MethylSeqR: An R package for efficient analysis of modified nucleotide data

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1 Abstract

DNA methylation regulates a wide range of biological processes, including gene

expression, disease progression, and cell identity. Long-read technologies now enable

more comprehensive and accurate methylome analyses than ever before, but they are

hindered by the computational resources needed to analyze the massive datasets. Here,

we present the CH3 file format, which aids data storage and transfer by reducing file

sizes by more than 95%, and the MethylSeqR R package, which builds on the CH3

format and a database backend to enable a broad range of epigenetic analyses. Together,

these tools enable high-throughput methylation analysis while minimizing computational

resource requirements.

11 Background

DNA methylation plays vital roles in regulating cellular identity and function in humans

and other animals. In this process, methyl groups are added to the fifth carbon of cytosine

residues, typically within cytosine-guanine dinucleotide (CpG) contexts. Aberrant

methylation patterns can destabilize the genome and disrupt normal gene expression,

contributing to numerous human diseases and serving as important diagnostic

biomarkers[1,2]. Beyond human biology, DNA methylation offers insights into the cellular

processes across numerous other animal species[3]. Accurate detection and analysis of

DNA methylation patterns are therefore essential for understanding development, disease

mechanisms, and responses to environmental changes[4].

Traditional approaches to DNA methylation profiling have relied heavily on microarray

technologies such as the Illumina MethylationEpic Bead Chip. These arrays, while cost-

effective and high-throughput, are inherently limited in genomic coverage and

resolution[5]. More recent sequencing-based methods, including bisulfite sequencing and

enzymatic methyl-seq (EM-seq), have extended our ability to interrogate methylation at

single-base resolution. However, they introduce additional limitations, including biases

introduced during cytosine conversion and PCR amplification, as well as computational

analysis challenges due to the number of mismatches introduced by the conversion

process. In contrast, native methylation sequencing directly analyzes unamplified,

unconverted DNA, avoiding these limitations. Platforms such as Oxford Nanopore

Technologies (ONT) and PacBio have enabled native, PCR-free sequencing and

subsequent genome-wide detection of DNA modifications in real time and at high

resolution[6,7]. These third-generation technologies offer more comprehensive and unbiased

epigenetic analyses than array-based platforms and other traditional methods[8]. These

technologies present significant technical advantages in most use cases, especially when

sequencing genomes from non-model organisms or exploring genomic regions that are

poorly represented by commercial arrays[9]. A complete analysis of the performance of

ONT sequencing is covered in the accompanying paper (cosubmission citation).

Despite its promise, native methylation sequencing data remains challenging to work

with due to its large size and lack of streamlined analytical tools[10]. A single pass of the

human genome (1x) produces 3-5 gigabases (Gbases) of data. Thus, sequencing at deeper

coverages or with multiple samples can produce terabytes of information. Available

tools, such as NanoSpring, are built to address this issue by compressing raw sequencing

data[11]. However, these tools fall short in providing support for further analysis. Various

software packages, such as the widely used MethylKit, provide differential methylation

analysis capabilities, but were primarily developed for short-read bisulfite sequencing

data[12]. Consequently, their performance is suboptimal for analyzing other data types such

as nanopore sequencing[13]. As a result, there remains an urgent need for efficient tools for

downstream analysis of long-read, native DNA methylation data.

To address these gaps, we developed a two-part solution: a highly-efficient modified

nucleotide data storage format (CH3) that reduces nanopore methylation data size by 90-

95%, and MethylSeqR, a publicly available R package for fast, flexible downstream

analysis. MethylSeqR supports differential methylation analysis at single-base or regional

levels, includes built-in visualization tools, and offers customizable parameters for

diverse experimental designs. Here we present the package's workflow, features, and

performance.

1 Results

2 CH3 - Efficient DNA Modification Data Storage

Methylation sequencing generates large volumes of raw data, typically in the form of

binary alignment map (bam) files containing raw read sequences. When modified base

calling is enabled, two tags are added to each sequence record in the file: one for the

modified base call positions and a second with probabilities for modification at the

position. In order to analyze the data, these files undergo a series of pre-processing steps,

as summarized in **Figure 1a**. This process includes alignment, merging, sorting, and

indexing. Finally, base modification predictions—such as methylation events—can be

extracted and stored in .tsv files using tools like the modkit package

(https://github.com/nanoporetech/modkit). While convenient as a human-readable format,

these plain-text files are often unmanageably large, presenting challenges for downstream

analysis, storage, and sharing.

Figure 1: Specification for the CH3 file format. A) The general workflow used to

generate a CH3 file. B) Sample CH3 file contents. C) Memory comparison between CH3

and modkit calls formats across 29 nanopore sequencing runs. Each bar represents the

total file size in gigabytes (GB) for a single sequencing run, with modkit calls files shown

in light blue and the corresponding CH3 files shown in red.

To address the limitations inherent in the standard methylation output format, we

developed the custom CH3 file format (named after the chemical formula for a methyl

group), a compact, efficient representation of base modification calls designed for

efficient compression and retrieval. This format contains all essential nucleotide

modification information, including the sample name, read ID, chromosome, 0-based

reference position, read length, query k-mer, call probability, call code, base quality, and

samtools flag value. The CH3 file specification is also extensible, so additional columns

can be added to meet unique needs. Internally, the CH3 format leverages the Apache

Parquet framework (https://parquet.apache.org/), enabling fast, column-based access and

easy use in many popular programming languages such as R, Python, C++, and Java. The

Parquet format reduces file size by using dictionary-based encoding for string columns,

and incorporates the zstd compression algorithm (https://github.com/facebook/zstd) to

efficiently represent data within the file while allowing for fast access. In addition to the

inherent efficiencies of the Parquet format, the CH3 file specification also relies on the

samtools flag, which encodes alignment classification (unaligned, primary, secondary, or

supplementary) and strand (+ or -) into a single integer instead of having multiple

columns for this information (**Figure 1b**), further reducing its size. Together, these

factors reduce the size of methylation data stored in TSV files to just 5–10% of their

original size, ensuring that large-scale epigenomic datasets can be stored, transferred, and

queried using minimal computational resources (**Figure 1c**).

MethylSeqR - An R Package for Analyzing Modified DNA

Current R packages for analyzing modified DNA have two major limitations. First, they

are built around a bisulfite conversion paradigm. Thus, they do not support direct

detection of modifications and are generally limited to methylation calls. However, third-

generation sequencing platforms, such as the Nanopore platform, can directly detect a

large number of modifications. Second, the amount of data generated from sequencing is

extremely large. For example, there are approximately 30 million CpG dinucleotides in

the human genome. At a sequencing depth of 10x, that is 300 million calls per sample.

This is orders of magnitude larger than the 900,000 data points generated by the Epic

Array and large enough to use the entire vector memory limit on machines with modest

hardware specifications.

To address both of these issues, we have created the MethylSeqR package (outlined in

Figure 2a). This package uses a DuckDB database backend, allowing users to conduct

large, complex analyses on a wide range of hardware[14]. The database is managed

internally to maintain ease of use and familiarity for users accustomed to using R's

conventional data structures and paradigms (e.g. piping) by managing the database

internally. The package also provides several built-in functions for working with the base

call data to facilitate common workflows, including QC, data summarization by position,

read, window, and region, and differential methylation analysis. An example pipeline

with explanations for each function is shown in **Figure 2b-c**. Below, we walk through the

major steps of an analysis workflow and highlight package functionality and outputs.

Figure 2: MethylSeqR Core Design and Workflow. A) Schematic of possible

MethylSeqR workflows, starting from the input of CH3 files and progressing through

database creation, summarization, analysis, and output. B) Example pipeline for a full

differential methylation pipeline using R's native pipe syntax. Pipeline begins with

creating a database, then summarizes data using sliding windows, and performs a

differential methylation analysis. Finally, significant windows are collapsed into merged

regions, and the results are exported to a user-defined path. C) The core functions used in

the example pipeline in Panel B, along with their required arguments and a brief

description.

Database Creation and Filtering

The first step in any MethylseqR pipeline is to create a database with all samples to be

included in the analysis (**Figure 2a**). The CH3 file format is designed to store the data

from a single sample, while the database combines multiple samples into a single data

structure, a calls table, for analysis (**Supplemental Figure 1**). The database is written

into the local directory as "filename.ch3.db", and is updated as tables are created within

it, allowing persistent storage of intermediate data structures and limiting the need to

rerun calculations between sessions or to maintain large amounts of data in memory

during multistep pipelines.

During this stage, the data can also be filtered for a variety of QC parameters, including

minimum read length, minimum call probability, and minimum base quality.

Additionally, users can choose to focus on specific types of modifications or

combinations of several modifications. For example, users importing data from CH3 files

containing methylation and hydroxymethylation can choose to use either modification,

combine both into a single call, or retain all modifications. This level of customization

empowers researchers to refine their workflows and explore epigenetic patterns in a way

that aligns with their biological questions and data characteristics.

1   **Summarizing DNA methylation information over positions, pre-defined**

2   **regions, or tiling windows**

The modification calls stored in the database can be summarized in many ways

(**Figure 2a**). MethylSeqR is able to summarize DNA methylation data across individual

genomic positions, predefined regions (such as promoters, CpG islands, or introns), or

sliding windows for further analysis. It also supports read-level summarization for

mixture analysis (described below). These summaries can then be used for downstream

analyses of these unique sites of interest. For more detailed explanations of all functions

within the package, refer to the MethylSeqR vignette.

Users have the accessibility to export any resulting summary tables directly into their R

environment for further exploration with the get_table() function. This includes data

summarized by positions, windows, regions, reads, or a differential methylation analysis.

Example summarization table outputs using a synthetic dataset are shown in

Supplemental Figure 2. Additionally, users may save the data table as a CSV file for

external use with export_table().

16   **Differential Methylation Analysis**

A differential methylation analysis can be performed to identify statistically

significant differences in DNA methylation levels at individual bases or genomic

windows between two different sample groups. This analysis is essential for detecting

epigenetic changes that may play roles in gene regulation, development, or disease

processes such as cancer and neurological disorders[15]. MethylSeqR provides multiple

methods for conducting differential methylation analysis, including logistic regression,

Fisher's exact test, and a rapid implementation of Fisher's test (r_fisher). MethylSeqR

generates differential statistics for each group, including modification fraction difference

and p-value calculations for regions. For an example output table, see **Supplemental**

Figure 3. The output of a differential methylation analysis can be plotted as a volcano

plot (**Figure 3a**) or a methylation correlation plot (**Figure 3b**).

To test the accuracy of the statistical analyses, we created a synthetic dataset with

known modification differences between example case and control groups. When

differential analysis was performed using MethylSeqR, our function accurately identified

all positions and their corresponding modification difference between the groups.

Figure 3: Example Output Plots for Differential Expression Analysis. A) Example

differential volcano plot generated with a custom MethylSeqR function. Each square

represents a genomic window/position. The x-axis is the methylation-fraction difference

between the case and control samples; the y-axis is the statistical significance. Density

correlates with darker colors, and points near the center indicate regions with little or no

difference. B) Case-vs-control fraction concordance plot built with a custom function.

Each location's modified fraction in the case (x-axis) is plotted against the corresponding

fraction in the control (y-axis). The background is a 2-D kernel-density surface:

red/yellow areas mark high point density, blue areas low density.

QC metrics: descriptive statistics, sample correlation, and PCA

In addition to its core analysis pipelines, MethylSeqR provides a suite of tools for whole

methylome characterization, including general quality control metrics, sample-level

comparisons, and dimensionality reduction. Users can visually assess key characteristics

of their methylation data using built-in plotting functions (**Figure 4**). Two primary

visualization options are available: read coverage per base, which provides insight into

sequencing depth and data reliability, and percent methylation per base, which reflects

the underlying biological signal. These metrics are stored in the package's core data

structures and are easily accessible for customized visualizations.

Figure 4: Descriptive Statistics and Quality Control Function Outputs. A) Percent

methylation per base graph: This histogram shows the distribution of CpG methylation

percentages across all bases for a sample. It provides an overview of the methylation

landscape, allowing users to quickly assess whether methylation values are skewed,

bimodal, or evenly distributed. B) Read coverage graph: This histogram displays the

distribution of CpG read coverage (on a log10 scale) per base. It helps evaluate the depth

and consistency of sequencing coverage, which is critical for assessing the reliability of

methylation calls. C) PCA plot: The principal component analysis (PCA) plot reduces

high-dimensional methylation data into two main components (PC1 and PC2) that

capture the largest variance. This visualization helps identify clustering of samples and

potential batch effects or outliers. D) Correlation plot: The sample correlation matrix

helps visualize the pairwise Pearson correlation coefficients between samples based on

methylation values. It quantifies the linear relationship between samples, helping assess

similarity and reproducibility across experimental conditions.

To compare methylation profiles across samples, MethylSeqR includes correlation plots

based on the Pearson correlation coefficient, a measure of the linear relationship between

two continuous variables. Additionally, a principal component analysis (PCA) function is

provided to explore global patterns in methylation data and detect clustering among

samples. PCA reduces the dimensionality of high-throughput data by projecting it into a

smaller set of orthogonal components that capture the greatest variance, helping reveal

biological trends or batch effects across the dataset.

Performance

MethylSeqR is designed to be computationally efficient and capable of analyzing large

datasets in practical runtimes. Thus, we tested the performance of several key analysis

steps on various dataset sizes. The most computationally intense step within the analysis

is summarization. The database is initially created with one row per methylation call.

These calls are then summarized into positions, reads, regions, or sliding windows across

all samples, which requires large amounts of database read and write calls. As shown in

Figure 5a, the time to conduct the summarization depends on both the type of

summarization and the size of the dataset. Importantly, however, the computation

completes for all dataset sizes, even when larger than the memory available. Differential

analysis also shows the same smooth increase in time without failing (**Figure 5b**).

Finally, we compared run times between an analysis server with 250 GB of RAM and 96

cpu cores compared to a personal computer with 16 GB of RAM and 8 cores (**Figure 5c**).

While run times were significantly longer on the personal computer, times were still

reasonable, and all analyses were completed.

Figure 5: MethylSeqR Performance on different hardware configurations. A)

Summarizing Data Time vs. Size for different data collection functions. This includes

summarizing methylation data by windows (100 bp step size), reads, genomic positions,

or regions. B) Differential Analysis Time vs. Size. This figure depicts a differential

analysis between three available options (regions, positions, and windows). These

functions were run on a personal computer with 16GB of RAM. C) Time vs. RAM

available for 4 functions on two different machines - one is a personal computer with

16GB of RAM available, while the other is a large server with 250GB of memory

available. The dataset consisted of 4 samples of 5x coverage.

Discussion

Analyses of DNA methylation and other nucleotide modifications have enormous

potential to provide novel biological insights into both natural processes and disease

mechanisms. While such analyses have been conducted for more than a decade, the

recent development of direct modified base calling on the NanoPore and PacBio

platforms has greatly increased genomic coverage of these assays. For example, the

current Epic Array has approximately 900 thousand of the approximately 30 million CpG

sites in the genome (approximately 3%). However, sequencing regularly provides

information on over 28 million sites (93%) (see cosubmission). This is a significant leap

forward that will provide significant insights missed by previous technologies.

Although the increased genomic coverage provided by third-generation sequencing is

very welcome, it also results in a significant increase in the size of the data files

produced, hindering analysis by requiring large amounts of computational resources and

specialized bioinformatics know-how to manage the data. Here, we have presented two

tools to address these issues: the CH3 file format and the MethylSeqR R package. These

tools, used together, will open up modified nucleotide analysis to more users and drive

wider adoption across the field.

One of the primary challenges with large datasets lies in the cost and complexity of

storing and transferring them—difficulties that will only intensify as data generation

continues to grow. Thus, we created a file format specification, CH3, that addresses these

problems by vastly decreasing the file size for modified base data. Analyses presented

here show that CH3 typically reduces file sizes by 95%. These reductions come from a

number of sources, including the removal of redundant and unnecessary data fields,

efficient compression algorithms, and careful selection of data types. File access and read

times are also improved by using column-based storage and internal indexing.

Importantly, the file specification is designed to be used with any modification code and

allows for additional columns to be added by the user to address specific use cases and to

future-proof the format. Thus, this file format will provide a strong foundation for future

studies on nucleotide modifications.

We also present in this paper the first R package designed specifically to work with

native modified base calling. As the first tool, we do not have any other tools to compare

to, but we were able to establish that the tool is able to handle large amounts of data and

provide accurate results using a synthetic dataset. We have also incorporated a large

number of functions to access and interact with the data, measure QC, and conduct

differential methylation analyses on positions, sliding windows, annotated regions, or

reads. As we look to the future, our team plans to further enhance MethylSeqR's

analytical capabilities by integrating sophisticated machine learning algorithms, such as

k-Nearest Neighbors (KNN) and regularized regression models, further advancing

modified base analysis in a number of use cases[16].

By dramatically reducing data storage requirements and offering an accessible, purpose-

built R package, our work lowers key technical barriers that have limited the broader

adoption of third-generation sequencing for epigenomic research. As the field continues

to evolve, we anticipate that these tools will not only accelerate discovery but also inspire

the development of new methodologies for understanding the biological roles of modified

nucleotides in health and disease.

Conclusions

The CH3 file format and MethylSeqR R package introduced in this study represent

important advances for the analysis of native DNA modifications using third-generation

sequencing. By addressing key challenges in data size, accessibility, and analytical

flexibility, these tools enable researchers to efficiently store, manage, and analyze high-

throughput methylation data across a range of hardware configurations. The CH3 file

format significantly reduces storage demands while preserving analytical utility, and

MethylSeqR provides a comprehensive and user-friendly R-based framework for

differential methylation analysis, quality control, and visualization. Together, they

remove critical technical barriers and empower broader use of native nucleotide

modification data across biological research. As interest in epigenetic mechanisms

continues to expand, these tools will serve as a foundational platform for scalable,

accessible, and reproducible studies of DNA methylation and other base modifications in

both health and disease contexts.

3 Methods

4 CH3 File Specification

To address the challenges of storing large-scale native base modification data, we

developed the CH3 file format—a compressed, columnar format based on Apache

Parquet. CH3 includes required fields such as read ID, chromosome, position,

modification type, and probability, and supports additional optional metadata. File

reduction is achieved via dictionary encoding and Zstandard compression, both natively

implemented as part of the Parquet standard. The format also supports fast, column-based

access and is compatible with MethylSeqR and common data science tools (e.g.,

DuckDB, PyArrow). Full specification details, including field definitions and encoding

rules, are available at https://github.com/Wasatch-Biolabs-Bfx/CH3.

14 MethylSeqR Implementation

15 MethylSeqR is implemented in R and SQL and structured around modular functions that

16 facilitate scalable, memory-efficient analysis of native base modification data. At its core,

17 the package uses a DuckDB backend to support disk-based storage and efficient SQL-like

18 querying of CH3-formatted data. Key functions are organized into workflow stages,

19 including database creation (create_ch3_database()), read filtering (filter_ch3_reads()),

summarization (summarize_ch3_positions(), summarize_ch3_windows(),

summarize_ch3_regions()), and differential methylation analysis (calc_ch3_diff()) using

either Fisher's exact test or logistic regression. Additional tools provide sample

correlation (calc_ch3_samplecor()), read classification (classify_ch3_reads()), quality

control, and dimensionality reduction via PCA. Each function is optimized to perform

reliably on systems with limited RAM and supports both single-sample and multi-sample

analysis workflows. Extensive helper functions manage database connections, cleanup,

and internal metadata. MethylSeqR supports piping syntax and outputs tidy data frames,

making it highly compatible with the broader tidyverse ecosystem. Full source code and

documentation are available at: https://github.com/Wasatch-Biolabs-Bfx/MethylSeqR.

Testing Data

We created a synthetic dataset to test the accuracy of our summarization functions and

differential analysis. We hope this will also enable a fast workflow through all steps of

the package. The positions are divided into deliberately chosen effect-size bands: eight

groups with absolute methylation-fraction shifts of ±0.90, ±0.50, ±0.25 and ±0.10 (60

loci per band), and two mid-fraction bands (0.25–0.50 and 0.50–0.75; 120 loci each). We

also included randomized noise for more realistic visuals. This dataset contains two CH3

files and is also publicly available for users to create a database and test the package

under the inst/extdata/ directory in the GitHub repository.

1 Performance Benchmarking

Performance testing was conducted on two markedly different hardware configurations to

assess scalability and resource utilization. The first environment was a MacBook Pro

equipped with an M3 processor and 16 GB of RAM, representing a typical end-user

system. The second environment was a high-performance server with 250 GB of

available memory and an AMD Threadripper processor with 96 cores, providing

substantially greater computational capacity.

8 Declarations

Software Availability and Requirements:

Project name: MethylSeqR

Project home page: https://github.com/Wasatch-Biolabs-Bfx/MethylSeqR

Operating system(s): Platform independent (works on Windows, macOS, and Linux

wherever R is available).

Programming language: R

Other requirements: R package dependencies include: arrow, DBI, dplyr, dbplyr,

duckdb, duckplyr, ggplot2, progress, tidyr, utils, readr (Imports). Optional for

vignettes/docs: knitr, rmarkdown (Suggests).

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Exception) provided for internal research use by commercial entities.

Any restrictions to use by non-academics: Internal evaluation and research only.

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Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The dataset analyzed during the current study is available in the MethylSeqR repository

through the Wasatch-Biolabs-Bfx organization, at https://github.com/Wasatch-Biolabs-

Bfx/MethylSeqR.

Competing interests

JH and TJ are cofounders of Wasatch Biolabs, which is currently applying for patents for

the CH3 file format and MethylSeqR package.

Authors' contributions

HZ and JH wrote the code for the MethylSeqR package, as well as writing this

manuscript. RM and AJ assisted in the development of the package and writing of this

paper. IS assisted in writing and figure generation. ES assisted in writing and editing the

manuscript. TJ helped oversee the project and provided guidance for methylation analysis

standards.

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b)
```
ch3_db <- make_ch3_db(
        ch3_files = "/desktop/ch3_files",
        db_name = "my_data") |>
    summarize_ch3_windows() |>
    calc_ch3_diff(
        call_type = "windows",
        cases = c("sperm"),
        controls = c("blood")) |>
    collapse_ch3_windows() |>
    export_ch3_table(
        table ="mod_diff_windows",
        out_path = "/desktop/results")
```

c)

Function	Required Arguments	Description
make_ch3_db	ch3_files db_name	Processes a directory of ch3 files and creates a database containing filtered and structured methylation call data.
summarize_ch3_windows	ch3_db	Summarizes methylation data by creating sliding windows over the genome
calc_ch3_diff	ch3_db cases controls	Calculates differential methylation between specified case and control groups
collapse_ch3_windows	ch3_db	Identifies and merges nearby significantly differentially methylated windows after a differential methylation analysis is called

`run_ch3_qc`(ch3_db)