Library-free methylation sequencing with bisulfite padlock probes

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Targeted quantification of DNA methylation allows for interrogation of the most informative loci across many samples quickly and cost-effectively. Here we report improved bisulfite padlock probes (BSPPs) with a design algorithm to generate efficient padlock probes, a library-free protocol that dramatically reduces sample-preparation cost and time and is compatible with automation, and an efficient bioinformatics pipeline to accurately obtain both methylation levels and genotypes from sequencing of bisulfite-converted DNA.

We have previously developed bisulfite padlock probes for the specific and parallel digital quantification of DNA methylation1. Recently, we enhanced BSPPs for improved flexibility and multiplexing capability. These improvements have contributed to recent findings in mouse and human pluripotent stem cells2–5.

First, target selection and probe design is crucial for BSPPs. To aid in the design of efficient padlock probes for bisulfite analysis, we developed a program called ppDesigner. It accepts as input the genome of any organism, a user’s list of arbitrary targets and user-desired probe constraints matching requirements of the experimental protocol. It in silico ‘bisulfite-converts’ the genome (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 (Supplementary Fig. 1). We had previously trained this network using data from probes for exomic targets6 based on seven properties. Using bisulfite capture data from the first BSPPs1, we refined the network 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 sapiens2,3, Mus musculus4 and Drosophila melanogaster5.

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’ protocol8 to multiplexed BSPP capture (Fig. 1). This method eliminates five steps from Illumina’s library-construction protocol such that multiplexed libraries can be generated from DNA in only four steps (Supplementary Fig. 1). Using multiplexed primers with 6–base pair (bp) barcodes, we have routinely generated libraries for 96 samples in 96-well plates and sequenced all at once in a single Illumina HiSeq flowcell. Additionally, we designed barcodes to process 384 samples per batch. 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 (Supplementary Tables 1 and 2). 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.

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, we developed an analysis pipeline for read mapping and methylation quantification, called bisReadMapper (Supplementary Fig. 2). In previous padlock probe studies, reads had been mapped only against target regions owing to the computational requirements of sequence alignment1. In contrast, we designed bisReadMapper 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 methylation9.

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but also allows accurate sample tracking in large-scale experiments. Finally, bisReadMapper can call methylation levels at both CpG and non-CpG sites.

To test our assay, we generated a genome-scale probe set based on our previous results and new information about differential methylation.1,10–12 We targeted our new design for evaluation of methylation at genomic locations known to contain differentially methylated regions or differentially methylated sites (DMSs).10–13, transcriptional repressor CTCF binding sites and DNase I–hypersensitive regions. We also targeted all microRNA genes and all promoters for human US National Center for Biotechnology Information reference sequence (RefSeq) genes. Using ppDesigner, we designed ~330,000 padlock probes that covered 140,749 non-overlapping regions with a total size of 34 megabases. We performed capturing experiments and end-sequencing, and found that these probes were slightly more specific (~96% on-target) and uniform than previous probes1 (Supplementary Fig. 3). To improve uniformity, we normalized the experimental capturing performance of these probes using subsetting and suppressor oligonucleotides as described previously.1 We could characterize roughly 500,000 CpG sites with ~4 gigabases of sequencing reads, and additional sites became callable with deeper sequencing (Supplementary Fig. 4 and 5).

We used these probes 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, we sequenced on average ~3.66 gigabases 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, we used the genomic regions enrichment of annotations tool14 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 (Supplementary Fig. 6).

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 $R = 0.97–0.98$, Supplementary Fig. 7a,b). 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 DNA $^{12}$ (Pearson’s correlation coefficient $R = 0.95$, Supplementary Fig. 8).

Our assay has very low technical variability. We performed the assay on over 150 samples in 96-well plates; the yield for each was similar (Supplementary Fig. 9). 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 (Supplementary Fig. 7c–e). 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 us to characterize roughly 20,000 SNPs for each sample with an accuracy of 96% or better. This allowed us to unambiguously track samples, which is crucial for projects involving large sample sizes.

Our library-free BSPP method is flexible for different study designs. Whereas our genome-scale probe set allows global profiling on thousands of samples, a focused assay is often 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, we designed a second set of 3,918 probes 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.15 We acquired the oligonucleotides from a second vendor (LC Sciences). Even with shorter capturing sequences (40 bp total for capturing arms rather than 50 bp on average, Supplementary Fig. 10) 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) we identified regions of aberrant methylation in induced pluripotent stem cells (Supplementary Fig. 11) and demonstrated that aberrant methylation continues further upstream and downstream than observed previously. 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.

This method can be implemented to aid in identifying the effects of DNA methylation in any organism by using the computational tools at http://genome-tech.ucsd.edu/public/Gen2_BSPP/.

METHODS
Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemethods/.

Note: Supplementary information is available on the Nature Methods website.

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AUTHOR CONTRIBUTIONS
K.Z. oversaw the project. D.D. and N.P. performed experiments and bioinformatic analyses. A.G. developed a probe design algorithm. R.S. and A.G. designed probes. H.-L.F. performed sequencing. D.D., N.P., A.G. and K.Z. wrote the manuscript.

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The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemethods/.

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ONLINE METHODS

Probe design. Probe design and read mapping algorithms, probe sequences and additional information are available at [http://genome-tech.ucsd.edu/public/Gen2_BSPP/](http://genome-tech.ucsd.edu/public/Gen2_BSPP/). A schematic for the padlock probes is illustrated in Supplementary Figure 10.

Bisulfite padlock probe production (oligonucleotides from Agilent). Libraries of oligonucleotides (~150 nucleotides (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 (Supplementary Table 3), and 50 µl of KAPA SYBG fast Universal 2x qPCR Master Mix (Kapabiosystems) at 95 °C for 30 s, 15–16 cycles of 95 °C for 3 s; 55 °C for 30 s; and 72 °C for 30 s, 5 cycles of 98 °C for 10 s; 54 °C for 30 s; and 72 °C for 30 s and 72 °C for 3 min. The resultant amplicons were digested with 100 U of Nb.BrsDI (10 U µl⁻¹, NEB) at 65 °C for 1 h. 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).

Capture-circles amplification (library-free BSPP protocol, Agilent oligonucleotides). Ten microliters of circularized DNA was amplified and barcoded in 100-µl reactions with 400 nM each of AmpF6.3Sol primer (Supplementary Table 3) and AmpR6.3 indexing primer (Supplementary Table 3), 0.4× SYBR Green I (Invitrogen) and 50 µl Phusion High-Fidelity 2x master mix (NEB) at 98 °C for 30 s, 5 cycles of 98 °C for 10 s; 52 °C for 30 s and 72 °C for 30 s, 15 cycles of 98 °C for 10 s; 60 °C for 30 s; and 72 °C for 30 s and 72 °C for 3 min. 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.

Capture-circles amplification (library-free BSPP protocol, LC Sciences oligonucleotides). 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 (Supplementary Table 3) and 50 µl KAPA SYBR fast Universal qPCR Master Mix at 98 °C for 30 s, 5 cycles of 98 °C for 10 s; 52 °C for 30 s and 72 °C for 30 s, 15 cycles of 98 °C for 10 s; 60 °C for 30 s; and 72 °C for 30 s and 72 °C for 3 min. 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 s, 4 cycles of 98 °C for 10 s; 54 °C for 30 s; and 72 °C for 30 s and 72 °C for 3 min. The primer barcode design for multiplexing. 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 (Supplementary Table 4).

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Bisulfite read mapping and data analysis. Bisulfite-converted data were processed as previously described. 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 (http://soap.genomics.org.cn/soapaligner.html) 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 (http://samtools.sourceforge.net/). Raw SNPs and methylation frequency files were computed from pileup counts. Methylation frequencies were called using a method described previously\(^1\).

Correlation of methylation between two samples. 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.

Analysis of methylation. 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 difference. Technical replicates did not show any differential methylation, and different cell types showed a large extent of differential methylation (~33%).