



Biomek i7 Hybrid NGS Workstation Automated Methylation Sequencing

Introduction

In mammalian genomes, approximately 70 to 80 percent of cytosine-guanine pairs contain methylated cytosine, including either 5-methylcytosine (5mC) or its oxidated form, 5-hydroxymethylcytosine (5hmC). These modifications regulate gene expression and affect a wide range of biological and pathological processes, including embryonic development and cancer susceptibility.^{1,2} Because methylated cytosine can be associated with either transcriptional silencing or gene activation, depending on genomic location, accurate 5mC and 5hmC detection is crucial for understanding many molecular pathways.¹

Currently, bisulfite sequencing is the standard method for mapping the methylated genome, or methylome. This technique relies on chemical conversion of unmethylated cytosines to uracil by sodium bisulfite treatment prior to sequencing. However, bisulfite library preparation exposes samples to extreme temperatures and pH changes, which damages DNA and leads to uneven genome coverage. As a result, bisulfite sequencing does not accurately capture the complete methylome.¹

The NEBNext® Enzymatic Methyl-seq workflow from New England Biolabs provides a much-needed alternative to bisulfite sequencing. It relies on the APOBEC enzyme to deaminate cytosines to uracils, and TET2 and an Oxidation Enhancer to enzymatically modify and protect 5mC and 5hmC from deamination. Unmethylated cytosines are sequenced as thymines and 5mC and 5hmC are sequenced as cytosines, allowing researchers to distinguish between modified and unmodified sites.¹

Although various high-throughput sequencing approaches to methylome mapping are possible with next generation sequencing (NGS), many existing methods rely on bisulfite conversion.³ The following experiments examine how the Beckman Coulter Biomek i7 Hybrid NGS Workstation can be combined with the NEBNext® Enzymatic Methyl-seq kit manual protocol for automated, high-throughput methylome mapping.

Methodology

Enzymatic methyl sequencing

To detect 5mC and 5hmC throughout the methylome, the NEBNext® Enzymatic Methyl-seq workflow uses an enzymatic pathway involving TET2 oxidation and APOBEC deamination. Compared to traditional sodium bisulfite conversion, this pathway results in less DNA damage and more uniform GC coverage.

In summary, the NEBNext® Enzymatic Methyl-seq manual protocol starts with genomic DNA and performs the following steps:

- Genomic DNA shearing to 300 base pairs
- End repair, tailing, and adaptor ligation
- Oxidation of 5mC and 5hmC with TET2 and Oxidation Enhancer to protect modified cytosines from deamination
- Deamination of unmethylated cytosine to uracil with APOBEC enzyme
- PCR amplification of library
- Sequencing of amplified methylome library

Automated method

To automate the NEBNext® Enzymatic Methyl-seq protocol, the Biomek i7 Hybrid NGS Workstation (**Figure 1**) was incorporated into the workflow. The Biomek i7 Hybrid NGS Workstation is designed with efficient workflow planning and ease of use in mind. It is an ideal platform for automating all steps of medium- to high-throughput NGS workflows, from extraction through library construction. To reduce hands-on time while increasing throughput and productivity, multichannel and Span-8 pipetting heads are included in the Biomek i7 Hybrid NGS Workstation, as well as standard on-deck process control elements such as shakers, Peltiers for temperature control, and thermocyclers. The automated NEBNext® Enzymatic Methyl-seq method utilizes one static Peltier for cooling reagents, a shaking Peltier to maintain the sample plate during processing, and an optional on-deck thermocycler for PCR amplification and incubations. To reduce the risk of cross-contamination, the method uses filter tips for all transfers.

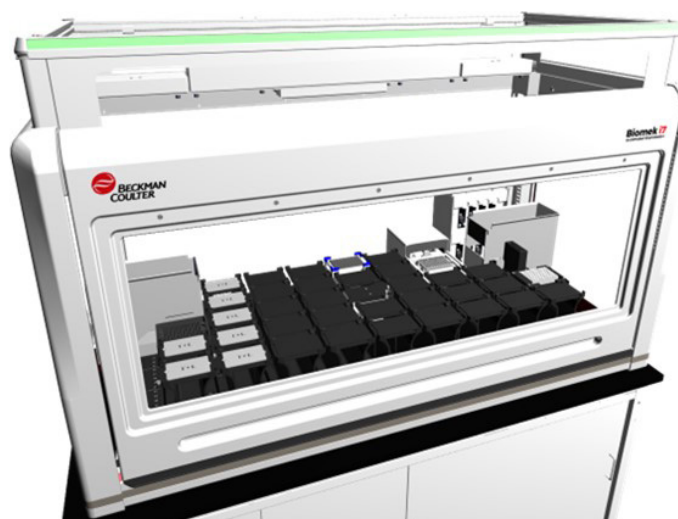


Figure 1. The Biomek i7 Hybrid NGS Workstation.

For methyl sequencing with the Biomek i7 Hybrid NGS Workstation, the automated protocol comprises several logical start/stop points built-in based on the manual protocol recommendations. These sections can be run independently or as part of a larger workflow and allow for more efficient workflow planning while enabling a completely walk-away solution (**Figure 2**).

Optional start points include but are not limited to:

- prior to oxidation of 5mC and 5hmC
- prior to deamination with APOBEC
- prior to PCR amplification

Safe stop points include but are not limited to:

- after end repair and adaptor ligation
- after enzymatic treatment and cleanup steps
- after PCR amplification and library cleanup

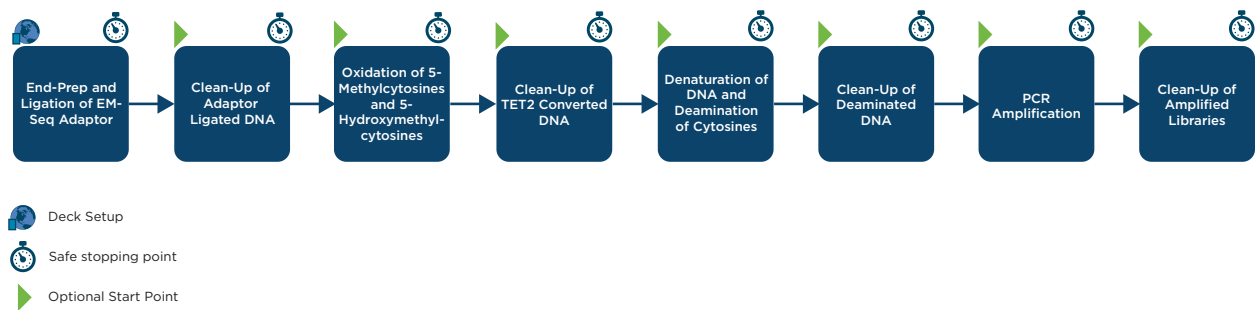


Figure 2. New England Biolabs NEBNext® Enzymatic Methyl-seq Kit automation workflow on the Biomek i7 Hybrid NGS Workstation.

The automated method is divided into eight sections that can be run independently or collectively. Optional start points have been built-in for seven of the eight sections, while safe stop points exist after each section.

The workflow is suited for up to 96 samples per run, requiring only 30 minutes of hands-on time for setup. The application can be launched using the Biomek Editor software. Alternatively, the application may be launched using Biomek Method Launcher software, which provides an easy-to-use launch experience while also utilizing the Biomek system cameras to confirm labware placement using DeckOptix Final Check. The application also features an HTML-driven Method Option Selector which permits protocol configuration flexibility, including:

- Selecting the number of samples to be processed
- Selecting the number of method sections to run based on the established safe stop points
- Selection of the Index Transfer Starting Column
- Selecting the number of PCR amplification cycles

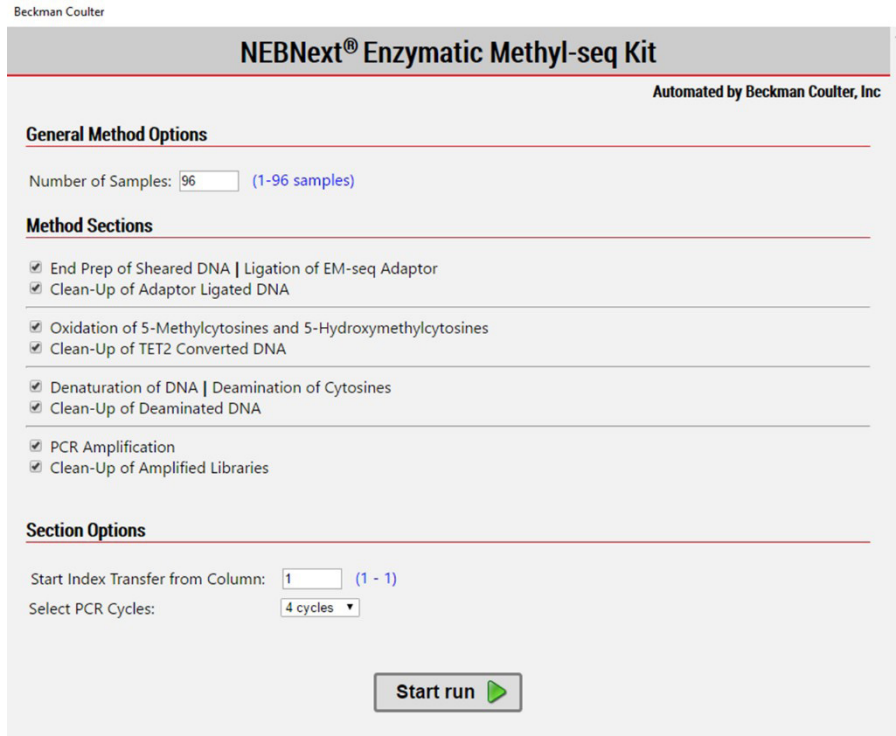


Figure 3. New England Biolabs NEBNext® Enzymatic Methyl-seq Kit automation workflow on the Biomek i7 Hybrid NGS Workstation Method Option Selector.

The software is also capable of providing clear instructions for labware setup and reagent volume requirements, based on the configuration of the protocol inputs.

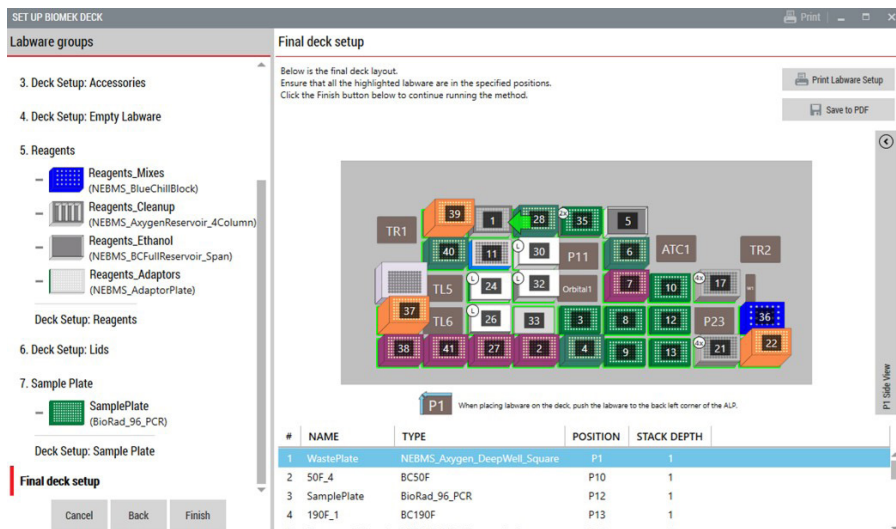


Figure 4. Guided Labware Setup of the New England Biolabs NEBNext® Enzymatic Methyl-seq Kit automation workflow on the Biomek i7 Hybrid NGS Workstation.

Experimental design

An automation run involving genomic DNA isolated from 94 human blood samples was performed on the Biomek i7 Hybrid NGS Workstation at Dana-Farber Cancer Institute’s Molecular Biology Core Facilities. Library preparation was performed entirely on the Biomek i7 Hybrid NGS Workstation. In a second experiment, four libraries using DNA isolated from human cell lines were produced using the automated method. These libraries were more deeply sequenced using an Illumina NovaSeq sequencer and aligned back to the human reference genome. All bioinformatic analysis was performed by the Dana Farber Cancer Institute Molecular Biology Core Facilities.

Results

To examine the accuracy and efficiency of the automated NEBNext® Enzymatic Methyl-seq protocol, two experiments were performed on the Biomek i7 Hybrid NGS Workstation at Dana-Farber Cancer Institute’s Molecular Biology Core Facilities. As mentioned before, one automation run involved genomic DNA isolated from 94 human blood samples, and in a second experiment, four libraries using DNA isolated from human cell lines were produced with the automated method.

In the first experiment, 200 ng of genomic DNA was utilized for each library. Positive and negative methylation controls—pUC19 and lambda DNA, respectively—were included with the genomic DNA samples as recommended in the manual protocol. Following library preparation on the Biomek i7 Hybrid NGS Workstation, libraries were assayed on the Agilent TapeStation 4200 using a D1000 ScreenTape. The majority of libraries were first sequenced on an Illumina NextSeq 550 using a 1x75 bp sequencing run with a High Output sequencing kit and aligned back to the pUC19, lambda DNA, and human reference genome (hg38) to generate a first-pass assessment of methylation conversion rates.

After library preparation, 93 out of 94 libraries passed TapeStation analysis, with an average library yield of 4.3 ng/μL and an average library size of 472 bp (**Figure 5**). 86 of these 93 libraries were then sequenced on the NextSeq 550, generating approximately 2 million reads per library.

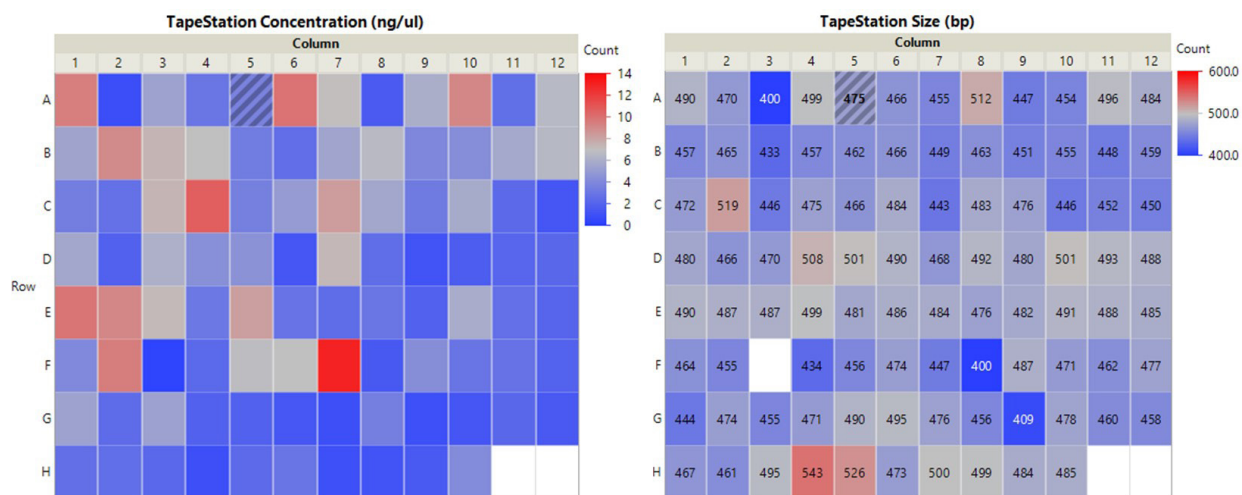


Figure 5. Agilent TapeStation concentration (left) and library size (right). Libraries were assayed on the Agilent TapeStation using a D1000 ScreenTape to determine size and concentration. A single library failed TapeStation analysis. The average library yield and size were 4.3 ng/μL and 472 bp, respectively.

The pUC19 positive methylation control showed high conversion rates of 5mC and 5hmC bases (greater than 98%) while the lambda DNA negative methylation control showed conversion rates of less than 0.003% for unmethylated cytosine bases (**Figure 6**), indicating successful conversion of methylated cytosine bases only.

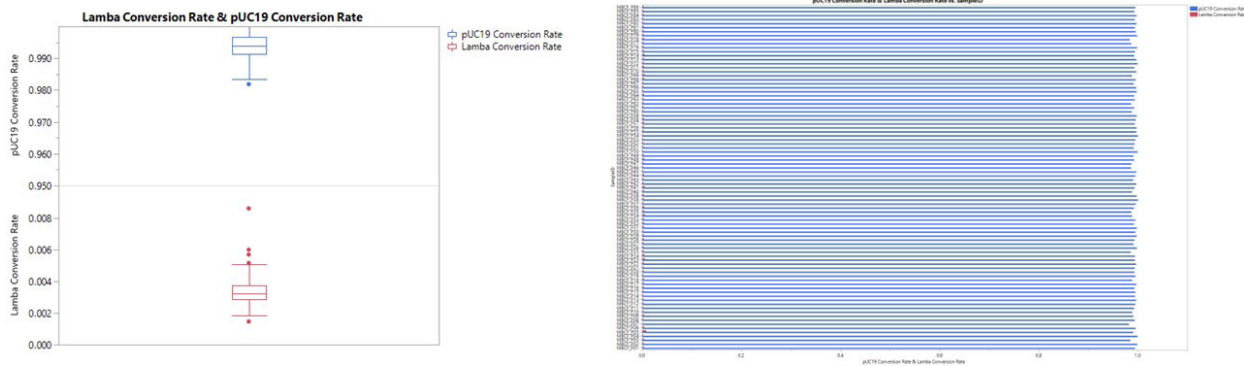


Figure 6. pUC19 and lambda DNA conversion rates. 86 libraries were lightly sequenced on a NextSeq 550 using a High Output kit (1x75 bp) and aligned back to hg38. pUC19 positive control DNA showed conversion rates greater than 98%, while negative control lambda DNA showed very low rates of conversion (0.003%).

In the second experiment, all four libraries passed TapeStation analysis and were subsequently sequenced on the Illumina NovaSeq. Following sequencing, over 99% of reads in each library aligned back to the human reference genome, with a median genome coverage of 21X. Percent GC content was similar across all four libraries (**Table 1**).

Sample	Percent Aligned to Reference Genome	Median Coverage	Genome Regions with \geq 30X Coverage	Percent Duplicates	Percent GC Content
MBCF_CL1	99.6%	13X	11.5%	18.5%	26%
MBCF_CL2	99.9%	28X	45.7%	28.2%	24%
MBCF_CL3	99.9%	23X	25.5%	23.8%	24%
MBCF_CL4	99.9%	21X	21.7%	23.8%	25%

Table 1. Deep sequencing metrics for four cell line Methyl-seq libraries.

Conversion rates of the pUC19 positive methylation control were high in all samples (>98.9%), while the lambda DNA negative methylation control conversion rates were 1.3% or lower. Cell line genomic DNA methylation was estimated to be 65% or higher for all cell line libraries (**Table 2**).

Sample	gDNA Percent Methylation	Lambda Percent Methylation (unmethylated control)	pUC19 Percent Methylation (methylated control)
MBCF_CL1	66.24%	1.36%	99.23%
MBCF_CL2	66%	0.34%	99.14%
MBCF_CL3	65.74%	0.24%	99.02%
MBCF_CL4	65.91%	0.31%	98.96%

Table 2. Methylation estimates for four cell line Methyl-seq libraries.

Conclusion

The Biomek i7 Hybrid NGS Workstation automates the NEBNext® Enzymatic Methyl-seq kit manual protocol, facilitating efficient methylome sequencing. The generated methylome libraries yield high-quality sequencing results, featuring excellent read integrity and successful methylation conversion.

References

1. R. Vaisvila et al., “Enzymatic methyl sequencing detects DNA methylation at single-base resolution from picograms of DNA,” *Genome Res*, 31(7):1280-89, 2021.
2. Y. Li, T.O. Tollefsbol, “DNA methylation detection: bisulfite genomic sequencing analysis,” *Methods Mol Biol*, 791:11-21, 2011.
3. Y. Zhang, A. Jeltsch, “The application of next generation sequencing in DNA methylation analysis,” *Genes (Basel)*, 1:85-101, 2010.

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