGATGAGCCT CCAAC	358
Ind324	AGGCAA	CAAGCAGAAGACGGCATACGAGATAGGCAAGCTAGGAACGATGAGCCT CCAAC	359
Ind325	TGAGCC	CAAGCAGAAGACGGCATACGAGATTGAGCCGCTAGGAACGATGAGCCT CCAAC	360
Ind326	TGAGAA	CAAGCAGAAGACGGCATACGAGATTGAGAAGCTAGGAACGATGAGCCT CCAAC	361
Ind327	GTACAG	CAAGCAGAAGACGGCATACGAGATGTACAGGCTAGGAACGATGAGCCT CCAAC	362
Ind328	ACGGAG	CAAGCAGAAGACGGCATACGAGATACGGAGGCTAGGAACGATGAGCCT CCAAC	363

Barcode ID	Barcode	Primer	SEQ ID NO:
Ind329	ACATAC	CAAGCAGAAGACGGCATACGAGATACATACGCTAGGAACGATGAGCCT CCAAC	364
Ind330	CAGCCA	CAAGCAGAAGACGGCATACGAGATCAGCCAGCTAGGAACGATGAGCCT CCAAC	365
Ind331	TGTCAA	CAAGCAGAAGACGGCATACGAGATTGTCAAGCTAGGAACGATGAGCCT CCAAC	366
Ind332	TATTGG	CAAGCAGAAGACGGCATACGAGATTATTGGGCTAGGAACGATGAGCCT CCAAC	367
Ind333	AGACCG	CAAGCAGAAGACGGCATACGAGATAGACCGGCTAGGAACGATGAGCCT CCAAC	368
Ind334	GCCAAC	CAAGCAGAAGACGGCATACGAGATGCCAACGCTAGGAACGATGAGCCT CCAAC	369
Ind335	ATGTAG	CAAGCAGAAGACGGCATACGAGATATGTAGGCTAGGAACGATGAGCCT CCAAC	370
Ind336	CGCTAC	CAAGCAGAAGACGGCATACGAGATCGCTACGCTAGGAACGATGAGCCT CCAAC	371
Ind337	CACTCG	CAAGCAGAAGACGGCATACGAGATCACTCGGCTAGGAACGATGAGCCT CCAAC	372
Ind338	GCTATT	CAAGCAGAAGACGGCATACGAGATGCTATTGCTAGGAACGATGAGCCT CCAAC	373
Ind339	CTCCAC	CAAGCAGAAGACGGCATACGAGATCTCCACGCTAGGAACGATGAGCCT CCAAC	374
Ind340	GTTAGC	CAAGCAGAAGACGGCATACGAGATGTTAGCGCTAGGAACGATGAGCCT CCAAC	375
Ind341	AAACCT	CAAGCAGAAGACGGCATACGAGATAAACCTGCTAGGAACGATGAGCCT CCAAC	376
Ind342	CTAGAT	CAAGCAGAAGACGGCATACGAGATCTAGATGCTAGGAACGATGAGCCT CCAAC	377
Ind343	ACCGTC	CAAGCAGAAGACGGCATACGAGATACCGTCGCTAGGAACGATGAGCCT CCAAC	378
Ind344	TCACCC	CAAGCAGAAGACGGCATACGAGATTCACCCGCTAGGAACGATGAGCCT CCAAC	379
Ind345	GAAGAT	CAAGCAGAAGACGGCATACGAGATGAAGATGCTAGGAACGATGAGCCT CCAAC	380
Ind346	TGGCTC	CAAGCAGAAGACGGCATACGAGATTGGCTCGCTAGGAACGATGAGCCT CCAAC	381
Ind347	GGTTAT	CAAGCAGAAGACGGCATACGAGATGGTTATGCTAGGAACGATGAGCCT CCAAC	382
Ind348	ACACTT	CAAGCAGAAGACGGCATACGAGATACACTTGCTAGGAACGATGAGCCT CCAAC	383
Ind349	GGAAG A	CAAGCAGAAGACGGCATACGAGATGGAAGAGCTAGGAACGATGAGCC TCCAAC	384
Ind350	TACGTG	CAAGCAGAAGACGGCATACGAGATTACGTGGCTAGGAACGATGAGCCT CCAAC	385
Ind351	CTTGAC	CAAGCAGAAGACGGCATACGAGATCTTGACGCTAGGAACGATGAGCCT CCAAC	386
Ind352	ATGCCC	CAAGCAGAAGACGGCATACGAGATATGCCCGCTAGGAACGATGAGCCT CCAAC	387
Ind353	ATTCAC	CAAGCAGAAGACGGCATACGAGATATTCACGCTAGGAACGATGAGCCT CCAAC	388
Ind354	GCTTAC	CAAGCAGAAGACGGCATACGAGATGCTTACGCTAGGAACGATGAGCCT CCAAC	389
Ind355	TTCTGC	CAAGCAGAAGACGGCATACGAGATTTCTGCGCTAGGAACGATGAGCCT CCAAC	390

Barcode ID	Barcode	Primer	SEQ ID NO:
Ind356	TGTATG	CAAGCAGAAGACGGCATACGAGATTGTATGGCTAGGAACGATGAGCCT CCAAC	391
Ind357	TGACGC	CAAGCAGAAGACGGCATACGAGATTGACGCGCTAGGAACGATGAGCCT CCAAC	392
Ind358	GTGTTA	CAAGCAGAAGACGGCATACGAGATGTGTTAGCTAGGAACGATGAGCCT CCAAC	393
Ind359	CATCGA	CAAGCAGAAGACGGCATACGAGATCATCGAGCTAGGAACGATGAGCCT CCAAC	394
Ind360	TGAGGT	CAAGCAGAAGACGGCATACGAGATTGAGGTGCTAGGAACGATGAGCCT CCAAC	395
Ind361	GGTCCA	CAAGCAGAAGACGGCATACGAGATGGTCCAGCTAGGAACGATGAGCCT CCAAC	396
Ind362	TATGCT	CAAGCAGAAGACGGCATACGAGATTATGCTGCTAGGAACGATGAGCCT CCAAC	397
Ind363	TTCATC	CAAGCAGAAGACGGCATACGAGATTTCATCGCTAGGAACGATGAGCCT CCAAC	398
Ind364	CCTCTG	CAAGCAGAAGACGGCATACGAGATCCTCTGGCTAGGAACGATGAGCCT CCAAC	399
Ind365	CCAAGG	CAAGCAGAAGACGGCATACGAGATCCAAGGGCTAGGAACGATGAGCCT CCAAC	400
Ind366	TAATGC	CAAGCAGAAGACGGCATACGAGATTAATGCGCTAGGAACGATGAGCCT CCAAC	401
Ind367	AGGGCA	CAAGCAGAAGACGGCATACGAGATAGGGCAGCTAGGAACGATGAGCCT CCAAC	402
Ind368	TAGAGA	CAAGCAGAAGACGGCATACGAGATTAGAGAGCTAGGAACGATGAGCCT CCAAC	403
Ind369	AGCACA	CAAGCAGAAGACGGCATACGAGATAGCACAGCTAGGAACGATGAGCCT CCAAC	404
Ind370	AGAGTC	CAAGCAGAAGACGGCATACGAGATAGAGTCGCTAGGAACGATGAGCCT CCAAC	405
Ind371	ACTCAA	CAAGCAGAAGACGGCATACGAGATACTCAAGCTAGGAACGATGAGCCT CCAAC	406
Ind372	GTGACG	CAAGCAGAAGACGGCATACGAGATGTGACGGCTAGGAACGATGAGCCT CCAAC	407
Ind373	GGTAGG	CAAGCAGAAGACGGCATACGAGATGGTAGGGCTAGGAACGATGAGCCT CCAAC	408
Ind374	CTGAAT	CAAGCAGAAGACGGCATACGAGATCTGAATGCTAGGAACGATGAGCCT CCAAC	409
Ind375	GTCTAC	CAAGCAGAAGACGGCATACGAGATGTCTACGCTAGGAACGATGAGCCT CCAAC	410
Ind376	TCGGAC	CAAGCAGAAGACGGCATACGAGATTCGGACGCTAGGAACGATGAGCCT CCAAC	411
Ind377	AACTAC	CAAGCAGAAGACGGCATACGAGATAACTACGCTAGGAACGATGAGCCT CCAAC	412
Ind378	AAGGTT	CAAGCAGAAGACGGCATACGAGATAAGGTTGCTAGGAACGATGAGCCT CCAAC	413
Ind379	AGGGAC	CAAGCAGAAGACGGCATACGAGATAGGGACGCTAGGAACGATGAGCCT CCAAC	414
Ind380	ACGCGA	CAAGCAGAAGACGGCATACGAGATACGCGAGCTAGGAACGATGAGCCT CCAAC	415
Ind381	TTGCTA	CAAGCAGAAGACGGCATACGAGATTTGCTAGCTAGGAACGATGAGCCT CCAAC	416
Ind382	ATAACG	CAAGCAGAAGACGGCATACGAGATATAACGGCTAGGAACGATGAGCCT CCAAC	417

Barcode ID	Barcode	Primer	SEQ ID NO:
Ind383	CCGGTA	CAAGCAGAAGACGGCATACGAGATCCGGTAGCTAGGAACGATGAGCCT CCAAC	418
Ind384	AGCTAG	CAAGCAGAAGACGGCATACGAGATAGCTAGGCTAGGAACGATGAGCCT CCAAC	419

[00171] Bisulfite read mapping and data analysis

[00172] The reference genome was computationally converted by changing all cytosines to thymines on the two strands separately. Sequencing reads were encoded by (i) predicting the mapping orientation, (ii) converting all predicted forward mapping reads by changing all cytosines to thymines and converting all predicted reverse complementary mapping reads by changing all guanines to adenines, the original reads are maintained. The bisulfite reads were then mapped to the converted reference separately using SOAP2Align with the parameters r = 0 (report uniquely mapped reads only), v = 2 (number of allowable mismatch), paired-end: m = 0 (minimal insert size), x = 400 (maximum insert size). Alignment files were then combined, and one alignment per read was selected. Original C calls were placed back into the alignment information. Alignments were then converted to pileup format using SamTools. Raw SNPs and methylation frequency files were computed from pileup counts. Methylation frequencies were called using a method described previously (Deng, J. et al. (2009) *Nat. Biotechnol.* 27: 353-360).

[00173] Correlation of methylation between two samples

[00174] To check whether methylation levels were similar between two samples, the Pearson's correlation was calculated on all CpG sites characterizable in both samples. First, a list of CpG sites with read depth of at least ten in both samples was generated. The methylation frequencies at these sites were obtained from bisReadMapper output and input into the statistical package R. Finally, Pearson's correlation for the two samples was computed using the cor() function.

[00175] Analysis of methylation

[00176] From the bisReadMapper output, the raw read counts showing methylation and lack of methylation were assembled for each line. Using these counts, a Fisher Exact Test with Benjamini-Hochberg multiple testing correction (false discovery rate = 0.01) was

carried out on each CpG site with minimum 10× depth coverage. This resulted in a set of DMSs between the two lines; at each of these sites, the methylation had at least 0.1 methylation level of difference. Technical replicates did not show any differential methylation, and different cell types showed a large extent of differential methylation (~33%).

Results and Discussion

[00177] These probes were used to analyze H1 embryonic stem cells (H1 ESCs), PGP1 fibroblasts and two technical replicates of PGP1 fibroblast–derived induced pluripotent stem cells (PGP1-iPSCs). For each sample, on average ~3.66 gigabases were sequenced and measured methylation for an average of 480,904 CpG sites. To assess whether these data could be used to identify potential epigenetic regulation of transcription, the Genomic Regions Enrichment of Annotations Tool ("GREAT"; McLean, C.Y. et al. (2010) *Nat. Biotechnol.* 28: 495-501) was used to predict the cis-regulatory potential of regions around captured CpG sites. In total, the padlock probes captured CpG sites in regions predicted to regulate 98% of RefSeq genes (Figure 13).

[00178] The data generated with BSPPs accurately represented the methylation status of the target regions. Methylation levels for the two technical replicates of PGP1-iPSCs were consistent both within a single batch and between separate batches (Pearson's correlation coefficient R = 0.97–0.98, Figure 14A-14B). Additionally, when we compared methylation levels between technical replicates, no CpG site was different by a Fisher Exact Test with Benjamini-Hochberg multiple testing correction (false discovery rate = 0.01, n = 439,090). In comparison, large fractions of sites were differentially methylated owing to either the process of nuclear reprogramming (27.9% DMSs between PGP1-iPSCs and PGP1 fibroblasts) or the difference in cell type (31.3% DMSs between PGP1 fibroblasts and H1 ESCs) with the same criteria (false discovery rate = 0.01, n = 444,111 and 359,290, respectively). Our BSPP results with H1 ESCs were consistent with the published whole-genome sequencing of bisulfite-converted DNA12 (Pearson's correlation coefficient R = 0.95, Figure 15).

[00179] This assay has very low technical variability. The assay was performed on over 150 samples in 96-well plates; the yield for each was similar (Figure 16). Approximately 10% of CpG sites were targeted separately on each strand, allowing low-

quality datasets with poor correlation between these built-in technical replicates to be identified (Figure 14C-14E). As our BSPP assay measures absolute methylation, no normalization is necessary as long as the internal replicates are consistent. Therefore, many datasets, even those generated in different laboratories, can be directly compared without batch effects, which is important for case-control studies on large samples or for meta-analyses. Additionally, the SNP-calling feature of bisReadMapper allowed for characterization of roughly 20,000 SNPs for each sample with an accuracy of 96% or better. This allowed unambiguous tracking of samples, which is crucial for projects involving large sample sizes.

[00180] The library-free BSPP method is flexible for different study designs. Whereas the genome-scale probe set allows global profiling on thousands of samples, a focused assay is necessary to follow up on tens to hundreds of candidate regions identified in genome-scale scanning. Such an assay needs to be customizable to different genomic targets, scalable to a very large sample size (1,000–100,000 samples), and inexpensive. To additionally test the flexibility, a second set of 3,918 probes (LC Sciences) was designed to evaluate the methylation state 1 kbp upstream and downstream of 120 genomic regions previously known and confirmed by BSPP to carry aberrant methylation in induced pluripotent stem cells (Lister, R. et al. (2011) *Nature* 471(7336): 68-73). Even with shorter capturing sequences (40 bp total for capturing arms rather than 50 bp on average, Figure 17) and a 100-fold smaller target size, an average of 56% of mappable bases were on-target, equivalent to an enrichment factor of ~6,500. With the data from three cell lines (H1 ESCs, PGP1 fibroblasts and PGP1-iPSCs) regions of aberrant methylation were identified in induced pluripotent stem cells and it was found that aberrant methylation continues further upstream and downstream than observed previously.

[00181] This analysis demonstrated that a focused probe set can be used to validate specific regions of interest identified in global scanning using either our genome-wide probe set or other methods.

Example 3: Improvements to ppDesigner

[00182] Several key improvements were made to both the probe design algorithm and the ppDesigner software implementing it. These changes allow for improved generation of low-bias padlock probes for bisulfite sequencing applications and additional customizability

in probe parameters such that probes can be synthesized from additional oligonucleotide vendors and utilized in additional experimental protocols.

[00183] The previous version of the probe design algorithm utilized one neural network to generate the optimal set of padlock probes for both normal and bisulfite-converted DNA targets. However, this network was specifically designed using results from probe experiments without bisulfite conversion. In the improved algorithm, a second neural network was added specifically for bisulfite-converted DNA based solely on bisulfite probe experiments. This network contains two hidden layers with 10 and 12 nodes, respectively (Figure 18), and accepts 25 pieces of information as input rather than 7. These 25 factors (Table 8) were specifically chosen for their ability to influence padlock probe capturing efficiency on bisulfite-converted DNA.

[00184] Table 8: Factors used by neural network to predict probe capturing efficiency

Factors used in Bisulfite Padlock Probe Design	Factors used in Genomic Padlock Probe Design
Extension Arm A%	Target Folding Energy
Extension Arm G%	Target GC Content
Target A%	Ligation Arm Melting Temperature
Target T%	Extension Arm Melting Temperature
Target G%	Ligation Arm Length
Number of "GG" Dinucleotides in Ligation Arm	Extension Arm Length
Number of "AT" Dinucleotides in Extension Arm	Target Length
Number of "GG" Dinucleotides in Extension Arm	
Number of "AA" Dinucleotides in Target	
Number of "AT" Dinucleotides in Target	
Number of "TA" Dinucleotides in Target	
Number of "GT" Dinucleotides in Target	
Number of "GA" Dinucleotides in Target	
Ligation Arm Terminal Dinucleotide	
Extension Arm Terminal Dinucleotide	
Target 5' Terminal Dinucleotide	
Ligation Arm Length	
Target Length	
Overall Melting Temperature	

[00185] Significant new factors include the counts of each DNA dinucleotide in the target region and the dinucleotides surrounding the ligation site. The use of two neural networks for the two separate capturing conditions allows for a more efficient prediction of probe efficiency, reducing capturing bias and experimental cost.

[00186] In addition, new user interface improvements were implemented to better facilitate these protocols. Several simple "default" setting profiles were added to allow the probe designer to more easily account for limitations in oligonucleotide synthesis from

various vendors (including Agilent Technologies and LC Sciences). Additional parameters were also added, allowing for easy implementation of the new library-free padlock probe protocol, including allowing the user to set a fixed total arm size and providing the specialized library-free linker sequence.

CLAIMS

What is claimed is:

1. A method of designing probes or primers for sequencing a target nucleic acid molecule, comprising:
selecting one or more inputs associated with efficiency of the probe or primer;
selecting a target nucleic acid sequence;
generating a first library of probe or primer sequences that comprise a target capturing sequence that is complementary to the target nucleic acid sequence;
determining the efficiency of each probe or primer sequence in the first library by using an algorithm comprising the one or more selected inputs;
ranking the probe or primer sequences in the first library by efficiency;
extracting the probe or primer sequences having the highest efficiency to generate a second library; and
adding a linker sequence to each of the probe or primer sequences in the second library.

2. The method of claim 1, further comprising synthesizing the probe or primer.

3. The method of claim 1, wherein the probe is a padlock probe.

4. The method of claim 1, wherein the one or more inputs comprise target length, target folding energy, target GC content, extension arm A%, extension Arm G%, target A%, target T%, target G%, number of "GG" dinucleotides in ligation arm, number of "AT" dinucleotides in extension arm, number of "GG" dinucleotides in extension arm, number of "AA" dinucleotides in target, number of "AT" dinucleotides in target, number of "TA" dinucleotides in target, number of "GT" dinucleotides in target, number of "GA" dinucleotides in target, ligation arm terminal dinucleotide, extension arm terminal dinucleotide, target 5' terminal dinucleotide, ligation arm melting temperature, extension arm melting temperature, ligation arm length, extension arm length, local single-stranded folding energy of the target, and dinucleotides present at the extension site and ligation site during probe capture.

5. The method of claim 1, wherein the target nucleic acid sequence is derived from a human.

6. The method of claim 1, wherein the target-capturing sequence includes a ligation arm and an extension arm.

7. The method of claim 1, wherein the target-capturing sequence contains one or more CpG dinucleotides.

8. The method of claim 6, wherein the target-capturing sequences in the first library contain all possible methylation state combinations of the one or more CpG dinucleotides.

9. The method of claim 5, wherein the extension arm comprises one or more priming sites for amplification of the target nucleic acid sequence.

10. The method of claim 8, wherein the one or more priming sites are universal priming sites.

11. The method of claim 1, wherein the target capturing sequence includes one or more restriction sites.

12. The method of claim 1, wherein the algorithm comprises one or more neural networks.

13. The method of claim 12, wherein the one or more neural networks comprise the one or more inputs.

14. The method of claim 13, wherein the one or more neural networks comprise seven or more inputs.

15. The method of claim 1, wherein the method further comprises, after the extracting, pooling the non-extracted probe or primer sequences and repeating the steps of generating the library of probe or primer sequences, determining the efficiency of each probe or primer sequence by using the algorithm, ranking the probe or primer sequences by efficiency, and extracting the probe or primer sequences having the highest efficiency.

16. The method of claim 1, wherein the linker sequence is common to each probe or primer sequence in the second library.

17. An apparatus comprising at least one processor and at least one memory including code which when executed by the at least one processor provides operations comprising:
selecting one or more inputs associated with efficiency of the probe or primer;
selecting a target nucleic acid sequence;
generating a first library of probe or primer sequences that comprise a target capturing sequence that is complementary to the target nucleic acid sequence;
determining the efficiency of each probe or primer sequence in the first library by using an algorithm comprising the one or more selected inputs;
ranking the probe or primer sequences in the first library by efficiency;
extracting the probe or primer sequences having the highest efficiency to generate a second library; and
adding a linker sequence to each of the probe or primer sequences in the second library.

18. The apparatus of claim 17, wherein the operations further comprise synthesizing the probe or primer.

19. The apparatus of claim 17, wherein the probe is a padlock probe.

20. The apparatus of claim 17, wherein the one or more inputs comprise target length, target folding energy, target GC content, extension arm A%, extension Arm G%, target A%, target T%, target G%, number of "GG" dinucleotides in ligation arm, number of "AT" dinucleotides in extension arm, number of "GG" dinucleotides in extension arm, number of "AA" dinucleotides in target, number of "AT" dinucleotides in target, number of "TA" dinucleotides in target, number of "GT" dinucleotides in target, number of "GA" dinucleotides in target, ligation arm terminal dinucleotide, extension arm terminal dinucleotide, target 5' terminal dinucleotide, ligation arm melting temperature, extension arm melting temperature, ligation arm length, extension arm length, local single-stranded folding

energy of the target, and dinucleotides present at the extension site and ligation site during probe capture.

21. The apparatus of claim 17, wherein the algorithm comprises one or more neural networks.

22. The apparatus of claim 21, wherein the one or more neural networks comprise the one or more inputs.

23. The apparatus of claim 22, wherein the one or more neural networks comprise seven or more inputs.

24. The apparatus of claim 17, wherein the operations further comprise, after the extracting, pooling the non-extracted probe or primer sequences and repeating the steps of generating the library of probe or primer sequences, determining the efficiency of each probe or primer sequence by using the algorithm, ranking the probe or primer sequences by efficiency, and extracting the probe or primer sequences having the highest efficiency.

25. A computer-readable storage medium including code, which when executed by at least one processor provides operations comprising:
selecting one or more inputs associated with efficiency of the probe or primer;
selecting a target nucleic acid sequence;
generating a first library of probe or primer sequences that comprise a target capturing sequence that is complementary to the target nucleic acid sequence;
determining the efficiency of each probe or primer sequence in the first library by using an algorithm comprising the one or more selected inputs;
ranking the probe or primer sequences in the first library by efficiency;
extracting the probe or primer sequences having the highest efficiency to generate a second library; and
adding a linker sequence to each of the probe or primer sequences in the second library.

26. The computer-readable storage medium of claim 25, wherein the operations further comprise synthesizing the probe or primer.

27. The computer-readable storage medium of claim 25, wherein the probe is a padlock probe.

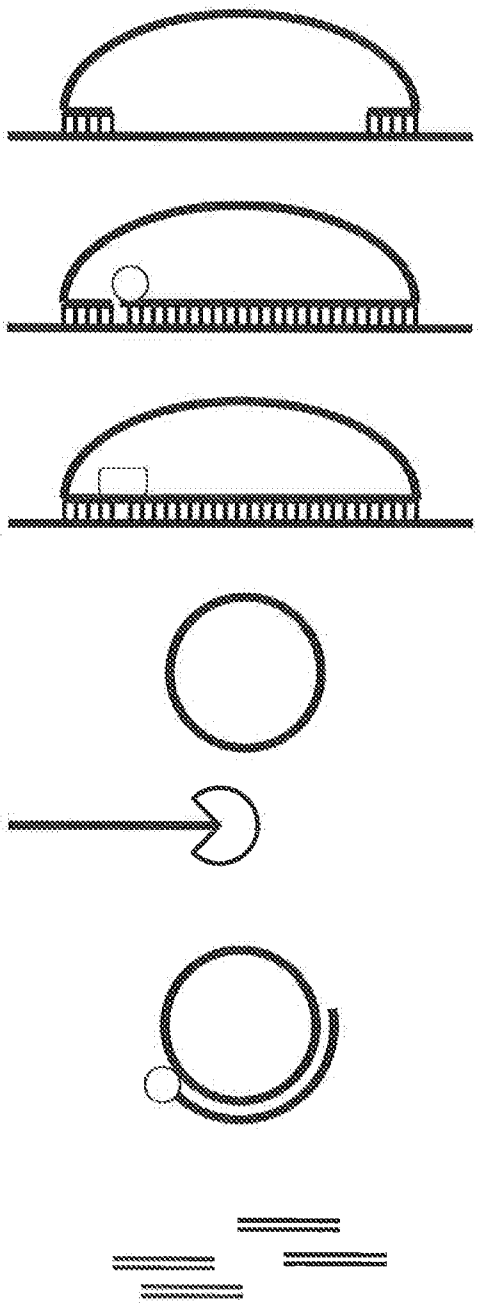
28. The computer-readable storage medium of claim 25, wherein the one or more inputs comprise target length, target folding energy, target GC content, extension arm A%, extension Arm G%, target A%, target T%, target G%, number of "GG" dinucleotides in ligation arm, number of "AT" dinucleotides in extension arm, number of "GG" dinucleotides in extension arm, number of "AA" dinucleotides in target, number of "AT" dinucleotides in target, number of "TA" dinucleotides in target, number of "GT" dinucleotides in target, number of "GA" dinucleotides in target, ligation arm terminal dinucleotide, extension arm terminal dinucleotide, target 5' terminal dinucleotide, ligation arm melting temperature, extension arm melting temperature, ligation arm length, extension arm length, local single-stranded folding energy of the target, and dinucleotides present at the extension site and ligation site during probe capture.

29. The computer-readable storage medium of claim 25, wherein the algorithm comprises one or more neural networks.

30. The computer-readable storage medium of claim 29, wherein the one or more neural networks comprise the one or more inputs.

31. The computer-readable storage medium of claim 30, wherein the one or more neural networks comprise seven or more inputs.

32. The computer-readable storage medium of claim 25, wherein the operations further comprise, after the extracting, pooling the non-extracted probe or primer sequences and repeating the steps of generating the library of probe or primer sequences, determining the efficiency of each probe or primer sequence by using the algorithm, ranking the probe or primer sequences by efficiency, and extracting the probe or primer sequences having the highest efficiency.



Binding arms hybridize to single-stranded genomic DNA around a target region

DNA Polymerase fills in gap between binding arms with complementary sequence

DNA Ligase circularizes the gap-filled probe molecule

ssDNA Exonuclease is used to digest all linear DNA; circularized probes are protected

Rolling Circle Amplification or Polymerase Chain Reaction is used to generate linear complementary region

Polymerase Chain Reaction is used to generate high-throughput DNA sequencing library

FIGURE 1

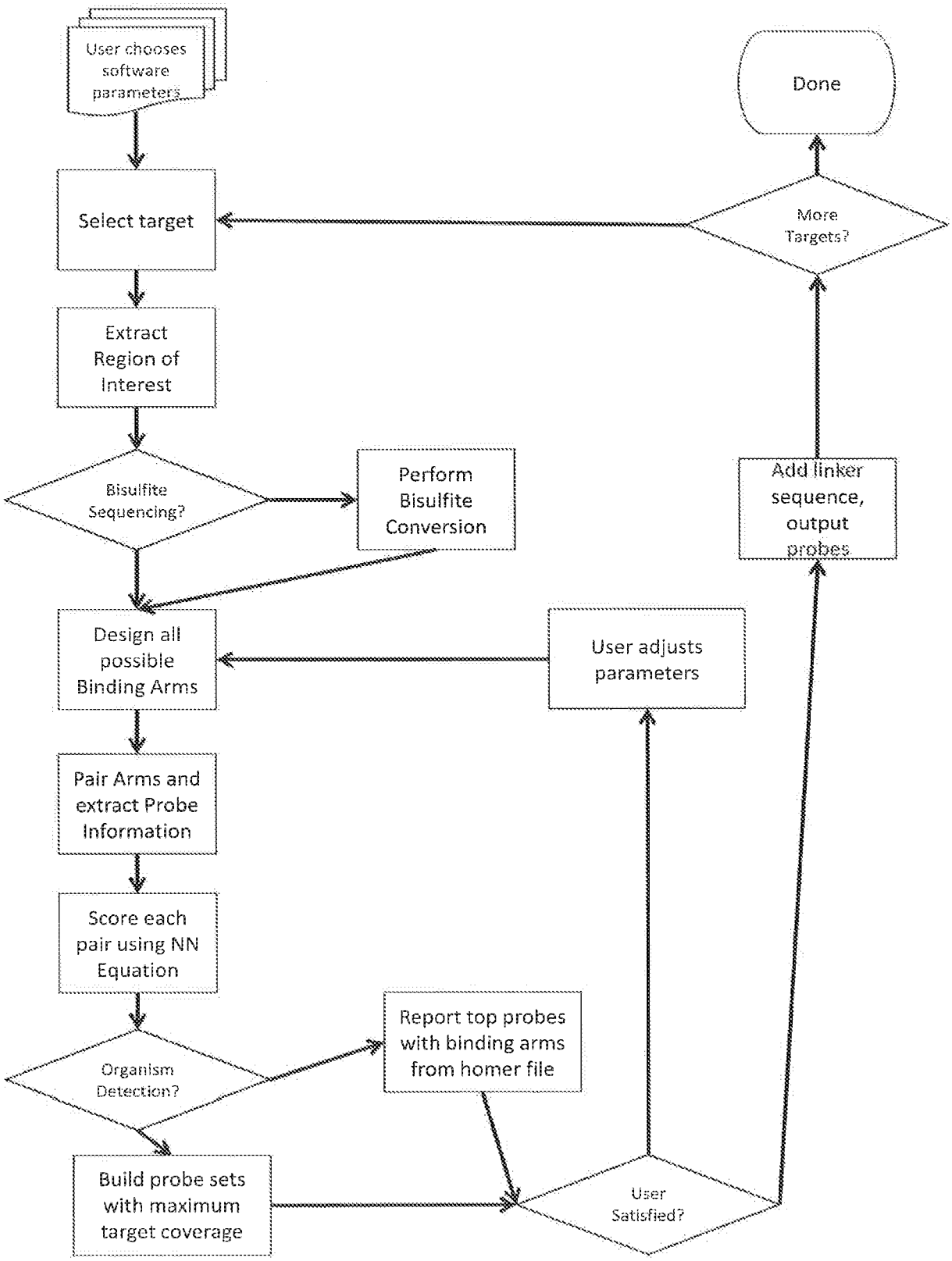
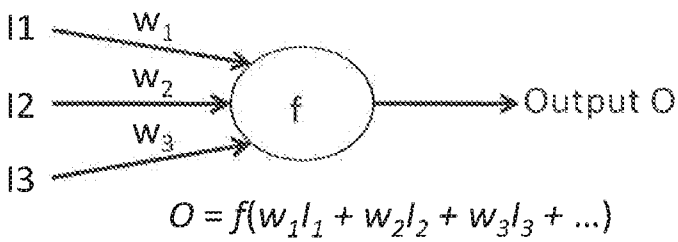
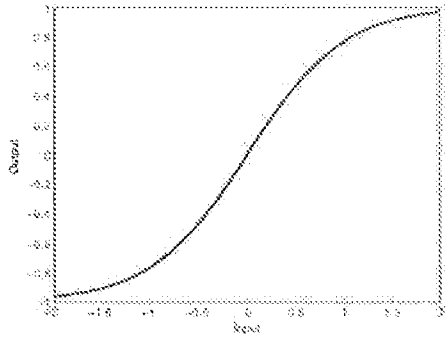

User chooses software parameters
Done
Select target
More Targets?
Extract Region of Interest
Bisulfite Sequencing?
Perform Bisulfite Conversion
Add linker sequence, output probes
Design all possible Binding Arms
User adjusts parameters
Pair Arms and extract Probe Information
Score each pair using NN Equation
Report top probes with binding arms from homer file
Organism Detection?
Build probe sets with maximum target coverage
User Satisfied?

FIGURE 2

Each neuron accepts several inputs, calculates a weighted sum, and outputs a scalar value between zero and one.





By putting 26 neurons together in a "Neural Network," an equation modeling probe efficiency was generated. The optimal weight terms (*w*'s) were selected using data from hundreds of thousands of padlock probe capturing experiments.

Inputs:
1. Target length
2. Target GC content
3. Binding Arm Melting Temperature
4. Each Binding Arm Length
5. Local single-stranded Minimum Folding Energy of Target

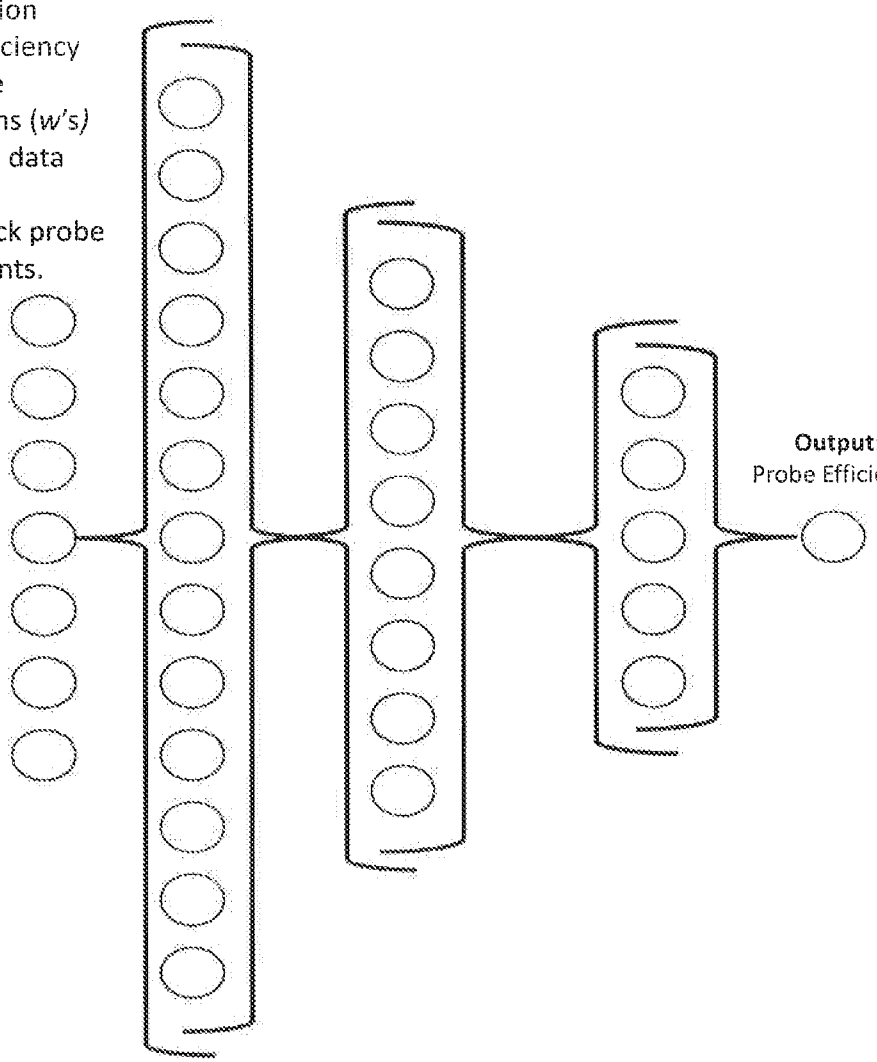
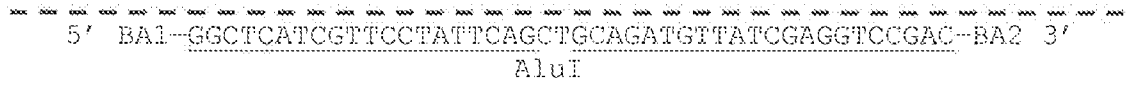


FIGURE 3

Two Example Linker Sequence Designs

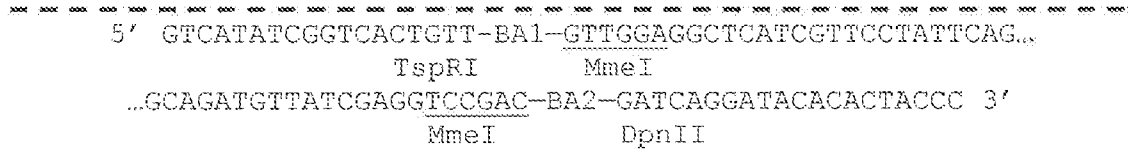


Key:

BA1 – Binding Arm 1 Sequence
BA 2 – Binding Arm 2 Sequence
Underlined Regions – Common PCR Primer Sites
AluI – AluI restriction endonuclease site

Features:

- Each binding arm is attached to two back-to-back common primers for PCR amplification
- AluI Restriction Endonuclease Site in the center of the linker; allows linearization of circular probe molecules without amplification



Key:

BA1 – Binding Arm 1 Sequence
BA 2 – Binding Arm 2 Sequence
Underlined Regions – Common PCR Primer Sites
MmeI – MmeI restriction endonuclease site
TspRI and DpnII – TspRI and DpnII restriction endonuclease sites

Features:

- Each binding arm is surrounded by flanking sequence: probes are inactive until TspRI and DpnII treatment
- MmeI restriction endonuclease site allows removal of binding arms 1 and 2 after capture, leaving only target behind

FIGURE 4

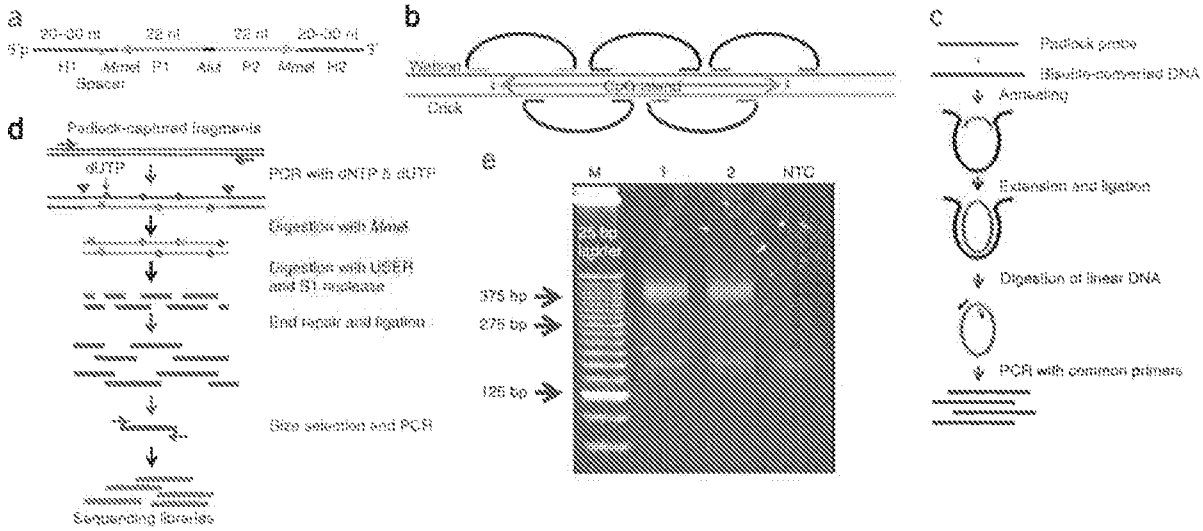

a
20-30 nt
22 nt
22 nt
20-30 nt
5'p
3'
H1
MmeI
P1
Ad2
P2
MmeI
H2
Space
b
Watson
Crick
c
Padlock probe
Bisulfite-converted DNA
Annealing
Extension and ligation
Digestion of linear DNA
PCR with common primers
d
Padlock-captured fragments
dUTP
PCR with dNTP & dUTP
Digestion with MmeI
Digestion with USER and S1 nuclease
End repair and ligation
Size selection and PCR
Sequencing libraries
e
M
1
2
NTC
375 bp
275 bp
125 bp

FIGURE 5

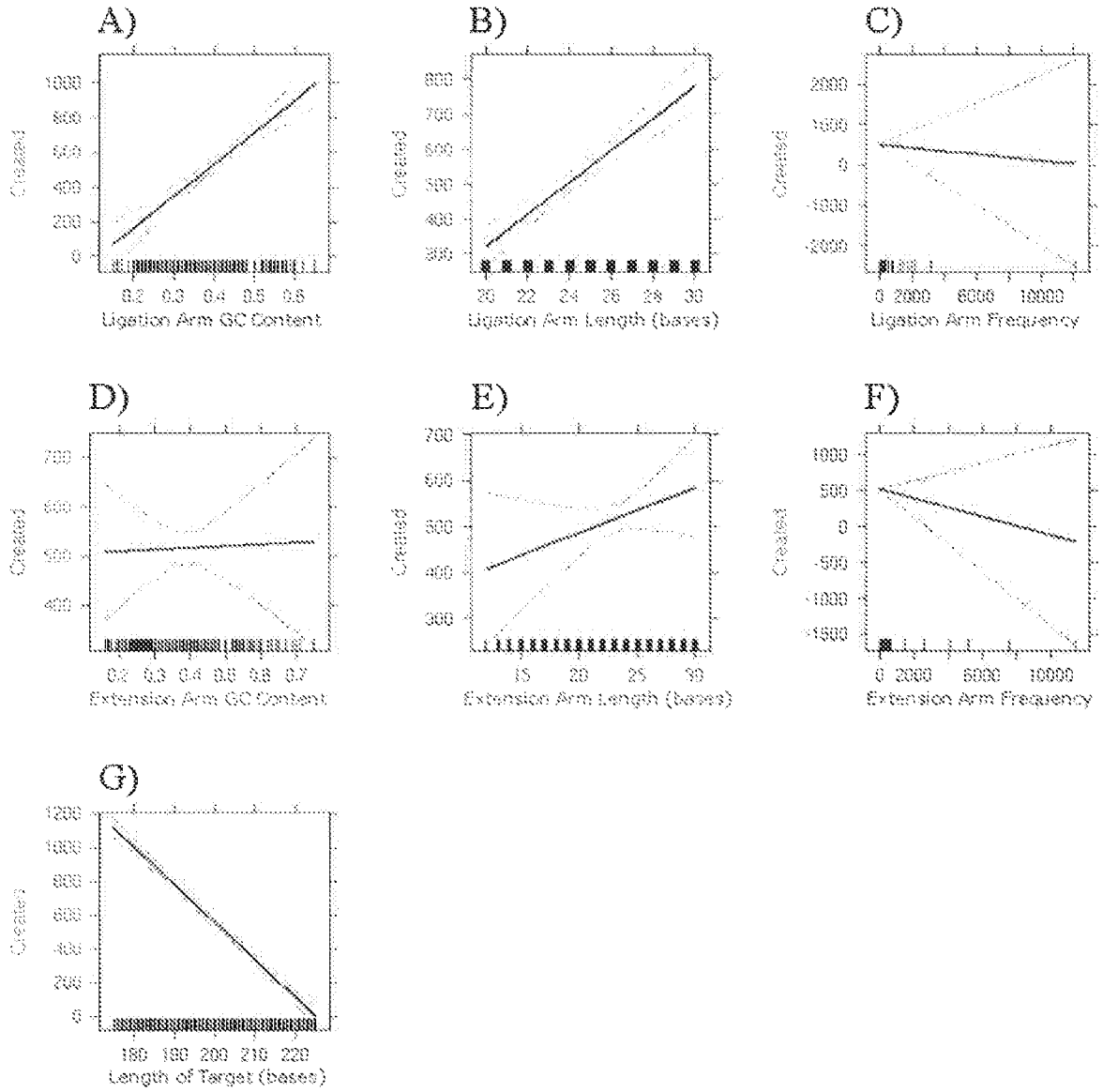

A)
Created
1000
800
600
400
200
0
0.2 0.3 0.4 0.5 0.6
Ligation Arm GC Content
B)
Created
800
700
600
500
400
300
20 22 24 26 28 30
Ligation Arm Length (bases)
C)
Created
2000
1000
0
-1000
-2000
0 2000 6000 10000
Ligation Arm Frequency
D)
Created
700
600
500
400
0.2 0.3 0.4 0.5 0.6 0.7
Extension Arm GC Content
E)
Created
700
600
500
400
300
15 20 25 30
Extension Arm Length (bases)
F)
Created
1000
500
0
-500
-1000
-1500
0 2000 6000 10000
Extension Arm Frequency
G)
Created
1200
1000
800
600
400
200
0
180 190 200 210 220
Length of Target (bases)

FIGURE 6

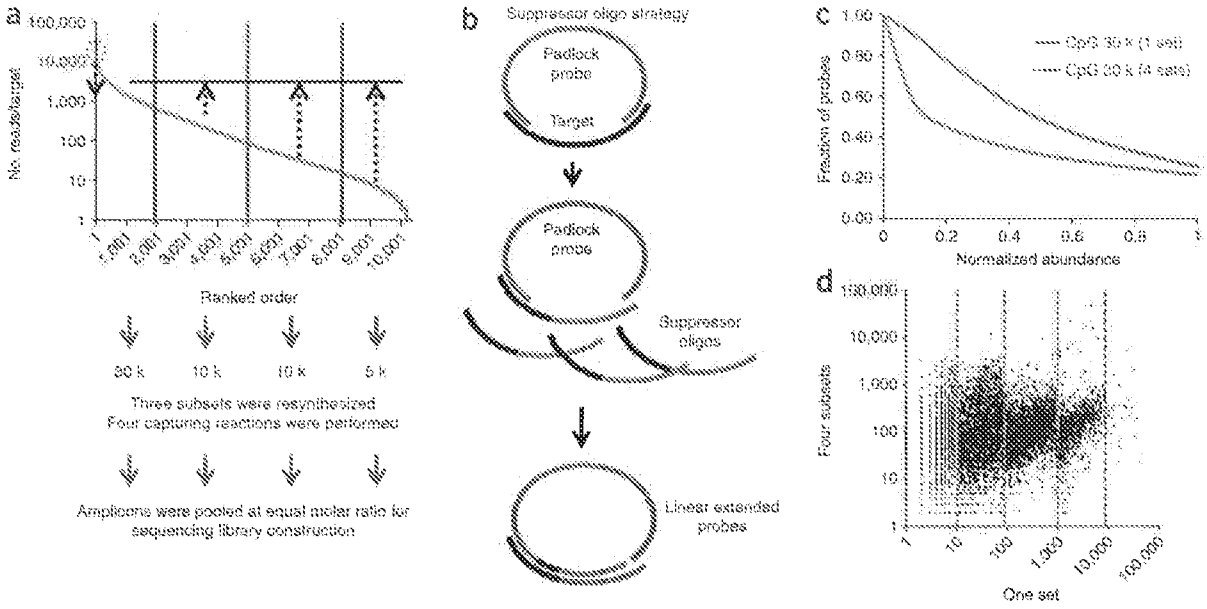
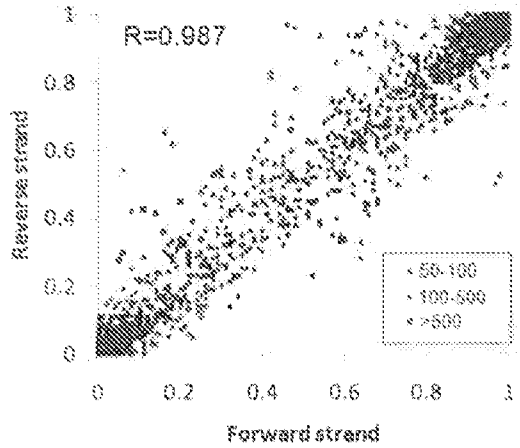
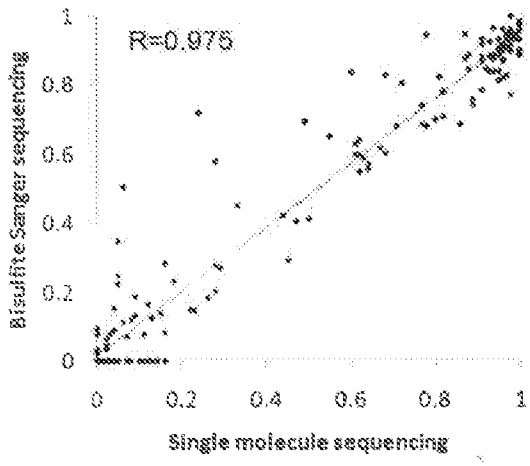

a
No. reads/target
Ranked order
30 k
10 k
10 k
5 k
Three subsets were resynthesized
Four capturing reactions were performed
Amplicons were pooled at equal molar ratio for sequencing library construction
b
Suppressor oligo strategy
Padlock probe
Target
Padlock probe
Suppressor oligos
Linear extended probes
c
Fraction of probes
Normalized abundance
CpG 30 k (1 set)
CpG 30 k (4 sets)
d
Four subsets
One set

FIGURE 7

A


R=0.987
Reverse strand
Forward strand
50-100
100-500
>500
0
0.2
0.4
0.6
0.8
1

B


R=0.975
Bisulfite Sanger sequencing
Single molecule sequencing
0
0.2
0.4
0.6
0.8
1

C

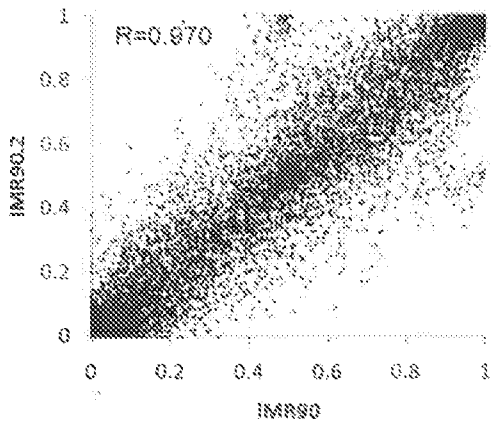

R=0.970
IMR90.2
IMR90
0
0.2
0.4
0.6
0.8
1

FIGURE 8

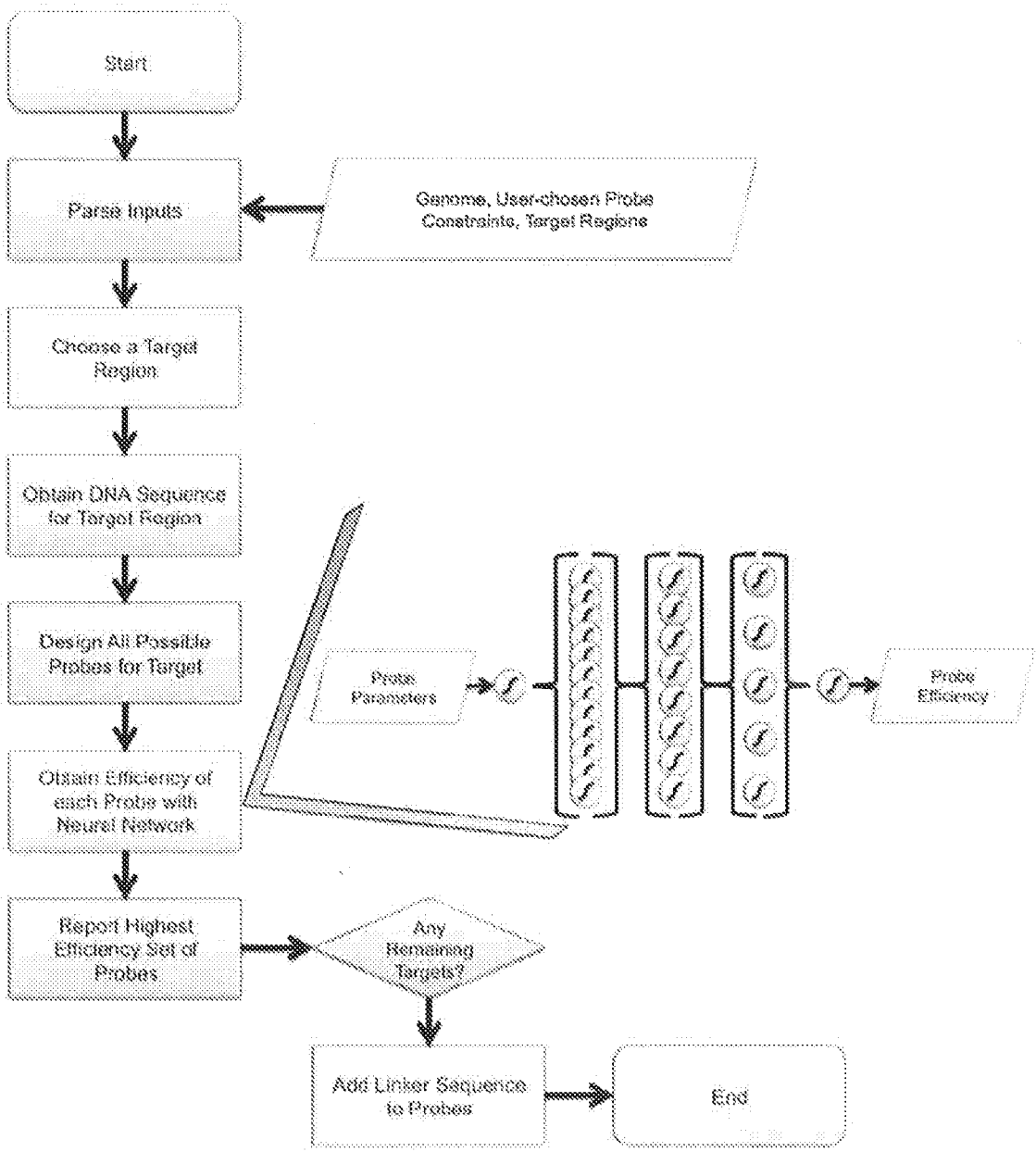

Start
Parse Inputs
Genome, User-chosen Probe Constraints, Target Regions
Choose a Target Region
Obtain DNA Sequence for Target Region
Design All Possible Probes for Target
Obtain Efficiency of each Probe with Neural Network
Probe Parameters
Probe Efficiency
Report Highest Efficiency Set of Probes
Any Remaining Targets?
Add Linker Sequence to Probes
End

FIGURE 9

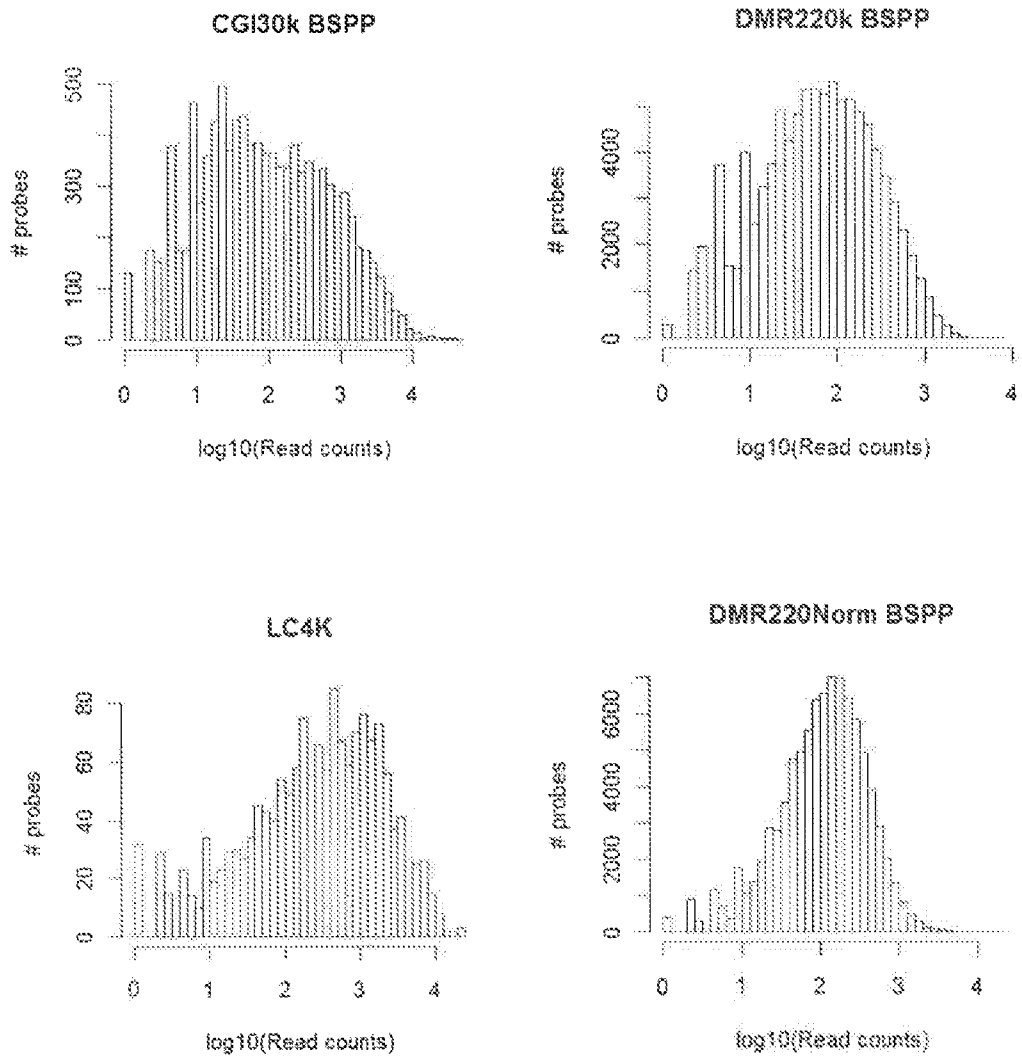

CGI30k BSPP
probes
0
100
300
500
0
1
2
3
4
log10(Read counts)
DMR220k BSPP
probes
0
2000
4000
0
1
2
3
4
log10(Read counts)
LC4K
probes
0
20
40
60
80
0
1
2
3
4
log10(Read counts)
DMR220Norm BSPP
probes
0
2000
4000
6000
0
1
2
3
4
log10(Read counts)

FIGURE 10

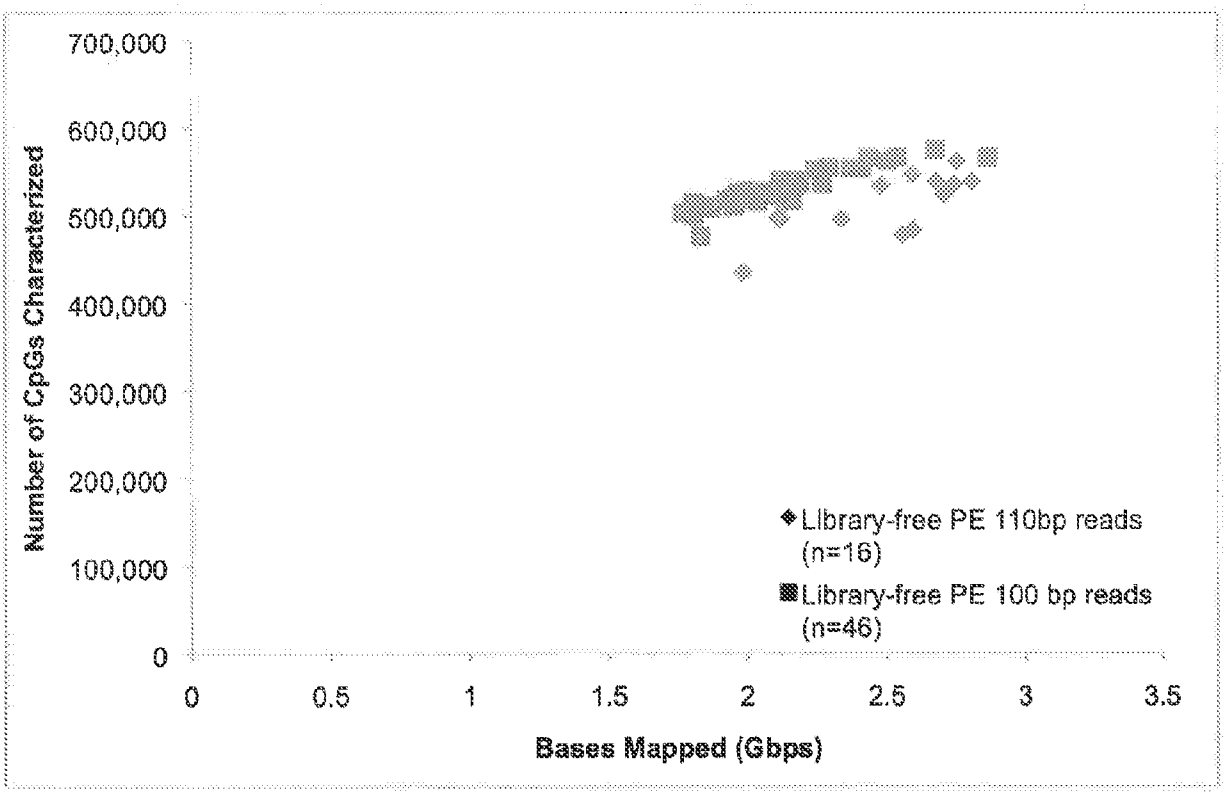

700,000
600,000
500,000
400,000
300,000
200,000
100,000
0
Number of CpGs Characterized
0
0.5
1
1.5
2
2.5
3
3.5
Bases Mapped (Gbps)
Library-free PE 110bp reads (n=16)
Library-free PE 100 bp reads (n=46)

FIGURE 11

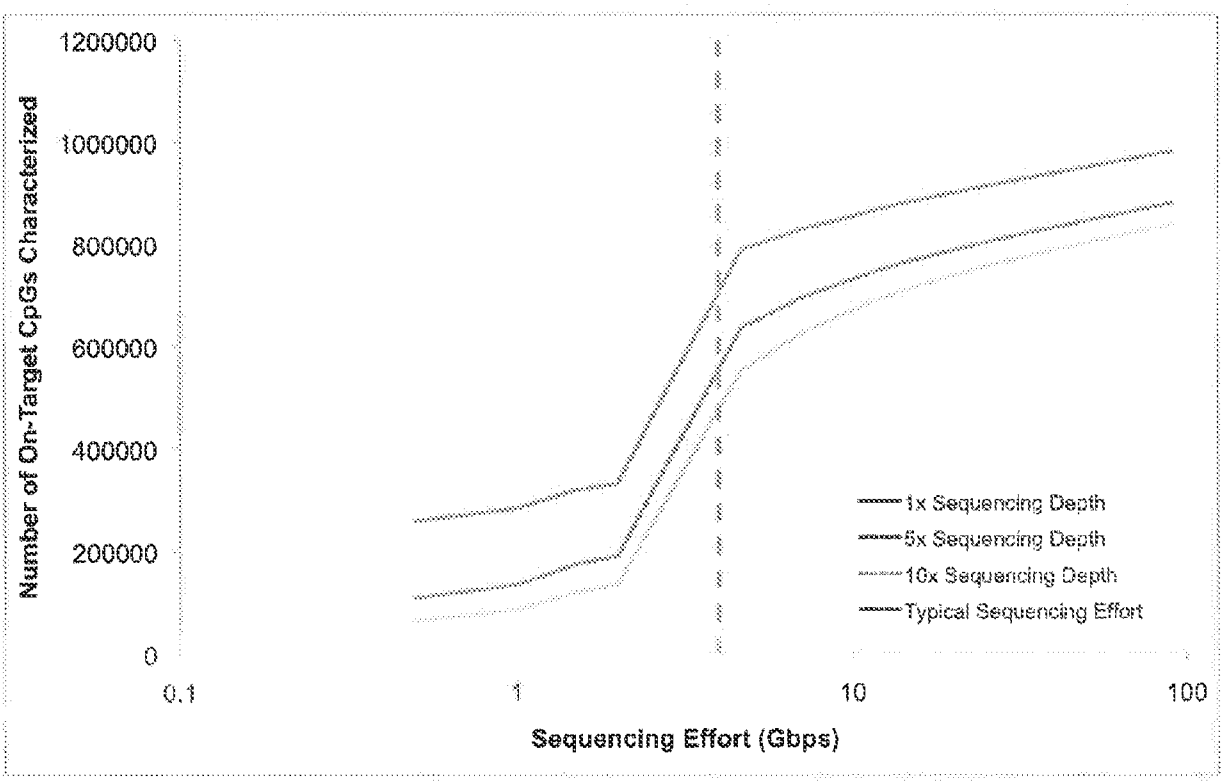

Number of On-Target CpGs Characterized
1200000
1000000
800000
600000
400000
200000
0
0.1
1
10
100
Sequencing Effort (Gbps)
1x Sequencing Depth
5x Sequencing Depth
10x Sequencing Depth
Typical Sequencing Effort

FIGURE 12

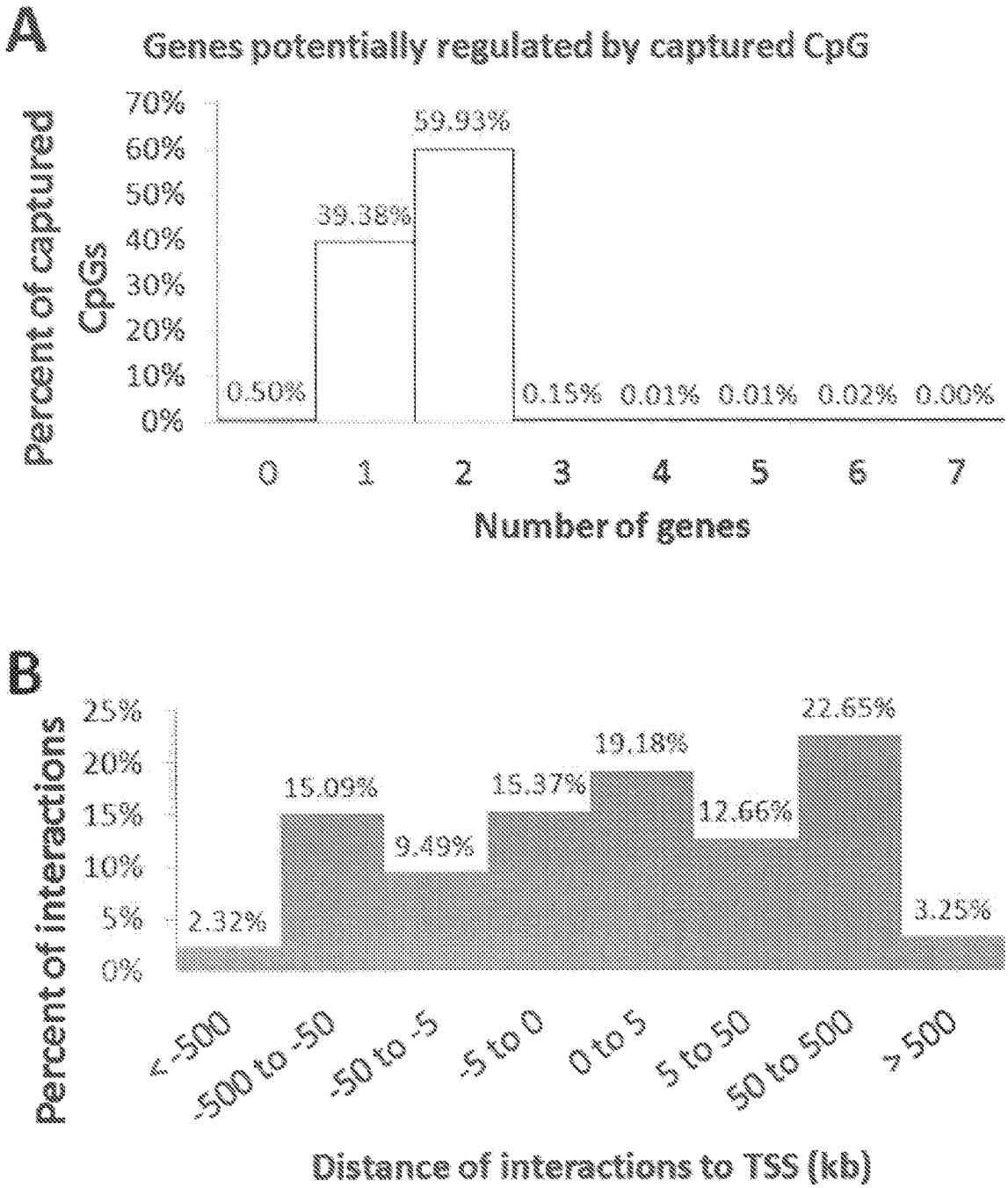

A
Genes potentially regulated by captured CpG
Percent of captured CpGs
70%
60%
50%
40%
30%
20%
10%
0%
0.50%
39.38%
59.93%
0.15%
0.01%
0.01%
0.02%
0.00%
0
1
2
3
4
5
6
7
Number of genes
B
Percent of interactions
25%
20%
15%
10%
5%
0%
2.32%
15.09%
9.49%
15.37%
19.18%
12.66%
22.65%
3.25%
< -500
-500 to -50
-50 to -5
-5 to 0
0 to 5
5 to 50
50 to 500
> 500
Distance of interactions to TSS (kb)

FIGURE 13

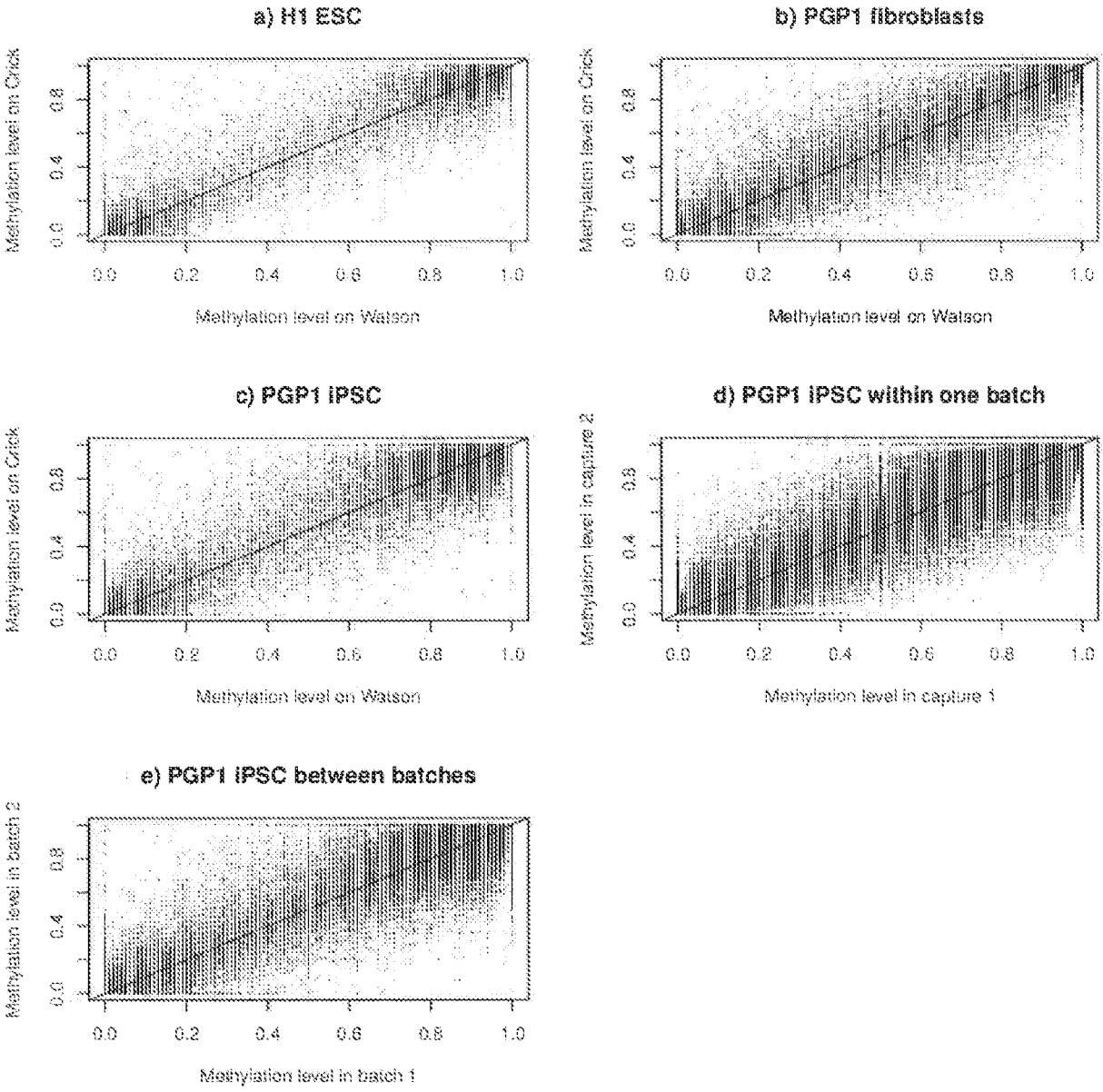

a) H1 ESC
Methylation level on Crick
Methylation level on Watson
b) PGP1 fibroblasts
Methylation level on Crick
Methylation level on Watson
c) PGP1 iPSC
Methylation level on Crick
Methylation level on Watson
d) PGP1 iPSC within one batch
Methylation level in capture 2
Methylation level in capture 1
e) PGP1 iPSC between batches
Methylation level in batch 2
Methylation level in batch 1
0.0
0.2
0.4
0.6
0.8
1.0

FIGURE 14

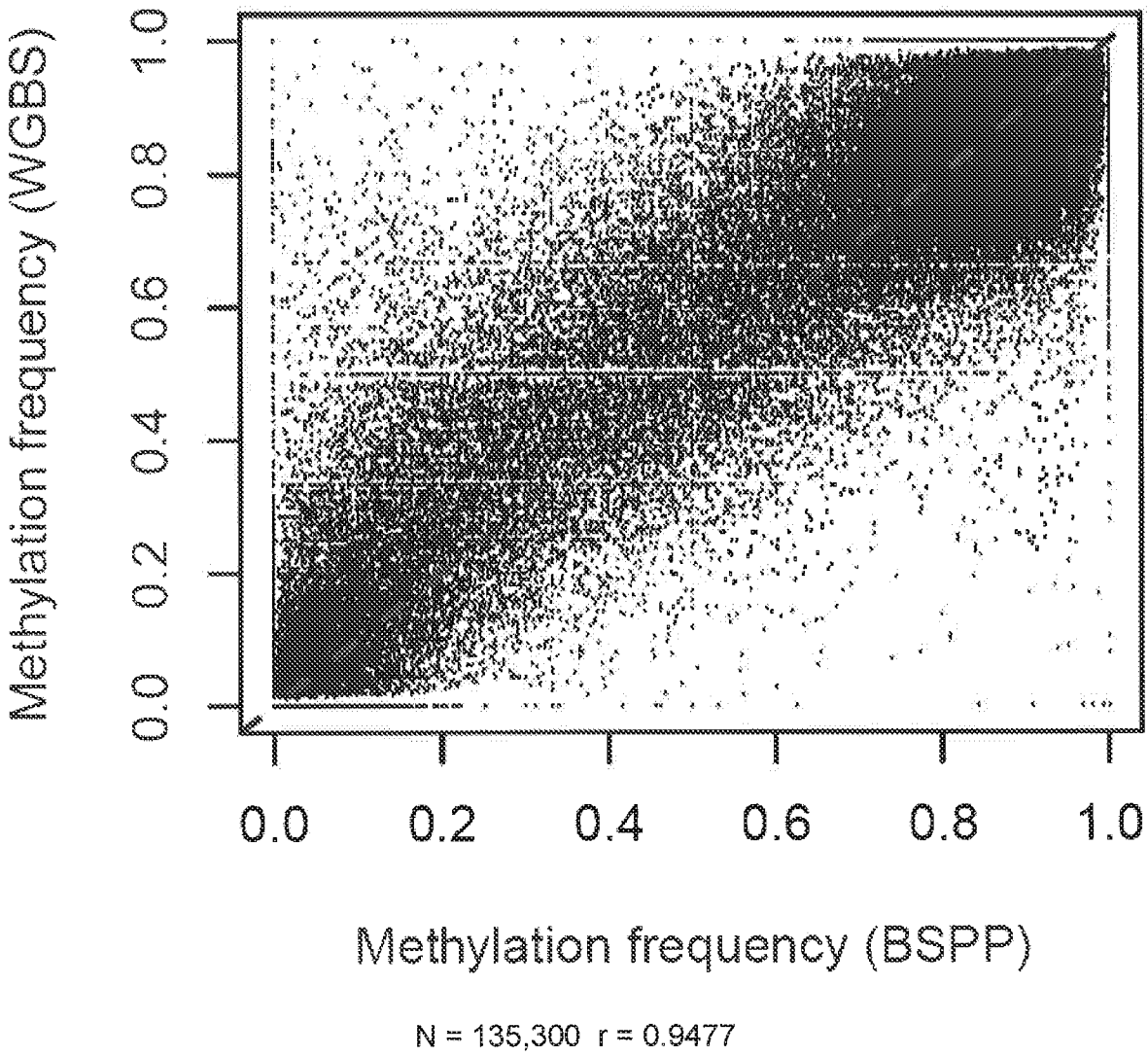

Methylation frequency (WGBS)
0.0
0.2
0.4
0.6
0.8
1.0
0.0
0.2
0.4
0.6
0.8
1.0
Methylation frequency (BSPP)
N = 135,300 r = 0.9477

FIGURE 15

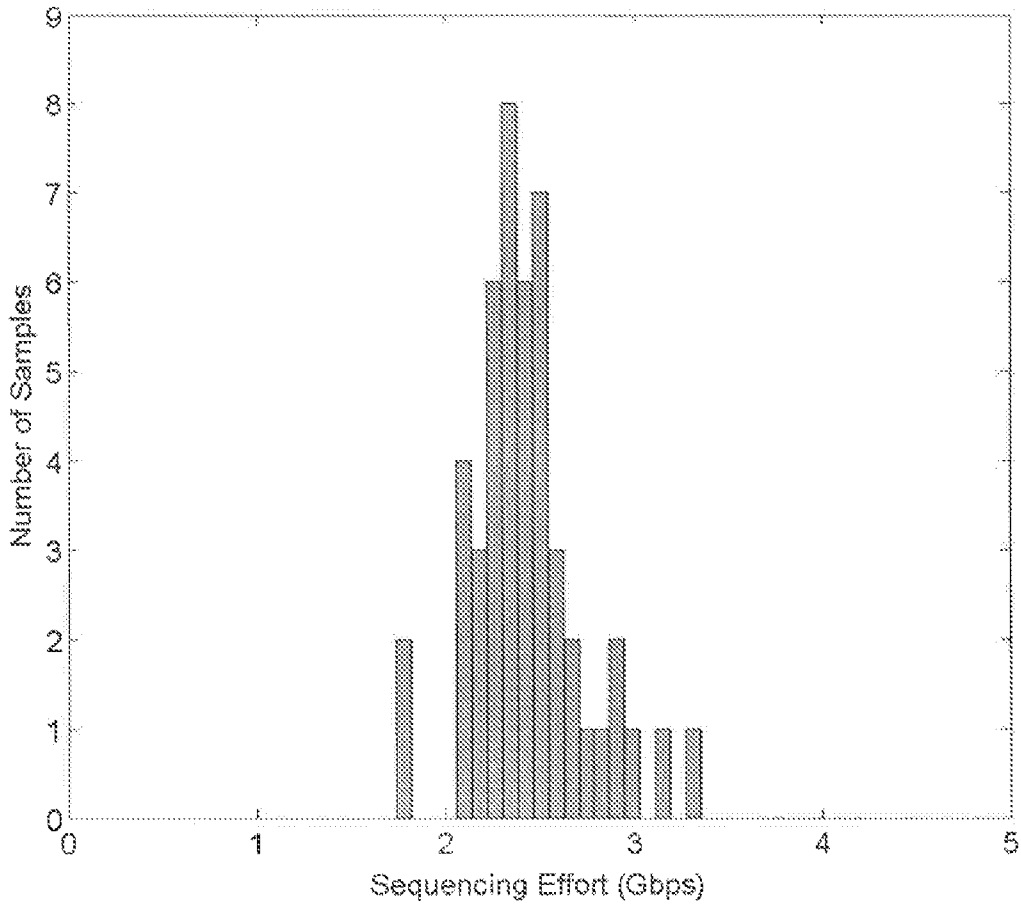


FIGURE 16

A. Agilent Padlock Probe
Amplification Primer
Ligation Arm
Linker Sequence
Extension Arm
Amplification Primer
B. LC Sciences Padlock Probe
Amplification Primer
Ligation Arm
Linker Sequence
Extension Arm
Amplification Primer

FIGURE 17

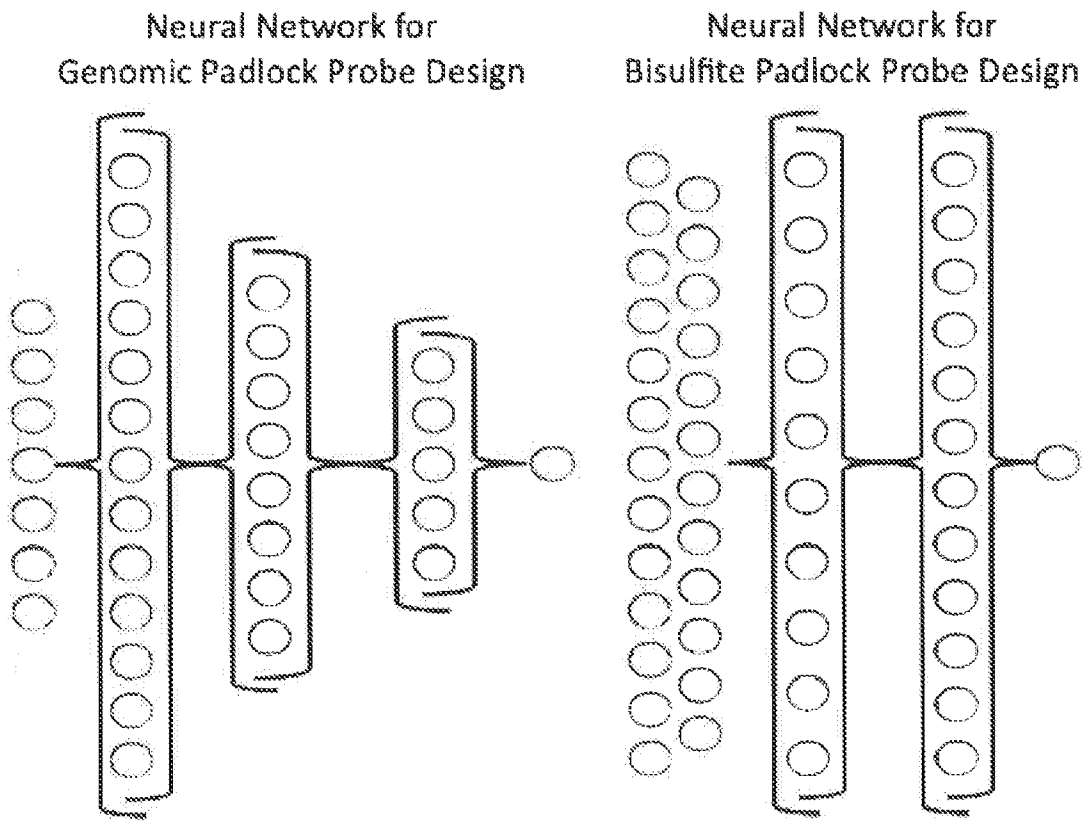

Neural Network for
Genomic Padlock Probe Design
Neural Network for
Bisulfite Padlock Probe Design

FIGURE 18

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2012/035227

A. CLASSIFICATION OF SUBJECT MATTER

C12Q 1/68 *(2006.01)*
C12N 15/00 *(2006.01)*
G01N 33/50 *(2006.01)*

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12Q 1/68, C12N 15/00, G01N 33/50

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

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

CIPO, DEPATISnet, DWPI, EAPATIS, EMBL, EPO-Internal, Esp@ce, Esp@cenet, Google, KIPRIS, PAJ, PubMed, RUPTO, ScienceDirect, SIPO, USPTO, WIPO, VINITI, NCBI, PatSearch, BD neopublikovannie zayavki, BD neopublikovannie zayavki RF, BD pateninie dokumenti SNG, BD patentnie dokumenti SNG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	RU 2318875 C1 (FEDERALNOE GOSUDARSTVENNOE UCHREZHDENIE NAUKI "GOSUDARSTVENNII NAUCHNII TSENTR VIRUSOLOGII I BIOTEKHNOLOGII "VEKTOR" FEDERALNOI CLUZHBI PO NADZORU V SFERE ZASCHITI PRAV POTREBITELEI I BLAGOPOLUCHIYA CHELOVEKA, FGUN GNTS VB "VEKTOR" ROSPOTREBNADZORA) 10.03.2008, p. 3, 4, claims 2, 3	1-32
A	EP 1813682 A1 (SYSMEX CORPORATION) 01.08.2007	1-32

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
09 July 2012 (09.07.2012)	23 August 2012 (23.08.2012)
Name and mailing address of the ISA/ FIPS Russia, 123995, Moscow, G-59, GSP-5, Berezhkovskaya nab., 30-1 Facsimile No. +7 (499) 243-33-37	Authorized officer O. Kolontaevskaya Telephone No. (495)531-65-15

Form PCT/ISA/210 (second sheet) (July 2009)