Sparse Sequencing permits accurate and efficient quantification of genome-wide cytosine modification levels

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ABSTRACT

5-methylcytosine (5mC) and 5-hydroxymethycytosine (5hmC) play crucial roles in epigenetic gene regulation, with dynamic levels in development and disease. Current methods for quantifying 5mC and 5hmC levels genome-wide are typically limited in either throughput or accuracy. Using computational down-sampling of deeply-sequenced data sets, we demonstrate that sparse base-resolution sampling (<0.24% of the mouse genome) can precisely measure mammalian genomic 5mC and 5hmC levels (error <5%), even when levels are low (<0.3%). We then demonstrate that Sparse Sequencing (Sparse-Seq) using chemical and enzymatic methods shows high accuracy and less variability than mass spectrometry-based methods in global quantification. The added ability of Sparse-Seq to dissect sequence context permitted resolution of distinct time-courses for the emergence of 5mCpH versus 5hmCpG in developing brains. Sparse-Seq offers a highly accessible and broadly useful approach for quantifying both 5mC and 5hmC levels, allowing for economical and high-throughput analysis of epigenomic dynamics.

INTRODUCTION

5-methylcytosine (5mC) is a critical epigenetic DNA modification in mammalian genomes, most commonly found in cytosine-guanine (CpG) dinucleotide contexts. 5mC is known to typically act as a repressive mark, with key roles in development and differentiation, imprinting, X-chromosome inactivation, and the suppression of mobile genetic elements (1). The discovery of the ten-eleven translocation (TET) family of enzymes has triggered the more recent focus beyond 5mC to include 5-hydroxymethylcytosine (5hmC), a prevalent and previously underappreciated mark in many cell types (2, 3). Oxidation of 5mC by TET enzymes initiates the process of DNA demethylation, whereby the repressive 5mC marks are erased (4, 5). At the same time, the high level and stability of 5hmC in certain cell types have suggested that this epigenetic modification also has its own regulatory roles, which can oppose those of 5mC in many regards (6-8).

In the study of the biological roles for 5mC and 5hmC, significant insights have been gained by localizing and quantifying these DNA modifications (9, 10). Base-resolution sequencing approaches can be critical to discriminating locus-specific roles for 5mC and 5hmC, however, such approaches can be cost-prohibitive at genome-wide scales, particularly when there are a large number of biologically important samples to be compared. In these cases, profiling the changes in global (genome-wide) levels of 5mC and 5hmC has been regarded as more accessible and highly revealing for the study of both disease and development. For example, genome-wide levels of 5mC and 5hmC have shown roles for TET enzymes in early zygotic reprogramming and in primordial germ cell development (11, 12). In post-mitotic tissues such as the brain, levels of 5hmC track with development, reaching levels as high as 20% of those of 5mC, with demonstrated roles in learning and memory (7, 13, 14). In the genomes of cancer cells, global 5mC or 5hmC levels can serve as biomarkers in cancer detection or prognosis (15-17), and aberrant global methylation or hydroxymethylation has also been shown in non-cancerous processes, including stroke and neurodegenerative diseases (18-20).

Given the biological significance and medical implications of 5mC and 5hmC, it is essential to have accurate and sensitive methods for quantification of these cytosine modifications in genomic DNA (gDNA) samples (**Figure 1, left**). While several approaches for measuring cytosine modification levels are available, the most commonly employed involve immunochemistry, mass spectrometry, or sequencing-based techniques (**Figure 1, right)**. Immunochemistry-based techniques such as ELISA with antibodies against 5mC and 5hmC can be facile to implement. However, they are less quantitative than liquid chromatography-tandem mass spectrometry (LC-

MS/MS), which has been generally regarded as the 'gold standard' for the measurement of global cytosine modification (21-23). With LC-MS/MS, gDNA is purified and degraded to nucleosides. The nucleosides are then separated by LC, identified by MS/MS, and quantified by comparison to standards. While highly specific and sensitive, the requirement for relatively high input amounts of DNA (typically >500 ng) for LC-MS/MS, and the technical requirements for protocol optimization and instrument availability can limit its accessibility. Iterations to LC-MS/MS methods have sought to reduce the input requirements, however, several limitations remain. For one, the requirement for a different standard for each DNA modification can lead to systematic errors. Furthermore, due to the obligate degradation of DNA to individual nucleosides prior to analysis, LC-MS/MS cannot report the genomic locations or sequence context of these modifications, an issue of note as DNA modifications in CpG versus non-CpG contexts hold different biological implications.

Sequencing of gDNA samples offers a common alternative for accurately measuring global DNA modification levels. Most efforts center around bisulfite sequencing (BS-Seq), whereby unmodified cytosines are chemically deaminated by sodium bisulfite and next-generation sequencing (NGS) is used to resolve unmodified and modified bases at base resolution (24). Conventional BS-Seq itself is unable to resolve 5mC and 5hmC, although methods coupling bisulfite to enzymatic oxidation (TAB-Seq), chemical oxidation (oxBS-Seq), or enzymatic deamination (bACE-Seq) have been developed to distinguish these two most prevalent modifications (25-28). As bisulfite-mediated deamination can lead to degradation of input DNA samples, non-destructive alternatives have been recently developed, including all-enzymatic methods for profiling cytosine modifications such as enzymatic-methylation sequencing (EM-Seq, 5mC + 5hmC), APOBEC-coupled epigenetic sequencing (ACE-Seq, 5hmC alone), Direct Methylation sequencing (DM-Seq, 5mC alone), or hybrid enzymatic-chemical methods such as TET-assisted pyridine borane sequencing (TAPS) (29-32).

The most rigorous application of sequencing involves whole-genome base-resolution analysis. However, such an approach can be cost-prohibitive, especially when base-resolution information is not absolutely required. Modifications to these protocols have, therefore, been employed to estimate global cytosine modification levels. Such approaches include repetitive element pyrosequencing, reduced representation bisulfite sequencing (RRBS), or immunoenrichment (MeDIP), although each approach brings different potential sets of bias to sampling of the genome (33-35). Similarly, analysis of gDNA with array-based assays can provide coverage of a large number of specific genomic sites. However, the array probes were designed to query some

genomic elements more than others (36), and aggregated measurements may not quantitatively extrapolate to whole-genome cytosine modification levels.

Recently, low-coverage BS-Seq has been suggested as a plausible alternative to deep whole-genome BS-Seq (WGBS) for estimating global DNA modification levels. Theoretical estimators led to the proposal that sequencing as few as 10,000 total cytosines in a CpG context could provide minimal margins of error for global levels of aggregate cytosine modifications (37). Motivated by these empirical calculations, we aimed to explore the potential generalizability of sparse sequencing (Sparse-Seq) as a facile, economical, and accurate method for estimating genome-wide levels of both 5mC and 5hmC. Here, using computational analysis, we first demonstrate that sparse sampling can theoretically be applied with high accuracy, even when modifications such as 5hmC drop to low levels. Guided by a framework from our theoretical analysis, we then experimentally benchmark Sparse-Seq against LC-MS/MS, with a specific emphasis on a combined chemical and enzymatic (bACE-Seq) approach that allows for resolution of 5mC and 5hmC. Our work shows that Sparse-Seq demonstrates fidelity and rigor when compared to LC-MS/MS, with additional advantages, including the ability to retain sequence context information inaccessible with LC-MS/MS. We therefore offer strong validation for Sparse-Seq as a powerful and highly accessible tool for the accurate and economical profiling of global changes in both methylation and hydroxymethylation in biological samples of interest.

MATERIAL AND METHODS

Computational processing and down-sampling

Murine brain, ESC, and human cell line ACE-seq, TAB-seq, and EM-seq data were acquired through GEO using the following accessions GSE48519, GSE63137, GSE116016, GSE47966, GSE195752. FASTQ reads were trimmed to remove sequencing adapter and aligned to the reference genome assembly (GRCh38 for human and GRCm38 for mouse), see below. Duplicate reads were masked using Picard tools (https://broadinstitute.github.io/picard/). DNA modification levels were extracted using bismark_methylation_extractor. Double counting from the overlapping mate reads was excluded.

To mimic real sparse sequencing data, the deep sequencing datasets were downsampled at the BAM file level to various degrees of sparsities evenly spaced in the power space from N=64 (2^6) to N=262,144 (2^{18}) reads. The sampled read numbers were then transformed to sequencing depth based on read length and genome size. Each downsampled BAM file went though the same

preprocessing steps including duplicate marking and methylation extraction. The cytosine modification levels at CpG, CpHpH, and CpHpG were aggregated.

Interpolation and construction of TAE estimator.

A total of 18 datasets (5 TAB-seq, 5 BS-seq, 4 ACE-seq and 4 EM-seq) were combined. Using this data set, loess regression (R stats package, degree = 1, span = 0.75) of the total analytical error was performed on $\log_2$-transformed genome coverage and cytosine modification levels. The regression model was then used to interpolate the TAE for genome coverage input ranging from 0.001% to 1% and total cytosine modification from 0% to 5%.

Mouse Genomic DNA extraction

Breeding stocks of C57BL/6J (The Jackson Laboratory, Bar Harbor, ME, USA) were maintained in a pathogen-free facility in a 12 hour light-dark cycle. All animals were housed in polysulfone cages and had access to drinking water and chow (Laboratory Autoclavable Rodent Diet 5010, LabDiet) *ad libitum*. Neonatal and P0 brains and postnatal cortices were isolated from mice at indicated ages and snap-frozen. Frontal cortices were obtained by slicing the adult brains coronally at Bregma 1 mm, and dissecting the frontal cortical tissue, being careful to avoid contamination with olfactory or putamen regions, under a dissection microscope. All animal work was conducted with the approval of the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania.

To isolate gDNA, brain was thawed and digested in lysis buffer (50mM Tris, pH8.0, 100mM EDTA, 0.5% SDS) with proteinase K (Sigma-Aldrich) overnight at 55°C. Genomic DNA was isolated using Phenol:Chloroform:Isoamyl Alcohol (Fisher BP17501-400) and ethanol precipitation. DNA samples resuspended in TE were stored at 4°C.

LC-MS/MS

The purified gDNA samples (250 ng) were digested using Nucleotide Digestion Mix (NEB) in a volume of 20 µL at 37 C for 16 h to get single nucleosides. 9 µL of the incubated digestion mix was then added 1 µL 1% FA to get a final solution of 0.1% FA. 5 µL of the sample or standard was injected into an HPLC-QQQ-MS/MS system. An Accucore Vanquish C18+ ultra-high performance liquid chromatography system (Thermo, 1.5 µm particle size, 50 mm length) was used to separate nucleosides with online mass spectrometry detection by the Atlis (Thermo Fisher Scientific) triple-quadrupole LC mass spectrometer. Nucleosides were separated starting with a

2 minute flow of 100% Buffer A (0.1% formic acid) followed by a 10 minute gradient of 0-4% Buffer B (30% acetonitrile, 0.1% formic acid) at 0.4 mL/min. Mass transitions for deoxynuclesides were C 228.093→112.05 *m/z*; 5mC 242.108→126.066 *m/z*; and 5hmC 258.103→142.061 *m/z*. Standard curves were generated using standard nucleosides ranging from 5.56 fmol to 92500 fmol for unmodified cytosine and 0.565 fmol to 9250 fmol for modified cytosines.

BS/bACE-Seq library prep

Purified mouse gDNA (200 ng) with 1 ng each of phiX, mCpG pUC19 (Zymo), and 5hmC T4 spike-in controls were sheared to approximately 350 bp using a Covaris instrument. Sheared gDNA was taken through end prep and adaptor ligation using the NEBNext Ultra II DNA Library Prep Kit (NEB). Custom-made pyrrolo-cytosine adaptors (IDT) were substituted for kit-supplied adaptors. Adapted samples were bisulfite treated using the Premium Bisulfite Kit (Diagenode) and eluted in nuclease-free water. Post bisulfite clean-up, each sample was split into two 10 µL aliquots. One aliquot was further purified with a 1.2x left-side SPRI purification (Beckman). The SPRI-purified aliquot was denatured and APOBEC3A-treated (NEB, E1725L). After A3A deamination, the sample was purified via SPRI beads (1.2x, left-sided). Quantitative PCR was used to determine the number of cycles for the indexing PCR. Indexing PCR was performed using the unique dual index primer pairs from NEBNext Multiplex Oligos for Enzymatic-Methyl Seq (NEB). After the indexing PCR, samples were purified with two 0.8x left-sided SPRI purifications. Libraries were quantified with a Qubit HS kit (Invitrogen,Q32851) and assessed for quality with an Agilent Bioanalyzer. The resulting libraries were then pooled and sequenced on an Illumina MiSeq using 150-bp read length on a nano or micro sequencing kit (Illumina).

EM-Seq library prep

Libraries were prepared using the NEBNext Enzymatic Methyl-seq kit (NEB, E7120L) in accordance with the manufacturer's instructions. Either 200 ng or 20 ng of mouse gDNA with unmodified lambda and CpG-methylated pUC19 control DNA was sheared to 350 bps using a Covaris sonicator and used as input. HiFi HotStart Uracil+ Ready Mix (KAPA Biosystems, KK2801) was used to amplify the libraries following conversion and then subjected to a 0.8x left-sided SPRI purification, with elution in nuclease-free water to yield final libraries. Libraries were quantified by Qubit HS kit (Invitrogen, Q32851) and assessed for quality with an Agilent Bioanalyzer 2100. The resulting libraries were then sequenced on an Illumina MiSeq.

Sequencing data processing

Sequencing data were processed in single-end mode as previously described.(29) Raw reads were trimmed for base quality and adaptor sequences using Trim Galore! (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) and reads below a length of 20 bases were discarded. Pre- and post-trimmed data were visually examined for quality using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The trimmed reads were aligned to the reference genomes using Bismark (v0.22.3) with the following parameters -D 15 -R 2 -L 20 -N 0 –score_min L,0,-0.2.(38) To account for A3A failure due to double-stranded DNA, reads with three or more consecutive non-converted cytosines in the CpH context were removed using the filter_non_conversion script included in the Bismark package.

Statistical analysis

A one-way ANOVA followed by Tukey's range test was performed in R to compare methylation levels detected by different methods. Statistically significant differences in pair-wise comparisons are indicated by lowercase letters; groups with significant difference are assigned different letters, while groups with no statistical difference are assigned shared letter. n is defined as technical replicates. Exact value of n, significance, and effect size are reported in text and figure legend.

RESULTS

Computational down-sampling reveals sparse coverage permits accurate cytosine modification quantification

We started with a computational approach to assess the theoretical viability of Sparse-Seq pipelines before embarking on experimental endeavors. In this approach, to broadly profile across chemical and enzymatic methods and to be inclusive of both 5mC and 5hmC, we gathered data sets with whole genome profiles available (genomic depth, range 1X to 9.5X) using various epigenetic pipelines (BS-Seq, TAB-Seq, EM-Seq, and ACE-Seq) in multiple murine and human cell types (**Figure 2, Figure S1, Figure S2**). We theorized that samples with lower overall levels of DNA modifications, such as those profiling 5hmC, might require greater coverage for accurate determination of cytosine modification levels, so we ensured that the data sets we chose had a range of overall modification levels, including mouse embryonic stem cells (ESCs), where 5hmC can be <0.3% of the total cytosine content.

For each full genomic sequencing sample, we used the entire available high-depth data set to calculate the 'true' DNA modification level for 5mC+5hmC (BS-Seq, EM-Seq) or 5hmC alone (TAB-Seq, ACE-Seq). To model the process of our proposed Sparse-Seq, we then used computational down-sampling to select a random subset of the complete sequencing reads. We targeted various values for genomic coverage, ranging from 0.00023% to 0.96% of the whole genome (e.g. ~0.007 Mb to 26 Mb of the mouse genome). At each selected level of genomic coverage, down-sampling was performed a total of 100 times, and these 100 sets of reads were separately processed to calculate the level of cytosine modification for each sample. Across the 100 sampled replicates at each level of genomic coverage, we calculated the mean DNA modification level and standard deviation. Because Sparse-Seq in practice would involve a single sampling at a given level of genomic coverage, we also calculated the total analytical error (TAE, % bias + 1.96 x coefficient of variance), a metric that assesses the expectation of test sample accuracy relative to the mean when run on a sample a single time.

The results show that across the epigenetic sequencing pipelines, even at the lowest genomic coverage we tested (0.0023%), the mean DNA modification level estimated across the 100 down-samplings was in reasonable agreement with the 'true' level from the complete dataset (**Figure 2A**). As anticipated, however, variation is large at low levels of genomic coverage, but measurements converge as the level of genomic coverage increases (**Figure 2B**). For example, at 0.0019% genomic coverage, the BS-Seq and TAB-Seq datasets on both mouse ESCs and neocortex have an average TAE across samples of 24.8% (ranging from 14.2 to 37.4%). By contrast, when the genome coverage increases to 0.24%, the same datasets show a reduced TAE of 2.2% (ranging from 1.2 to 3.2%). This implies that when a single sample is profiled at 0.24% in sequencing depth, there is a 95% probability that the determined level of cytosine modification will deviate by less than 2.2% from the 'true' value, as defined by high-coverage, whole-genome sequencing. By comparing across methods, we observed similar patterns across chemical and enzymatic-based sequencing methods (**Figure S1, Figure S2**), highlighting that the accuracy of down-sampling is independent of the method of library construction. In the computational analysis, the TAE of each dataset correlates negatively with the overall modification level of the full dataset, supporting our hypothesis that Sparse-Seq would be less precise in samples with lower overall modification (at 0.24% coverage, TAB-Seq adult neocortex (5hmC = 1.2%) has a TAE of 2.3%, while TAB-Seq of ESCs (5hmC = 0.49%) has a TAE of 2.9%, **Figure 2B**). Nonetheless, even with lower overall levels of DNA modification, single sequencing

replicates at 0.24% of the genome are associated with a TAE <5% for measurement of either 5mC+5hmC or 5hmC alone across all methods.

Our theoretical calculations with computational down-sampling across different methods and samples thus isolated two key features – the percent coverage of the genome and the calculated mean DNA modification level – as instructive of the potential error associated with a single measurement of a given sample. Given the broad range of modification levels covered by our analysis, we next used computational interpolation to assess the landscape of TAE anticipated from measuring samples of different modification levels to various genome coverages (**Figure 2C**). As expected, the TAE prediction model integrating across the different data sets shows that the higher the modification level or genome coverage, the lower the TAE of the estimation. In anticipated applications of Sparse-Seq approaches, the end product of analysis would be a known percent coverage of the genome and an estimated modification level. Using the interpolated TAE chart, these two values can thus potentially be used to control the measurement error and yield confidence levels surrounding the calculated modification level.

Considering the generalizability of the above sparse sequencing approach in estimating cytosine modification levels and quantitatively controlling errors, we selected a target range of genomic depth coverages that would allow for accurate quantification and higher throughput analysis. A coverage level of 0.24% of the mouse genome is equivalent to sequencing approximately 6.5 million bases. With NGS read length of 150 bases, this level of genomic coverage would be equivalent to about 45,000 (45K) mapped reads. Given the wide range of flow cells available and the relative accessibility of sequencing to most laboratories, we considered that Sparse-Seq at this level of coverage could open opportunities for economical and efficient sequencing for a wide variety of applications, particularly if the methodology showed comparable performance to LC-MS/MS-based approaches.

Comparison of Sparse-Seq with multiple sequencing pipelines to LC-MS/MS

Based on the results from our computational work, we next moved to experimentally explore Sparse-Seq at a target of ~45K mapped reads per sample, with a rigorous comparison to LC-MS/MS based methods. Given that our objective was accurate quantification of both 5mC and 5hmC levels from genomic DNA samples, we chose to employ Sparse-Seq using the BS and bACE-Seq pipeline (27, 28). This approach combines bisulfite and enzymatic deamination to parse 5mC and 5hmC (**Figure 3A)**. Specifically, genomic DNA is subjected to chemical

deamination with bisulfite, which leads to the conversation of C, but not 5mC or 5hmC, separating unmodified C from modified C bases. Half of the sample is then subjected to enzymatic deamination with the DNA deaminase, APOBEC3A (A3A). A3A can readily deaminate the 5mC bases, but is unable to deaminate the 5hmC bases, which were converted to the bulky and deaminase-resistant base, cytosine 5-methylenesulfonate (CMS), after the bisulfite step. By comparison of the BS-Seq (5mC+5hmC read as C) with the bACE-Seq library (only 5hmC reads as C), the global level of both 5mC and 5hmC can be calculated. We focused on this approach as an accessible option for labs without access to LC-MS/MS given that BS-Seq is the most commonly employed approach for sequencing and the commercial availability of A3A.

To permit an assessment of biological and technical variation, we collected three independent biological samples of murine brain genomic DNA (gDNA) at four distinct time points in mouse development ranging from neonates (P0) to 26 weeks old (**Figure 3B**). We processed 250 ng of the gDNA with a standard LC-MS/MS protocol involving degradation to nucleosides and 200 ng of the gDNA with the Sparse BS and bACE-Seq pipeline, pooling the barcoded samples for analysis via NGS. For LC-MS/MS, the degraded gDNA was analyzed for detected masses and quantification was performed against a standard curve generated for each DNA modification. For NGS, we aimed for equivalent library pooling targeting similar depth across samples. We obtained a range of ~71-151K reads (average 108K), which, after alignment and de-duplication, yielded ~27-102K reads across samples (average 68K). Notably, while some samples thus fell below our goal of 45K, computational down-sampling suggested anticipated reliability (< 2.5% TAE), and our calculator can be used to estimate the specific TAE for any given sample based on the number of reads and the mean modification level. In comparing the global cytosine modification levels for both 5mC and 5hmC, we found strong concordance between the values determined using Sparse-Seq and LC-MS/MS (**Figure 3C**). Overall, the relative abundance of the individual modifications followed the same rank order for both methods, although Sparse-Seq appeared to consistently detect more 5hmC and less 5mC relative to the LC-MS/MS. Importantly, it is not clear which method would be more accurate in this regard. In LC-MS/MS, each modification is quantified against its own standard, while in Sparse-Seq, the modification levels are derived from comparison of C versus T calls at each site, a process that avoids potential compounded errors from multiple standard curves. Importantly, across samples, we also found consistently less variance among the samples processed with Sparse-Seq relative to LC-MS/MS, with a range of 1.5 to 6-fold higher variance for 5mC detection with LC-MS/MS. To assess for technical variation, we repeated LC-MS/MS analysis and Sparse-Seq on a set of the biological replicates and

observed higher consistency with Sparse-Seq, with a variance that was 2.1- to 4.0-fold lower than LC-MS/MS in 5mC detection (**Figure 3C**). The higher degree of technical variance observed in measurements from LC-MS/MS suggests at least one source of the greater overall variance observed with biological replicates using LC-MS/MS relative to Sparse-Seq.

Given the increased momentum towards enzymatic rather than chemical approaches to detect cytosine modifications (10), we also explored Sparse-Seq with an alternative all-enzymatic sequencing approach. We chose EM-Seq due to its commercial accessibility as a method for quantifying combined 5mC+5hmC levels, also recognizing that this orthogonal approach could serve as an added means to assess the relative accuracy of BS-Seq versus LC-MS/MS given their slight discordance. An added benefit of enzymatic library generation approaches, such as EM-Seq and ACE-Seq, is that these approaches require much lower starting input amounts of gDNA than either LC-MS/MS methods or bisulfite-based methods. To this end, given our interest in the technical variance of each method, we processed one biological gDNA sample from P0 and another from the 20-week timepoint with LC-MS/MS (250 ng input gDNA), Sparse BS-Seq (200 ng), and Sparse EM-Seq using either 200 ng or 20 ng input gDNA. After processing, the BS-Seq and EM-Seq data sets had a range of 10-90K reads (average 56K) which were used to calculate the levels of 5mC + 5hmC. By performing Tukey's HSD Test for multiple comparisons, we found that the two orthogonal sequencing methods (Sparse BS-Seq vs. EM-Seq) agreed more closely with each other than LC-MS/MS ($p < 0.0001$) with a large effect size ($\eta^2=0.94$) (**Figure 3C**). While all methods will have some biases, the consistency between the different enzymatic and chemical-based sequencing methods, relative to LC-MS/MS, highlights possible advantages in the accurate detection of modification levels. Regarding technical variance, LC-MS/MS showed 4.6-5.3% variability between independent technical measurements, while 1.9-2.5% variability was observed across Sparse-Seq samples. Overall, across analysis of biological and technical reproducibility, the data highlight the generalizability of Sparse-Seq across chemical and enzymatic methods as well as its high precision relative to LC-MS/MS at the tested sequencing depths.

Sparse Seq allows for biological insight from sequence context

One of the most notable disadvantages of LC-MS/MS for profiling cytosine modifications is that the approach erases all information on sequence context given the requirement for degradation of gDNA to nucleosides. While Sparse-Seq lacks whole genome base-resolution information, it

does allow for broader analyses of sequence context or mapping of DNA modification levels to specific genomic elements.

To explore the utility of Sparse-Seq in addressing open biological questions, we focused analysis on the developing brain. Prior work has shown that over the course of development, two different DNA marks accumulate on cytosine bases in post-mitotic neurons. First, there is an accumulation of 5mC in non-CpG (CpH) contexts (39), and second, an accumulation of 5hmC, although this modification is instead almost entirely confined to a CpG context (39, 40). Notably, based on single-cell profiling work either 5mCpH alone or 5hmCpG alone can be used to define cell identity and lineage in the brain, highlighting the importance of both of these epigenetic marks (28, 41). While both 5mCpH and 5hmCpG accumulate during brain development, the challenge and expense of base-resolution mapping have meant that the relative kinetics with which these modifications arise during prenatal and postnatal brain development remain incompletely characterized.

Given that Sparse-Seq is well-situated to assess DNA modification levels while retaining sequence context information, we collected murine brain gDNA samples at various points in development from E16 to 10 weeks of age, with 3 biological replicates for each timepoint (**Figure 4A)**. We then subjected the samples to the split Sparse BS-Seq and Sparse bACE-Seq approach to quantify both 5mC and 5hmC. To balance cost and accuracy, we targeted ~30K mapped reads and ran the samples on an Illumina Micro kit (resulting range: 11–54K mapped reads, average 29K). Consistent with prior studies, an accumulation of both methylation in CpH contexts (5mCpH) and hydroxymethylation in CpG contexts (5hmCpG) was observed. Tracking these modifications at more regular intervals between embryonic development and young adulthood with Sparse-Seq approach yielded several new insights. As anticipated, both 5mCpH and 5hmCpG accumulate with development, however the kinetic patterns of accumulation can differ. First, a surprisingly high amount of 5hmC can be observed in CpG contexts even in the earliest developmental time point collected, E16 (**Figure 4B**), consistent with an early role for TET enzymes in shaping brain epigenetic landscapes (7, 13, 14). 5hmC then continues to accumulate with 25% of the total increase from E16 to 10 weeks of age already detectable at the time of birth (P0). By contrast, non-CpG methylation (5mCpH) is at background levels at birth (P0), with a rapid increase that only occurs post-partum. Interestingly, both 5hmCpG and 5mCpH levels, however, appear to reach 90% of their maximal levels by 4 weeks after birth, suggesting that the epigenetic marks are largely established at that point in brain development. The shared and divergent patterns with

which these epigenetic marks arise highlight the likelihood of independent epigenetic roles for 5hmC and non-CpG methylation in neuronal development.

DISCUSSION

The ability to accurately, efficiently, and economically estimate the global level of DNA modifications in samples derived from cells or tissues is of high value. The levels of DNA modifications can be used to track cellular and developmental dynamics or to classify, diagnose, or prognosticate disease states. While multiple methods exist for obtaining estimates of global 5mC and 5hmC levels, the accessibility of sequencing makes Sparse-Seq a potentially generalizable solution to address this need.

Currently, LC-MS/MS-based approaches are generally regarded as the 'gold standard' for estimation of global levels of 5mC and 5hmC. To rigorously evaluate Sparse-Seq, we performed comparisons to LC-MS/MS, with our results indicating comparable quantification of C, 5mC, and 5hmC with less technical variability. Although our results were generally concordant, we also noted some systematic differences in some measurements. We postulate that with LC-MS/MS, systematic errors might be amplified, as each modification is quantified by comparison against its own standard curve. By contrast, in sequencing, modification levels are derived from internal comparisons, specifically the number of bases that read as C versus T at each aligned base across reads. If deeply-sequenced whole genome data sets are regarded as the most reliable means to estimate global modification levels, integrating across our computational and experimental data, a case can thus be made for Sparse-Seq as viable alternative to LC-MS/MS methods.

The preservation of sequence context information is a distinctive advantage of Sparse-Seq approaches over LC-MS/MS. In applying Sparse-Seq to dissect cytosine modification dynamics in the developing mouse brain, we were able to analyze the time course with which 5hmCpG and non-CpG methylation occur. While both modifications accumulate in a time-dependent manner and plateau before 6 weeks after birth, we find that 5hmC starts to accumulate in the prenatal brain, while non-CpG methylation accumulation only begins postnatally. On a related note, a strength of Sparse-Seq is that the library preparation approach is identical to that needed for high-depth sequencing. Thus, samples identified as being of high value after assessment of global modification levels, for example, the timepoints at which 5mC and 5hmC are most rapidly changing, can be preserved, and sequenced at high depth for further analysis of base-resolution

information. Similarly, as reads are mapped for Sparse-Seq, it may be possible to estimate 5mC and 5hmC levels in specific genomic elements (e.g. promoters or enhancers) to guide more detailed downstream analysis. Such sub-sampling would be associated with higher error rates, but the errors can potentially be imputed from the relative level of coverage of these genomic elements using the TAE error calculator.

Sparse-Seq offers a flexible method that can be used with different sequencing workflows to quantify desired modifications of interest with tunable sensitivity. We demonstrate that the method can be applied to resolve combined levels of 5mC + 5hmC (e.g BS-Seq, EM-Seq), or integrated to yield 5mC and 5hmC alone (e.g. TAB-Seq, bACE-Seq). Importantly, the extension to all-enzymatic approaches could be particularly valuable for the study of samples where DNA amounts are limiting. Enzymatic methods, such as EM-Seq and DM-Seq, are typically performed with low quantities of DNA at the nanogram level and have even been extended to the single-cell level, offering opportunities not readily accessible to LC-MS/MS (30, 32, 42). One associated limitation of Sparse-Seq is that the detection limit for given modifications can be impacted by both the accuracy of correct base calling in NGS as well as the rate of failures in proper C-to-T conversion for a given sequencing pipeline. However, these rates can typically be accurately estimated, for example, by examining the frequency of BS-Seq failure in the CpC context, where methylation is not commonly found in mammalian genomes. Notably, recent work has also suggested the possibility of sparse sampling of DNA methylomes through nanopore approaches, which may have different associated accuracy errors (43, 44). Although the validity of parsing 5hmC by nanopore has yet to be rigorously established, the principles we explored in short-read sequencing platforms could likely be extended to long-read approaches with sparse sampling.

In our estimation, the ease of access to sequencing platforms, relative to the need for more specialized expertise and equipment for LC-MS/MS, should support the facile and widespread adoption of Sparse-Seq. Given the scope of questions that could be informed by analysis of global 5mC and 5hmC levels, we believe that our results suggest that Sparse-Seq should now be viewed as supported by a solid theoretical and experimental foundation.

DATA AVAILABILITY

Analysis performed using previously developed software is detailed in the relevant methods section. All original code used for analyses in this paper have been deposited on GitHub (https://github.com/ChristianELoo/Sparse-Seq) and are publicly available as of the date of publication. NGS data have been deposited at GEO (GSE273521) and are publicly available as of the date of publication. Additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

AUTHOR CONTRIBUTIONS

Conceptualization, C.E.L, J.M.F., W.Zhou, and R.M.K.; Methodology, C.E.L., J.M.F., W.Zhou, R.M.K.; Software, W.Zhou; Investigation, C.E.L., J.M.F., J.Z.; Formal analysis, C.E.L., J.M.F., J.Z., W. Zhu, W.Zhou, R.M.K.; Writing - Original Draft, C.E.L., J.M.F., R.M.K.; Writing - Review and Editing, All Authors; Resources, M.S.B., C.K., W.Zhou, R.M.K.; Supervision – W.Zhou and R.M.K.

ACKNOWLEDGEMENTS

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CONFLICT OF INTERESTS

C.E.L and R.M.K. through the University of Pennsylvania have filed patent application(s) related to epigenetic sequencing pipelines.

FIGURE LEGENDS

Figure 1. Methods for detecting cytosine modifications. Structures of the nucleobase cytosine and its two most common mammalian genomic variants, 5-methylcytosine and 5-hydroxymethycytosine (left). Common methods for quantifying cytosine modifications in the genome and their potential strengths and limitations are noted (right).

Figure 2. Computational down-sampling confirms the theoretical viability of Sparse Sequencing for cytosine modification measurement. A) Data from whole genome sequencing in various murine cell types with either BS-Seq or TAB-Seq were randomly down-sampled to various levels of sparse genomic coverage. Box plots represent the cytosine modification levels determined across 100 replicates at each sparse coverage level for each whole genome dataset. Dashed lines mark the cytosine modification levels from the complete, deeply-sequenced whole genome dataset. Analogous analyses of whole genome data sets obtained by enzymatic methods are shown in **Figure S1 and S2**. **B)** Shown is the calculated total analytical error (TAE) at each level of down-sampling from the whole genome datasets. The TAE gives the error in measurement of the true mean relative to the measured value for a single measurement at a given level of down-sampling **C)** 18 total whole-genome data sets (5 BS-Seq, 4 EM-Seq, 4 TAB-Seq, and 4 ACE-Seq), the genomic coverage in down-sampling, the percent modification, and associate TAE were taken as a base data set. Loess regression was performed, and the regression model was then used to interpolate the TAE for genome coverage input ranging from 0.001% to 1% and total cytosine modification from 0% to 5%.

Figure 3. The accuracy, precision, and generalizability of Sparse Sequencing is confirmed with comparison to LC-MS/MS. A) Workflow for experimental validation of Sparse Sequencing using BS-Seq, bACE-Seq, and EM-Seq pipelines via comparison to LC-MS/MS using murine cortex gDNA. CMS, cytosine 5-methylenesulfonate; C*, modified cytosine protected from enzymatic deamination. **B)** Biological variance comparison of 5mC and 5hmC quantification using LC-MS/MS (left) and BS/bACE-Seq (right). Each of three biological replicates are shown (points), with bars representing the average of measurements, and standard deviations listed above the bars. **C)** Technical variance comparison of LC-MS/MS and Sparse-Seq. Data are from three technical replicates from a single sample from (**B**) at either P0 and 20 weeks using LC-MS/MS, BS-Seq, or EM-Seq at either of two sample inputs (200 ng or 20 ng), with mean and standard deviation provided for each set of technical replicates. A one-way ANOVA with Tukey's range

test, n=3; significant differences between groups are denoted using different letters, while groups with no significant differences share the same letter.

Figure 4. Sparse-Seq reveals 5mCpH and 5hmCpG dynamics during murine development
A) The developing mouse brain is known to accumulate 5mC in non-CpG (CpH) contexts and 5hmC in CpG context. Three biological replicates of brain specimens (whole brain for fetal and P0 stages and isolated cortex thereafter) taken every two weeks from E16 through 10 weeks were analyzed by BS/bACE Sparse-Seq and quantified for the level of modifications in each context. **B)** Levels (%) of 5mCpH and 5hmCpG in the developing mouse brain detected by BS/bACE Sparse-Seq. Data are from the replicates at each time point are shown as the mean, with standard deviation provided by error bars. Error bars not shown if smaller than the symbol representing mean value. At bottom is shown a heat map representation depicting the relationship between 5hmCpG and 5mCpG from minimal to maximal values over time.

Figure S1. Computational down-sampling applied to published ACE-Seq datasets.

A) Whole genome sequencing data sets sequenced with ACE-Seq in various murine cell types were down-sampled to sparse genomic coverage. Box plots represent the cytosine modification levels determined in each of 100 replicates at each sparse coverage level. Dashed lines mark the cytosine modification levels from the complete, deeply whole genome sequenced dataset. **B)** Shown is the calculated total analytical error (TAE) as a function of the downsampled coverage from the whole genome datasets.

Figure S2. Computational down-sampling applied to published EM-Seq datasets.

A) Whole genome sequencing data sets sequenced with EM-Seq in various murine cell types were down-sampled to sparse genomic coverage. Box plots represent the cytosine modification levels determined in each of 100 replicates at each sparse coverage level. Dashed lines mark the cytosine modification levels from the full, deeply whole genome sequenced dataset. **B)** Shown is the calculated total analytical error (TAE) as a function of the downsampled coverage from the whole genome datasets.

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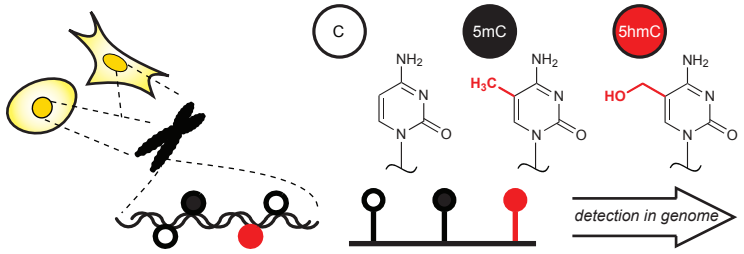
Figure 1



Immunochemistry	*qualitative*		
LC-MS/MS	*quantitative*	*high input requirement*	
Whole-genome Seqencing	*quantitative*	*low input requirement*	*expensive*
Sparse Seqencing	*quantitative*	*low input requirement*	*cost-effective*

Figure 2

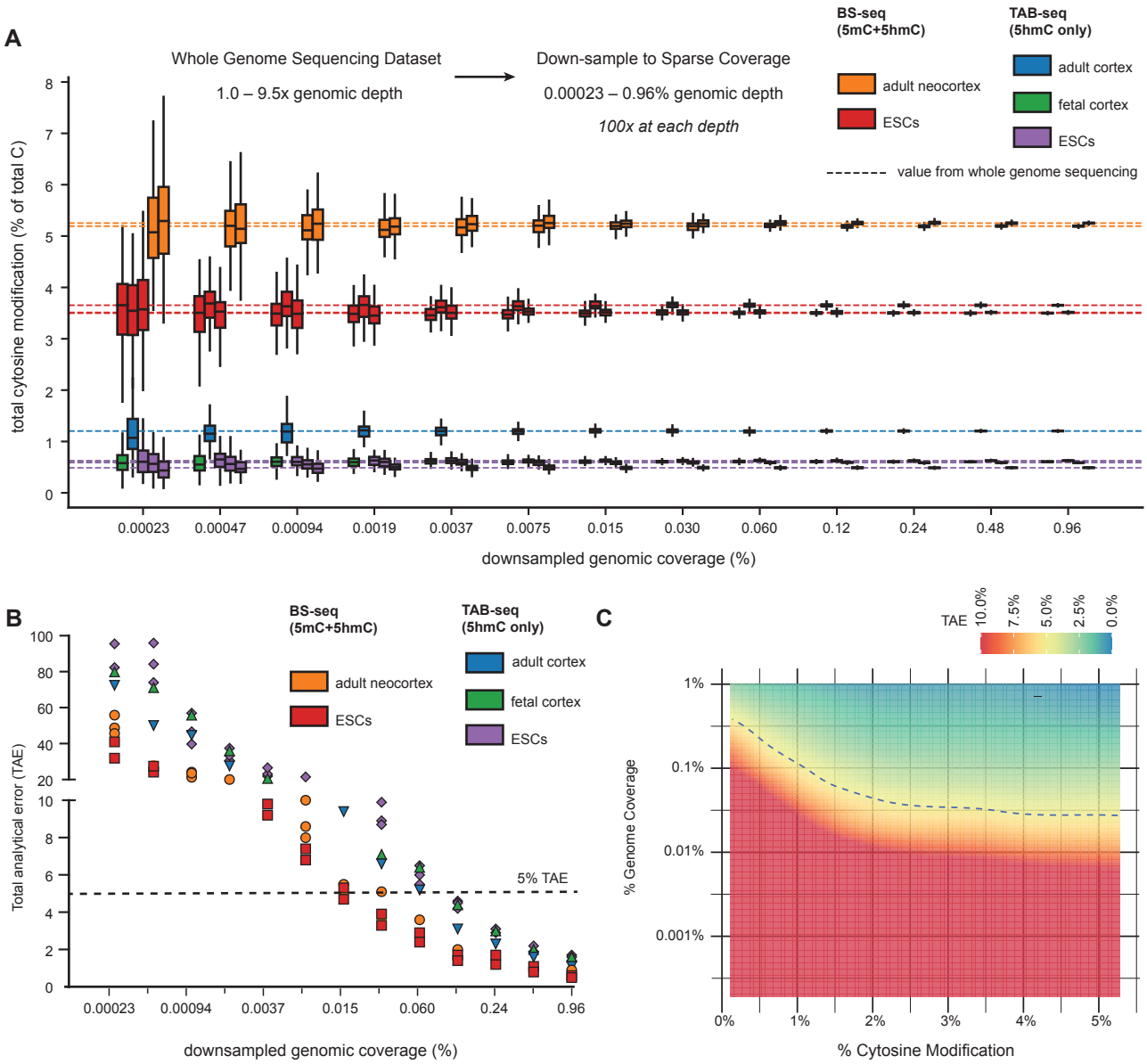

A
Whole Genome Sequencing Dataset
1.0 – 9.5x genomic depth
Down-sample to Sparse Coverage
0.00023 – 0.96% genomic depth
100x at each depth
BS-seq (5mC+5hmC)
adult neocortex
ESCs
TAB-seq (5hmC only)
adult cortex
fetal cortex
ESCs
value from whole genome sequencing
total cytosine modification (% of total C)
0.00023
0.00047
0.00094
0.0019
0.0037
0.0075
0.015
0.030
0.060
0.12
0.24
0.48
0.96
downsampled genomic coverage (%)
B
BS-seq (5mC+5hmC)
adult neocortex
ESCs
TAB-seq (5hmC only)
adult cortex
fetal cortex
ESCs
5% TAE
Total analytical error (TAE)
downsampled genomic coverage (%)
C
TAE
10.0%
7.5%
5.0%
2.5%
0.0%
% Genome Coverage
% Cytosine Modification

Figure 3

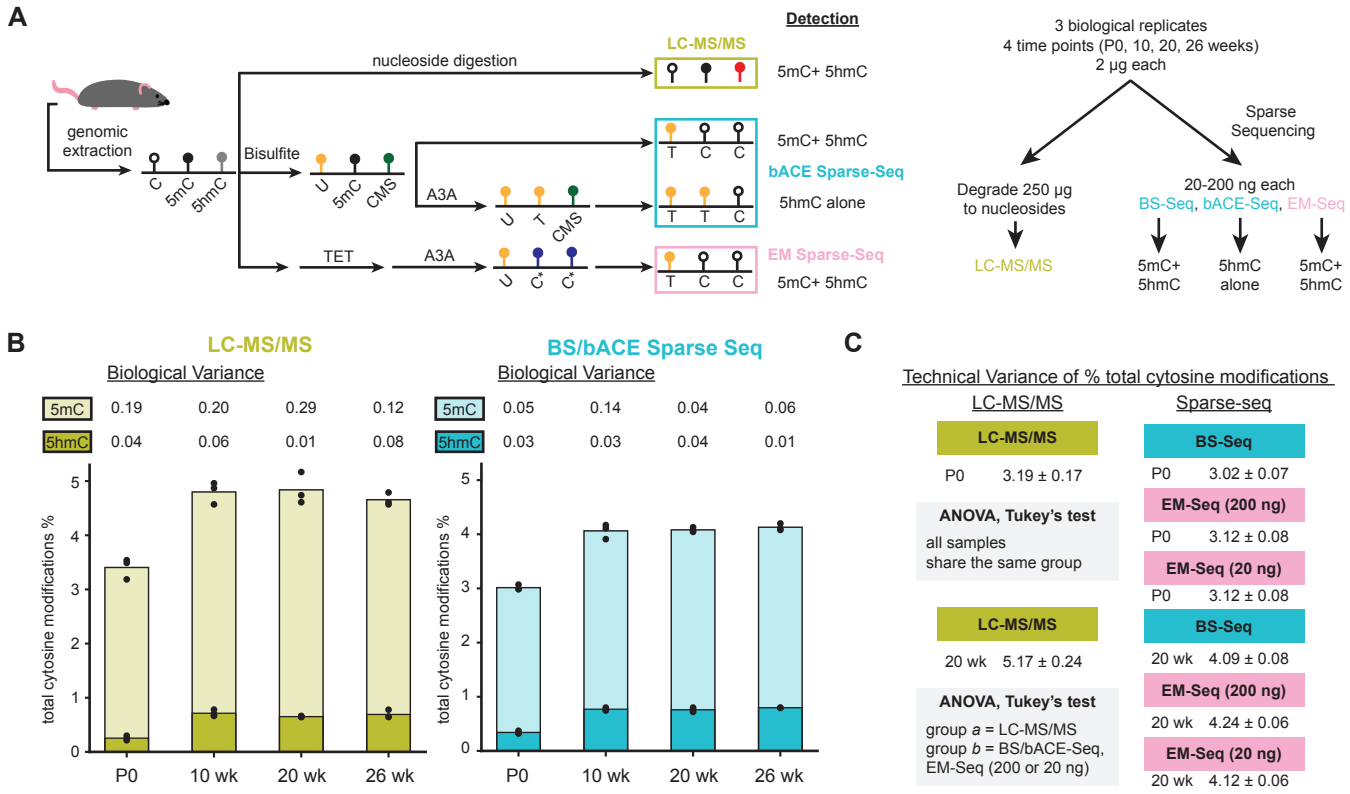

A
Detection
LC-MS/MS
nucleoside digestion
5mC+ 5hmC
genomic extraction
C
5mC
5hmC
Bisulfite
U
5mC
CMS
A3A
T C C
5mC+ 5hmC
bACE Sparse-Seq
U T CMS
T T C
5hmC alone
TET
A3A
U C* C*
T C C
EM Sparse-Seq
5mC+ 5hmC
3 biological replicates
4 time points (P0, 10, 20, 26 weeks)
2 µg each
Sparse Sequencing
Degrade 250 µg to nucleosides
20-200 ng each
BS-Seq, bACE-Seq, EM-Seq
LC-MS/MS
5mC+ 5hmC
5hmC alone
5mC+ 5hmC
B
LC-MS/MS
Biological Variance
5mC 0.19 0.20 0.29 0.12
5hmC 0.04 0.06 0.01 0.08
total cytosine modifications %
P0
10 wk
20 wk
26 wk
BS/bACE Sparse Seq
Biological Variance
5mC 0.05 0.14 0.04 0.06
5hmC 0.03 0.03 0.04 0.01
total cytosine modifications %
P0
10 wk
20 wk
26 wk
C
Technical Variance of % total cytosine modifications
LC-MS/MS
LC-MS/MS
P0 3.19 ± 0.17
ANOVA, Tukey's test
all samples share the same group
LC-MS/MS
20 wk 5.17 ± 0.24
ANOVA, Tukey's test
group a = LC-MS/MS
group b = BS/bACE-Seq, EM-Seq (200 or 20 ng)
Sparse-seq
BS-Seq
P0 3.02 ± 0.07
EM-Seq (200 ng)
P0 3.12 ± 0.08
EM-Seq (20 ng)
P0 3.12 ± 0.08
BS-Seq
20 wk 4.09 ± 0.08
EM-Seq (200 ng)
20 wk 4.24 ± 0.06
EM-Seq (20 ng)
20 wk 4.12 ± 0.06

Figure 4

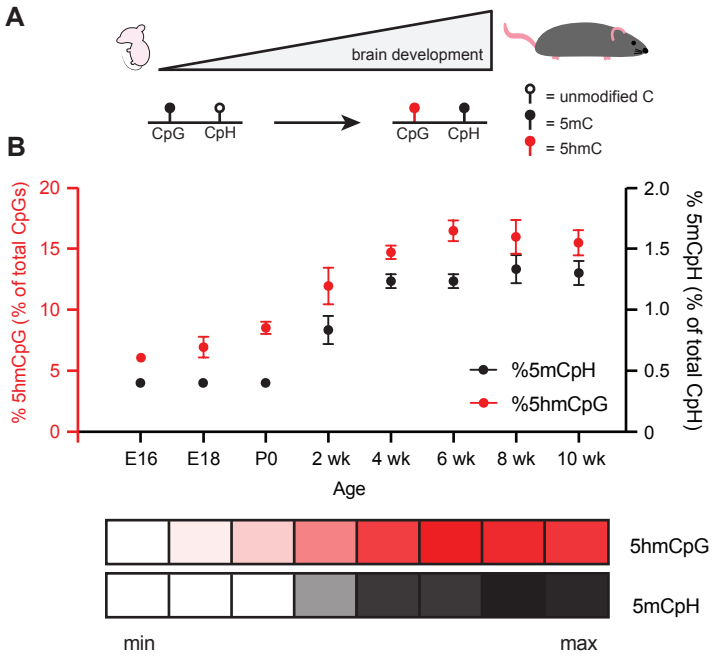

A
brain development
CpG
CpH
CpG
CpH
= unmodified C
= 5mC
= 5hmC
B
% 5hmCpG (% of total CpGs)
% 5mCpH (% of total CpH)
%5mCpH
%5hmCpG
E16
E18
P0
2 wk
4 wk
6 wk
8 wk
10 wk
Age
5hmCpG
5mCpH
min
max

Figure S1

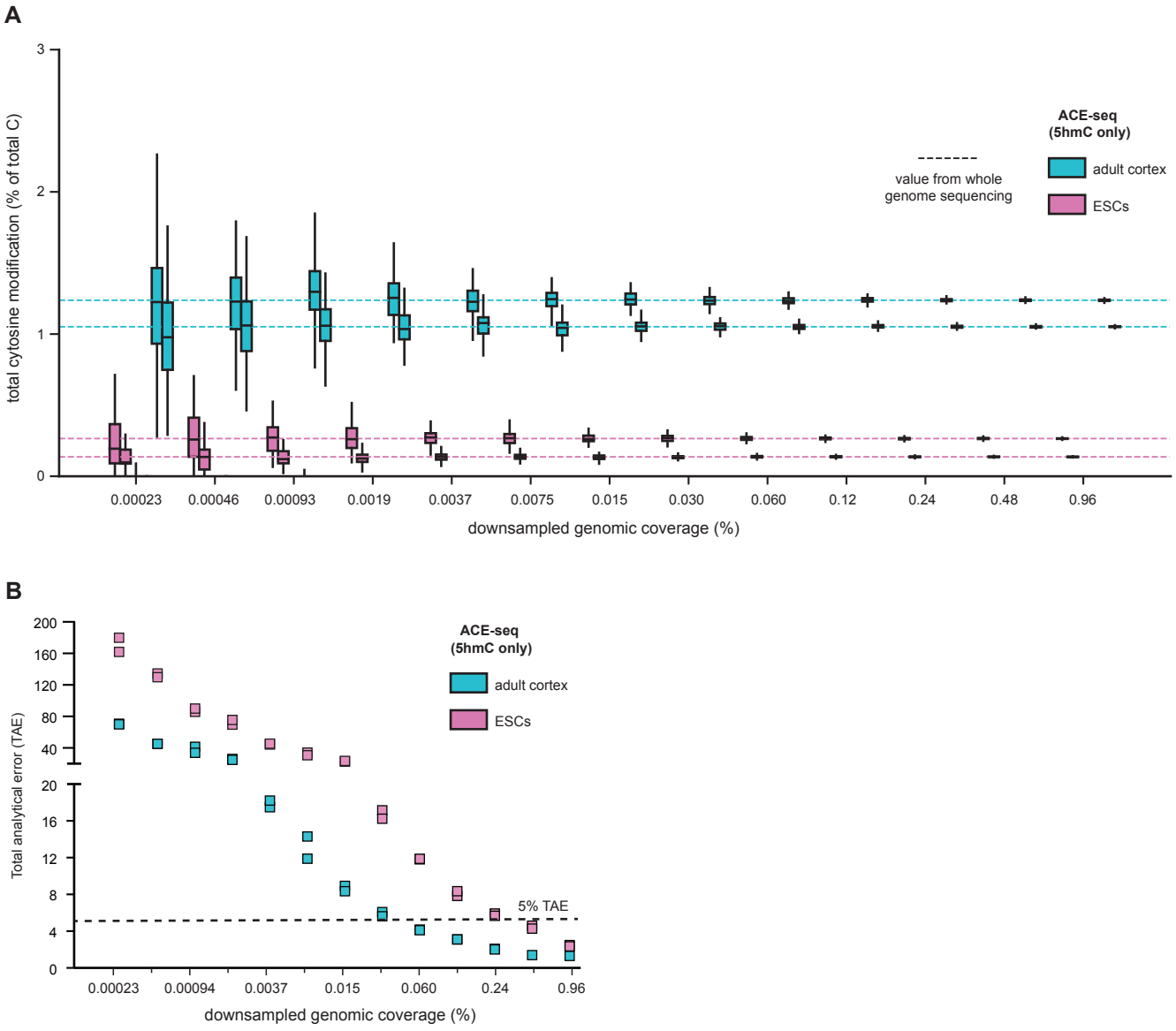
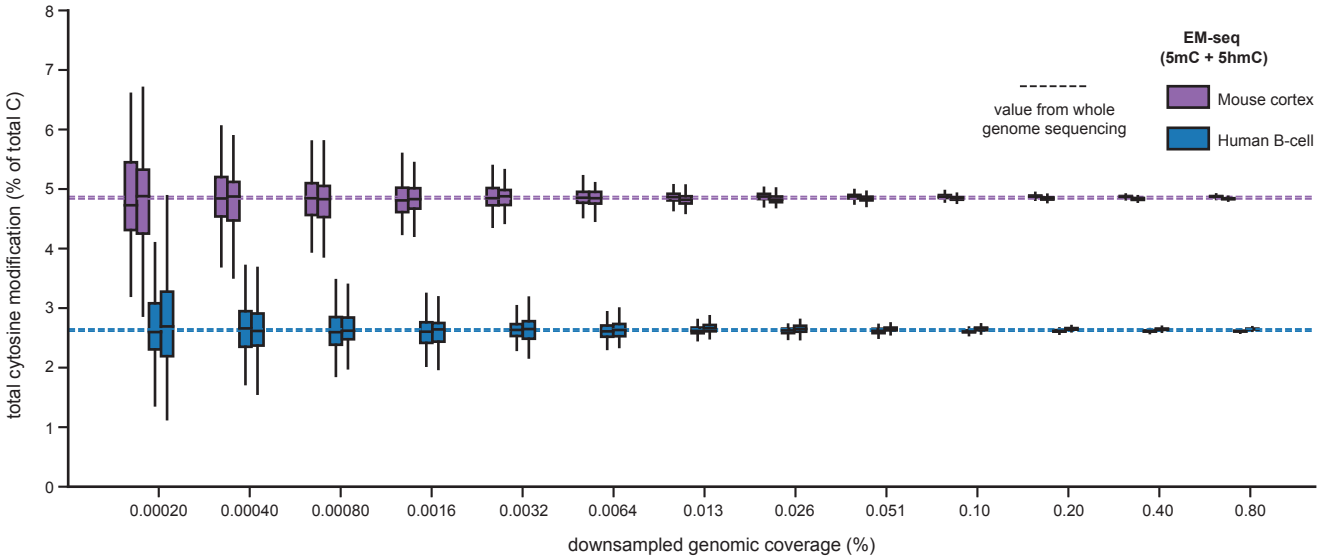
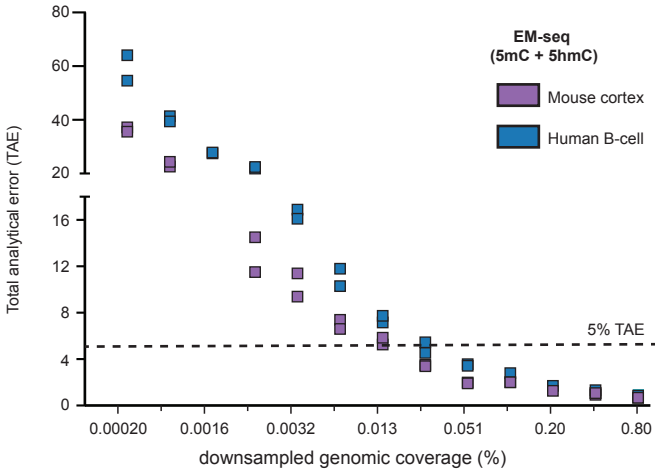

A
total cytosine modification (% of total C)
3
2
1
0
ACE-seq
(5hmC only)
value from whole
genome sequencing
adult cortex
ESCs
0.00023
0.00046
0.00093
0.0019
0.0037
0.0075
0.015
0.030
0.060
0.12
0.24
0.48
0.96
downsampled genomic coverage (%)
B
Total analytical error (TAE)
200
160
120
80
40
20
16
12
8
4
0
ACE-seq
(5hmC only)
adult cortex
ESCs
5% TAE
0.00023
0.00094
0.0037
0.015
0.060
0.24
0.96
downsampled genomic coverage (%)

Figure S2

A


EM-seq
(5mC + 5hmC)
value from whole genome sequencing
Mouse cortex
Human B-cell
total cytosine modification (% of total C)
0
1
2
3
4
5
6
7
8
0.00020
0.00040
0.00080
0.0016
0.0032
0.0064
0.013
0.026
0.051
0.10
0.20
0.40
0.80
downsampled genomic coverage (%)

B


EM-seq
(5mC + 5hmC)
Mouse cortex
Human B-cell
Total analytical error (TAE)
0
4
8
12
16
20
40
60
80
5% TAE
0.00020
0.0016
0.0032
0.013
0.051
0.20
0.80
downsampled genomic coverage (%)