Research Square

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Reduced Representation Nanopore Sequencing: A Cost-Effective Workflow for Accurate and High-Throughput Targeted DNA Methylation Profiling

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Abstract

Background

Conventional DNA methylation analysis methods rely on bisulfite conversion, which cannot distinguish between 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC), while nanopore-based whole-genome methylation analysis remains limited by the substantial sequencing throughput required, resulting in high per-sample costs.

Results

Here, we developed Reduced Representation Nanopore Sequencing (RRNS), a cost-effective workflow for accurate and high-throughput targeted DNA methylation profiling. RRNS enriches CpG-rich genomic regions through digestion with the restriction enzyme MspI and combines multiplex nanopore sequencing with direct detection of DNA modifications. By reducing the amount of sequencing required through targeted enrichment of CpG-rich regions, RRNS substantially lowers per-sample sequencing costs while maintaining accurate methylation profiling. Compared with the DNA methylation array, RRNS demonstrated high concordance (Pearson's r = 0.91–0.92) at shared CpG sites at a coverage threshold of > 10 and additionally detected numerous CpG sites not represented on the array.

Conclusions

These results demonstrate that RRNS provides an accurate and cost-effective approach for targeted DNA methylation profiling and expands the practical utility of nanopore-based DNA methylation analysis across a broad range of biological and biomedical research.

Background

DNA methylation is a fundamental epigenetic modification that plays important roles in gene regulation and is widely used as a biomarker in cancer, aging, and many other biological and clinical studies [1, 2]. Whole-genome bisulfite sequencing (WGBS) [3], reduced representation bisulfite sequencing (RRBS) [4, 5], and DNA methylation arrays [6–8] are commonly used methods for genome-scale DNA methylation analysis [9]. Although these methods have become standard approaches for methylation profiling, they remain relatively expensive on a per-sample basis, limiting their application in large-scale studies.

A major limitation of these methods is their reliance on bisulfite conversion [10], which cannot distinguish between 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) [11]. As a result, both DNA modifications are interpreted as methylated cytosines. Because 5hmC has distinct biological functions from 5mC, the inability to differentiate between these modifications may compromise the accuracy of methylation analyses, particularly in studies of aging and disease [11]. Nanopore

sequencing offers an attractive alternative because it directly detects DNA modifications from electrical current signals generated as DNA molecules pass through nanopores, eliminating the need for bisulfite conversion [12]. This enables discrimination between 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) while preserving native DNA.

Recent advances in nanopore sequencing technology and methylation-calling methods have established nanopore sequencing as a reliable platform for DNA methylation analysis [12–14]. Previous studies have demonstrated high concordance between nanopore-based methylation profiling and conventional methylation profiling methods, including whole-genome bisulfite sequencing (WGBS), reduced representation bisulfite sequencing (RRBS), and DNA methylation arrays [15–18]. In addition to accurate methylation quantification, nanopore sequencing enables direct analysis of native DNA without bisulfite conversion and has been increasingly applied to diverse areas of epigenomic research, including cancer biology, developmental biology, genomic imprinting, and clinical epigenetics [19]. Together, these advances indicate that nanopore sequencing has become a practical platform for DNA methylation analysis in both basic and translational research [19].

Despite these advances, whole-genome methylation analysis using nanopore sequencing remains relatively expensive because it requires substantial sequencing throughput, particularly for large mammalian genomes such as those of humans and mice. Therefore, there is a need for a cost-effective methylation profiling method that combines the ability to distinguish DNA modifications with sufficient throughput for routine biological studies. Here, we present Reduced Representation Nanopore Sequencing (RRNS), a targeted DNA methylation analysis workflow designed to balance analytical accuracy, cost-effectiveness, and throughput. RRNS enriches CpG-rich genomic regions using restriction enzyme digestion, followed by multiplex nanopore sequencing through sample barcoding. We evaluated the performance of RRNS by comparison with a DNA methylation array and assessed its accuracy, reproducibility, genomic coverage, and potential utility for targeted DNA methylation profiling.

Methods

Animals and ethical approval

Wild-type C57BL/6J male mice were obtained from Sankyo Labo Service Corporation, INC. (Edogawa-ku, Tokyo, Japan) and originally bred by Japan SLC, Inc. (Hamamatsu City, Shizuoka, Japan). All animal experiments were approved by the Keio University Institutional Animal Care and Use Committee (approval number: A2022-105). Mice were deeply anesthetized with isoflurane, and death was induced by diaphragm incision followed by perfusion prior to organ collection.

Establishment of intestinal epithelial organoids

Isolation and dissociation of stem cells from the normal intestinal epithelium of wild-type C57BL/6J male mice were performed as previously described [20]. Isolated epithelial cells were embedded in

Matrigel on ice (growth factor-reduced, phenol red-free; Corning, Corning, NY, USA) and seeded in 48-well plates. The Matrigel was polymerized for 10 min at 37°C, and overlaid with 250 µL/well basal culture medium (advanced Dulbecco's modified Eagle medium/F12 supplemented with penicillin/streptomycin, 10 mmol/L HEPES, Glutamax, 1 × N2, 1 × B27 [all from Thermo Fisher Scientific, Waltham, MA, USA], and 1 mmol/L N-acetylcysteine [Sigma-Aldrich, St. Louis, MO, USA]) containing the following optimized growth factor combinations: murine epidermal growth factor (Thermo Fisher Scientific), recombinant murine noggin (Peprotech, Rocky Hill, New Jersey, USA), Y-27632 (Selleck, Yokohama, Kanagawa, Japan), and R-spondin 1. Organoids were established from two male C57BL/6J mice, aged 13 (mouse 1) and 111 weeks (mouse 2). The establishment of intestinal epithelial organoids was deemed successful when cultures were maintained for over five passages.

DNA extraction

Passaged organoids were harvested using Cell Recovery Solution (Corning). After addition of the solution to each well, the plate was incubated on ice for 1 h. Organoids were centrifuged for 2 min, the supernatant was removed, and DNA was extracted using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions.

MspI digestion and size selection

The extracted DNA was digested with the restriction enzyme MspI (New England Biolabs Japan, Sumida-ku, Tokyo, Japan) at 37°C for 1 hour, according to the manufacturer's instructions. The restriction-enzyme-treated DNA was concentrated by ethanol precipitation, electrophoresed on a 1% agarose gel, and DNA fragments ranging in size from 40 to 300 bp were extracted from the gel using NucleoSpin Gel and PCR Clean-up (MACHEREY-NAGEL, 52355 Düren, Germany). DNA fragments of 40–300 bp were size-selected to enrich CpG-rich genomic regions generated by MspI digestion, following the principle of conventional RRBS[4, 5].

Library Preparation and Nanopore Sequencing

Purified MspI-digested DNA was used for library preparation using the Native Barcoding Kit 96 V14 (SQK-NBD114.96) (Oxford Nanopore Technologies, Oxford, OX4 4DQ, United Kingdom). Library preparation was performed according to the manufacturer's instructions, except that AMPure XP Beads (AXP) were used in a concentration of 1.2x for clean-up after barcode ligation and 0.9x for clean-up after adapter ligation. The prepared library was sequenced using the FLO-MIN114 (Oxford Nanopore Technologies) according to the manufacturer's instructions. MinKNOW (version 25.09.16) was used as the sequencing software.

DNA Methylation Array

DNA methylation profiling was performed in RIKEN Genesis Co., Ltd. (Kawasaki City, Kanagawa, Japan) using Infinium Mouse Methylation Beadchip kit (Illumina, San Diego, CA 92122, USA).

Nanopore Sequencing and DNA Methylation Array Analysis

Basecalling and modified base detection were performed using Dorado (v1.4.0) [21] with the HAC and 5mCG_5hmCG models on POD5 files generated by the nanopore sequencer using a workstation equipped with an AMD Ryzen Threadripper 9970X CPU (32 cores/64 threads), 128 GB RAM, and an NVIDIA RTX PRO 2000 Blackwell GPU (16 GB VRAM). The reads obtained were aligned to the mouse reference genome (GRCm39) using the Dorado aligner. Modified base calls were processed using modkit (v0.6.1) [22]. For the present study, only 5mC calls were extracted for downstream analyses. Markers located outside the standard chromosomes (autosomes, sex chromosomes, and the mitochondrial genome) were excluded from the analysis. Additionally, sites where 65,535 reads—the maximum depth supported by modkit—were observed were also excluded. The methylation β value at each CpG site was calculated as the proportion of methylated reads among all reads covering the site.

To validate the nanopore data, DNA methylation array data were obtained from intestinal organoids derived from the same individual. Four technical replicates were obtained for both mouse 1 and mouse 2. Probes with no missing values across all samples were used for analysis, and the β values for each methylation site on the array were calculated by taking the average for each unique methylation site. For each individual, the average of the four replicate β values was taken as the consensus value on the DNA methylation array. A total of 261,625 probes were analyzed. The probe manifest file provided by Illumina was converted to coordinates on GRCm39 using UCSC liftOver (version 357). Subsequently, based on the combination of chromosome number and coordinates, the methylation sites obtained from the nanopore data were matched to the probes on the DNA methylation array. For each unique Illumina probe ID, the numbers of methylated and unmethylated nanopore reads were tallied to calculate nanopore-derived β values, which were subsequently compared with DNA methylation array-derived β values.

All downstream analyses, including CpG site filtering, methylation β value calculation, and comparison between nanopore-derived and array-derived methylation values, were performed using custom scripts developed for this study and available on GitHub (https://github.com/DaigoOkada/RRNS).

Results

Sequencing Overview of RRNS

The RRNS workflow consisted of genomic DNA extraction, MspI digestion, size selection, library preparation, nanopore sequencing, and downstream methylation analysis (Fig. 1A).

A single nanopore flow cell operated for 61 h 9 min until flow cell end-of-life generated 15.08 Gb comprising 33.73 million reads, with an estimated read N50 of 411 bp (Table 1A). The estimated read-length distribution peaked at approximately 0.24–0.37 kb, with most reads ranging from approximately 0.11 to 0.75 kb (Fig. 1B), consistent with the expected fragment size after size selection (40–300 bp) together with barcode and adapter ligation (combined length of approximately 110 bp).

At a coverage threshold of > 50, RRNS detected 2,782 CpG sites (Table 1B). These high-confidence CpG sites were distributed across most chromosomes but were absent from chromosomes 7, 10, 19, and Y (Fig. 1C), indicating that high-coverage CpG sites were concentrated within a subset of genomic regions.

Table 1
A

Estimated bases	**15.08 Gb**
Reads generated	33.73 M
Estimated N50	411 bp
Run time	61 hours and 9 minutes
Run status	Flow Cell End of Life

The total output result from a single flow cell, which multiplexed both the mouse 1 and mouse 2 individual libraries.

Table 1
B

Chromosome types	Number of detected sites		Proportion of detected sites (Coverage > 50) (%)
	Coverage > 0	Coverage > 50	
chr1	509,400	12	0.4
chr2	581,535	167	6.0
chr3	391,695	46	1.7
chr4	555,837	11	0.4
chr5	557,006	25	0.9
chr6	415,779	218	7.8
chr7	503,828	0	0
chr8	464,663	6	0.2
chr9	415,659	933	33.5
chr10	415,333	0	0
chr11	525,169	74	2.7
chr12	351,650	18	0.6
chr13	350,891	10	0.4
chr14	318,245	109	3.9
chr15	342,316	8	0.3
chr16	255,299	70	2.5
chr17	362,402	835	30.0
chr18	256,525	4	0.1
chr19	234,614	0	0
chrX	153,500	9	0.3
chrY	76,771	0	0
chrMT	573	227	8.2

The number of sequenced sites at a coverage threshold of > 0 and a coverage threshold of > 50, as well as the proportion of sequenced sites at a coverage threshold of > 50.

Validation of RRNS by Comparison with the DNA Methylation Array

To evaluate the performance of RRNS, we compared its methylation measurements with those obtained using a DNA methylation array, a widely used platform for DNA methylation profiling. Validation was performed using intestinal organoids derived from two biologically independent mice.

RRNS showed strong agreement with the DNA methylation array across a range of coverage thresholds (Fig. 2A; Table 2A). At a coverage threshold of > 10, the Pearson correlation coefficient was 0.92 (N = 1,385) for mouse 1 and 0.91 (N = 3,023) for mouse 2, indicating high concordance between RRNS-derived and array-derived β values.

At a coverage threshold of > 10, 1,385 CpG sites in mouse 1 and 3,023 CpG sites in mouse 2 overlapped with probes represented on the DNA methylation array (Fig. 2B; Table 2B). These shared CpG sites represented only a small fraction of all CpG sites detected by RRNS (149,752 and 287,710 CpG sites, respectively), indicating that RRNS interrogated numerous CpG sites that were not represented on the DNA methylation array.

Table 2
A

Coverage threshold	Mouse 1			Mouse 2		
	Number of sites	Correlation coefficient	Mean error	Number of sites	Correlation coefficient	Mean error
> 0	119,394	0.75	0.23	110,102	0.75	0.23
> 5	11,181	0.90	0.13	15,178	0.89	0.13
> 10	1,385	0.92	0.12	3,023	0.91	0.12
> 20	27	0.88	0.13	60	0.81	0.17

The Pearson correlation coefficients and mean errors between the array β values and the nanopore β values for different coverage thresholds. Number of sites means the number of CpG sites detected by RRNS, which were also detected by the DNA methylation array.

Table 2
B

Coverage threshold	Number of sites detected in both methods (mouse 1)	Number of sites detected in both methods (mouse 2)
> 0	119,394	110,102
> 5	11,181	15,178
> 10	1,385	3,023
> 20	27	60
> 50	7	9
> 100	5	6
> 500	1	2

Number of sites detected in both methods means the number of CpG sites detected by RRNS, which were also detected by the DNA methylation array.

Discussion

In this study, we developed Reduced Representation Nanopore Sequencing (RRNS), a targeted DNA methylation analysis workflow based on nanopore sequencing, and demonstrated its reproducibility and performance through comparison with a conventional DNA methylation array. Our results indicate that RRNS enables accurate methylation profiling of CpG-rich regions while reducing sequencing cost and increasing throughput, making nanopore-based targeted methylation analysis more practical for routine epigenomic studies.

Recent advances in nanopore sequencing technology and methylation-calling algorithms have established nanopore sequencing as a reliable platform for direct DNA methylation analysis [12–14]. Nevertheless, routine application of nanopore methylation profiling remains challenging because whole-genome sequencing requires substantial sequencing throughput and consequently high consumable costs, particularly for large mammalian genomes. Thus, although the technical performance of nanopore methylation analysis has matured considerably, improving its cost-effectiveness remains an important challenge for large-scale biological and biomedical studies.

To address this practical limitation, we developed RRNS by combining restriction enzyme-based enrichment of CpG-rich regions with nanopore sequencing. Unlike conventional RRBS, which relies on bisulfite conversion [4, 5], RRNS preserves native DNA and retains the ability to directly detect DNA modifications while substantially reducing sequencing requirements. Although long-read sequencing is generally regarded as a major advantage of nanopore technology [13, 23], we intentionally adopted sequencing of short restriction fragments to maximize sequencing efficiency for targeted methylation profiling. This design enabled efficient analysis of CpG-rich regions while substantially reducing

sequencing cost per sample. Furthermore, by adjusting the degree of sample multiplexing according to the required sequencing depth, RRNS provides a flexible balance between cost, throughput, and methylation profiling performance.

Despite sequencing only a reduced representation of the genome, RRNS achieved high concordance with the DNA methylation array, with Pearson correlation coefficients exceeding 0.9 at shared CpG sites, demonstrating that accurate methylation profiling can be achieved using this targeted sequencing strategy. This observation is consistent with previous comparative studies reporting good agreement between nanopore-based and conventional DNA methylation profiling methods [15–18]. Notably, only a subset of the CpG sites detected by RRNS overlapped with probes represented on the DNA methylation array. Nevertheless, methylation levels at these shared CpG sites showed high concordance, whereas RRNS and the DNA methylation array also interrogated distinct sets of CpG sites owing to differences in genomic coverage and measurement strategies. This observation is also consistent with recent comparative studies showing that different methylation profiling platforms interrogate overlapping but distinct sets of CpG sites [15–18]. These findings suggest that RRNS complements existing DNA methylation analysis platforms rather than serving as a direct replacement for array-based methylation analysis.

This study has several limitations. First, RRNS does not provide genome-wide methylation profiling because it is designed to analyze CpG-rich regions generated by restriction enzyme digestion. Second, there is an inherent trade-off between sequencing depth, the number of detectable methylation sites, and cost per sample, depending on the degree of sample multiplexing. Therefore, the optimal number of samples to sequence simultaneously should be determined according to the study objective, sample species, and required methylation coverage. Third, RRNS requires a larger amount of input DNA than conventional DNA methylation analysis methods because it is a PCR-free protocol. Finally, validation was performed using intestinal organoids derived from only two mice. Although the primary objective of this study was technical validation of the workflow rather than assessment of biological variation, further evaluation using diverse tissues and larger sample cohorts will strengthen the general applicability of RRNS.

Despite these limitations, RRNS expands the practical utility of nanopore sequencing for epigenetic research [19]. By combining direct detection of native DNA modifications with reduced sequencing cost and improved throughput, RRNS provides a practical alternative for targeted DNA methylation analysis while complementing existing array-based approaches through analysis of additional CpG sites. RRNS may be particularly valuable for large-scale epigenomic studies, including biomarker discovery, cancer research, and aging research, where sequencing cost remains a major practical limitation.

Conclusions

We developed Reduced Representation Nanopore Sequencing (RRNS), a cost-effective workflow for targeted DNA methylation profiling of CpG-rich regions. RRNS demonstrated high concordance with DNA

methylation arrays at shared CpG sites while also interrogating a complementary set of CpG sites that were not represented on the array. By combining accurate methylation profiling with reduced sequencing cost, improved throughput, and direct detection of native DNA modifications without bisulfite conversion, RRNS provides a practical, scalable, and reliable platform for targeted DNA methylation analysis. This workflow expands the practical utility of nanopore sequencing for routine epigenetic research and may facilitate large-scale biological and biomedical studies in which sequencing cost is a major consideration.

Abbreviations

WGBS
Whole-genome bisulfite sequencing
RRBS
Reduced representation bisulfite sequencing
5mC
5-methylcytosine
5hmC
5-hydroxymethylcytosine
RRNS
Reduced representation nanopore sequencing

Declarations

Ethics approval and consent to participate

The study was approved by the Keio University Institutional Animal Care and Use Committee (approval number: A2022-105).

Consent for publication

Not applicable.

Competing interests

D.O. and Y.S. have received research grants from REGENESOME Inc. The authors declare that they have filed a patent application related to the method described in this manuscript.

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Author Contribution

K.H., and D.O. designed the experiments, performed the experiments and statistical analysis. K.S., M.K., and Y.S. supervised the work. M.I. and A.T. prepared and provided the computational workstation used for the data analysis. K.H. wrote and edited the manuscript. All authors read and approved the final manuscript.

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Not applicable.

Data Availability

The raw nanopore sequencing reads generated during the current study are available in the DDBJ Sequence Read Archive under BioProject accession number PRJDB42866. Processed DNA methylation datasets, including nanopore-derived methylation profiles and DNA methylation array beta values, are available in Zenodo at https://doi.org/10.5281/zenodo.21768504 [24]. The analysis scripts used for processing and analyzing the RRNS data are available at GitHub: https://github.com/DaigoOkada/RRNS.

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Figures

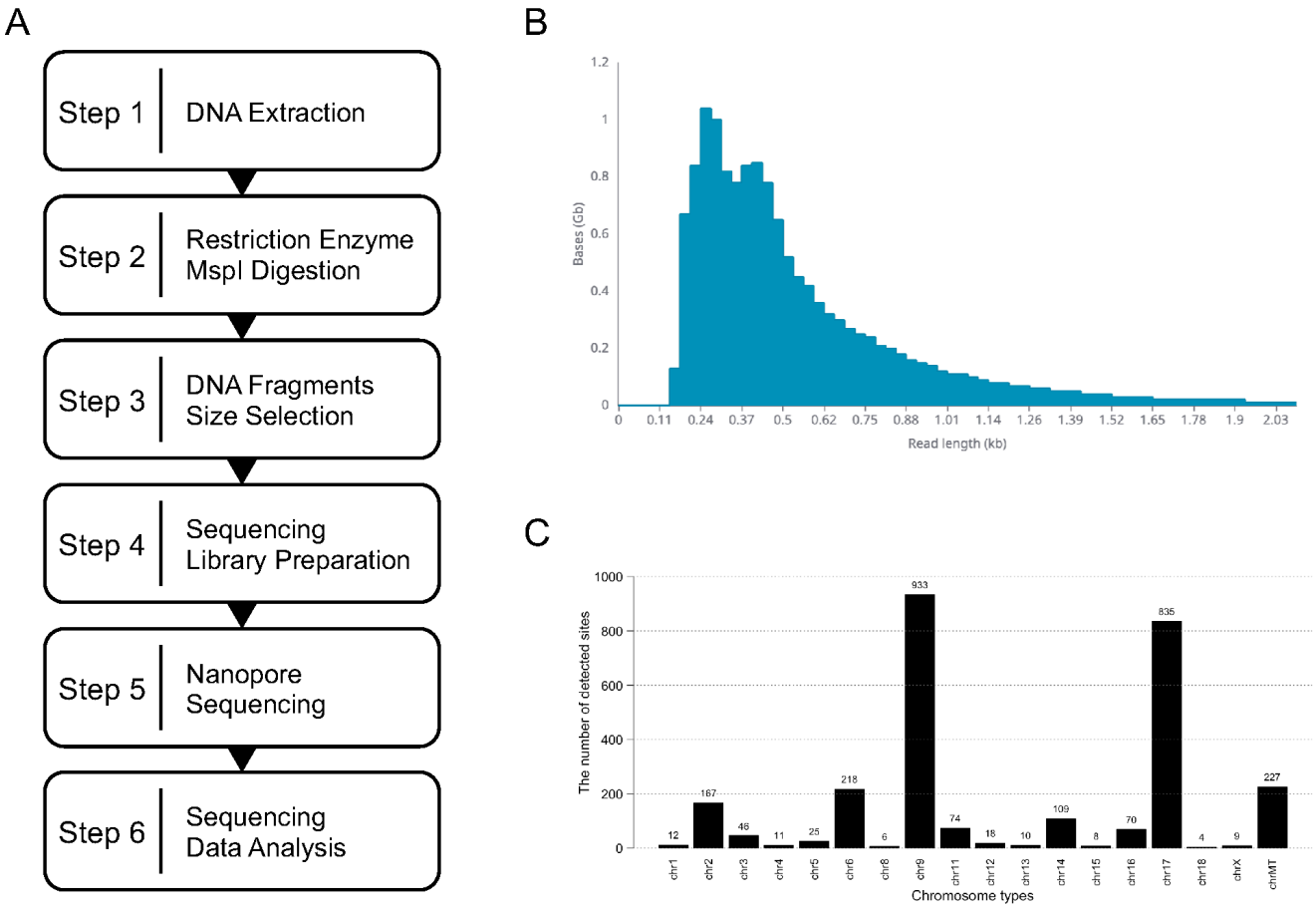


Figure 1

Overview of Reduced Representation Nanopore Sequencing (RRNS).

(A) Schematic overview of the RRNS workflow. Genomic DNA was extracted, digested with MspI, size-selected, subjected to library preparation, sequenced using nanopore sequencing, and analyzed for DNA methylation. (B) Estimated read-length distribution of nanopore sequencing reads. (C) Chromosomal distribution of CpG sites detected at a coverage threshold of > 50.

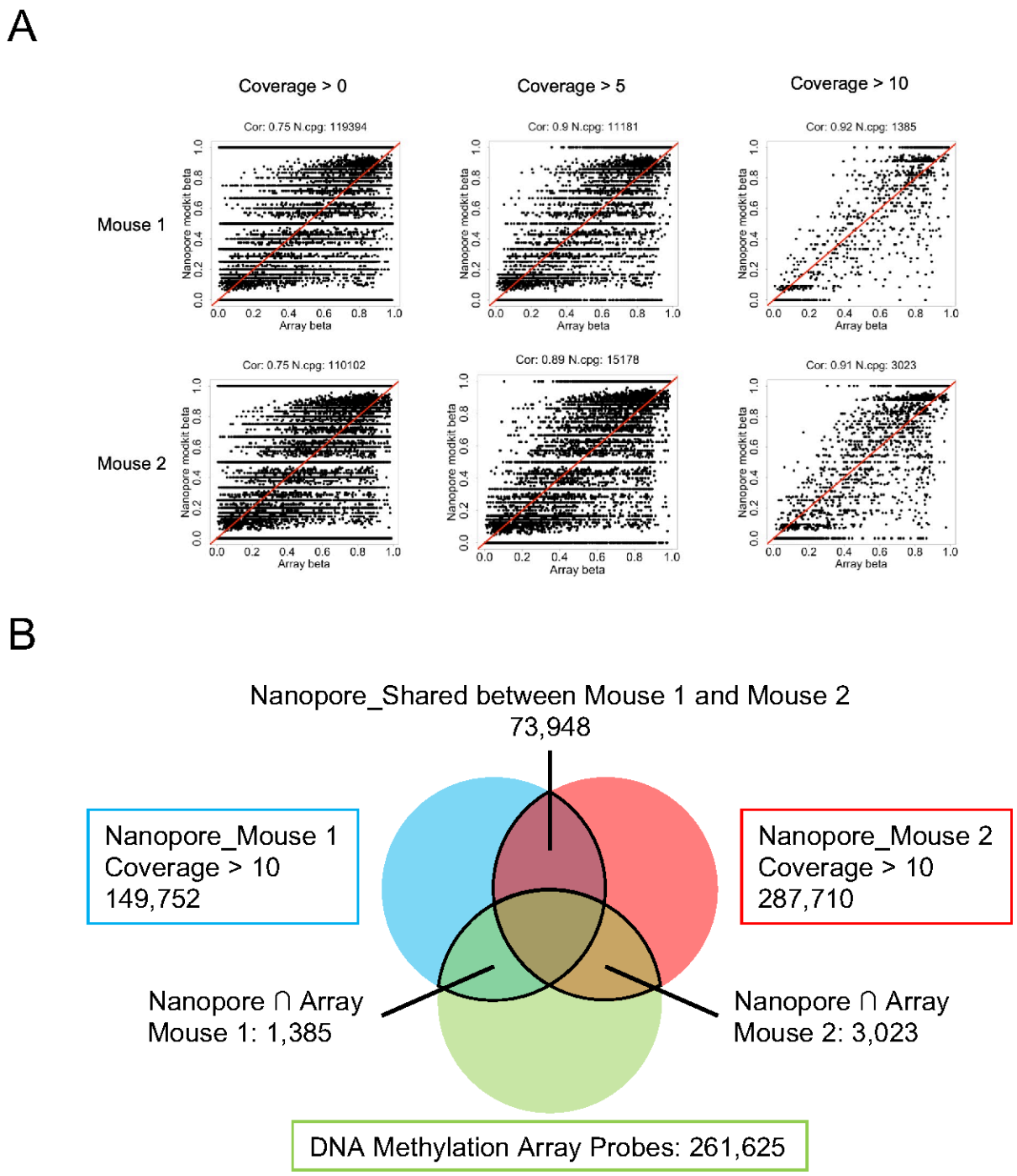


Figure 2

Validation of RRNS by comparison with the DNA methylation array.

(A) Correlation of CpG methylation β values measured by RRNS and the DNA methylation array. Pearson correlation coefficients (Cor) and the numbers of shared CpG sites (N.cpg) are shown. (B) Schematic

illustration of the overlap between CpG sites detected by RRNS and probes represented on the DNA methylation array.