INSTRUCTION MANUAL

# NEBNext® Enzymatic 5hmC-seq Kit

NEB E3350S/L

24/96 reactions

Version 1.1_2/24

## Table of Contents



## The Library Kit Includes

*The volumes provided are sufficient for preparation of up to 24 reactions (NEB #E3350S) or 96 reactions (NEB #E3350L).*
*The NEBNext Sample Purification Beads should be stored at room temperature and all other reagents should be stored at –20°C.*
*Colored bullets represent the color of the cap of the tube containing the reagent.*

- (lilac) Control DNA Unmethylated Lambda
- (lilac) Control DNA 5hmC T4
- (green) NEBNext Ultra II End Prep Reaction Buffer
- (green) NEBNext Ultra II End Prep Enzyme Mix
- (red) NEBNext Ultra II Ligation Master Mix
- (red) NEBNext Ligation Enhancer
- (red) NEBNext E5hmC-seq Adaptor
- (red) NEBNext Carrier DNA
- (white) Elution Buffer
- (yellow) NEBNext Glucosylation Reaction Buffer
- (yellow) UDP-Glucose
- (yellow) T4-BGT
- (yellow) Stop Reagent
- (orange) APOBEC
- (orange) Deamination Reaction Buffer
- (orange) Recombinant Albumin
- (blue) NEBNext Q5U® Master Mix

NEBNext Sample Purification Beads

## Required Materials Not Included

- Covaris® instrument and the required tubes or other fragmentation equipment
- NEBNext Primers for Epigenetics (Unique Dual Index Set 2B) NEB #E3392S (24 reactions) or Set 3 NEB #E3404S (96 reactions)
- PCR strip tubes or 96-well plates
- Formamide (Sigma #F9037-100 ml), Hi-Di™ Formamide (Thermo Fisher Scientific® #4401457) or 0.1 N NaOH. Formamide is preferred. If using NaOH, please see FAQ on NEB #E3350 FAQ page.
- 80% Ethanol
- 1X TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA), low TE (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA) or 10 mM Tris-HCl pH 7.5 or 8.0
- Nuclease-free Water

## Required Materials Not Included (Cont'd)

- Magnetic rack/stand, such as NEBNext Magnetic Separation Rack (NEB #S1515)
- Metal cooling block, such as Diversified Biotech® (#CHAM-1000)
- PCR machine
- Agilent® Bioanalyzer®, TapeStation® or other fragment analyzer and associated consumables

## Overview

The NEBNext Enzymatic 5hmC-seq (E5hmC-seq) Kit contains the reagents required to enzymatically modify and enable specific detection of 5-hydroxymethylcytosine (5hmC). The user-friendly workflow enables 5hmC identification from 0.1–200 ng of input DNA.

Each kit component must pass rigorous quality control standards, and for each new lot the entire set of reagents is functionally validated together by construction of indexed libraries that are sequenced on an Illumina® sequencing platform.

For larger volume requirements, customized and bulk packaging is available by purchasing through the Customized Solutions Team at NEB. Please contact custom@neb.com for further information.

**Figure 1. NEBNext E5hmC-seq workflow**

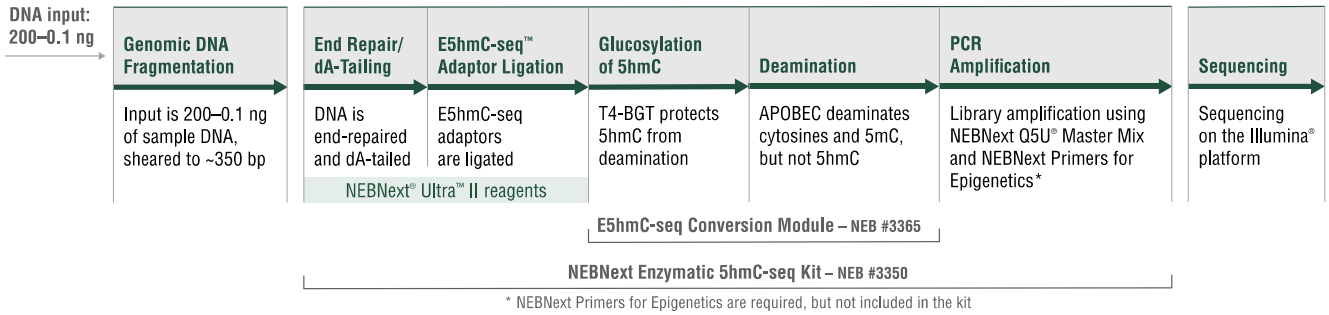


*Figure 1 is an overview of the E5hmC-seq workflow. First, a library is made by ligating the E5hmC-seq adaptor to fragmented, end repaired/dA-tailed genomic DNA. This is followed by two sets of enzymatic conversion steps to differentiate 5hmC from cytosines and 5mC. Finally, libraries are PCR amplified before sequencing. PCR primers can be purchased separately as NEBNext Primers for Epigenetics (NEB #E3392S – 24 reactions or NEB #E3304S – 96 reactions).*

**Figure 2. Overview of the E5hmC-seq conversion method**

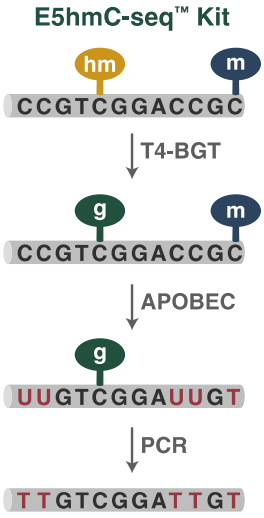


*Figure 2 shows the two-step enzymatic conversion process used to detect 5hmC. The first step uses T4-BGT to glucosylate 5hmC. This protects 5hmC but not 5mC or cytosines from deamination by APOBEC. The DNA sequence shown in the final step of Figure 2 is generated after PCR amplification. PCR primers can be purchased separately.*

# Protocol for E5hmC-seq Library Construction

**Symbols**

*This is a point where you can safely stop the protocol and store the samples prior to proceeding to the next step in the protocol.*

*This caution sign signifies a step in the protocol that has two paths leading to the same end point.*

●

*Colored bullets indicate the cap color of the reagent to be added.*

**Starting Material:** 0.1–200 ng double stranded DNA

### 1.1. DNA Preparation

1.1.1. **DNA and Control DNA:** The following table is a guide for the amount of ● (lilac) Control DNA Unmethylated Lambda and ● (lilac) Control DNA 5hmC T4 to be added to samples prior to E5hmC-seq library construction. Expected read numbers along with read length should be considered to ensure that enough controls are included for the user's individual sequencing goals.

**Table 1.1 Dilutions of control DNAs for a range of genomic DNA inputs. These are suitable for shallow/pre-sequencing (approx. 2–4 million paired reads), for example on a MiSeq®, prior to deeper sequencing (approx. 100–150 million paired reads) on the NovaSeq®, HiSeq® or NextSeq® Illumina platforms.**

| DILUTION OF ● (LILAC) UNMETHYLATED LAMBDA CONTROL AND ● (LILAC) 5hmC T4 CONTROL | | |
|---|---|---|
| **DNA Input Amount** | **Pre-sequencing** 2–4 Million Paired Reads | **Deep Sequencing** 100–150 Million Paired Reads |
| 0.1 ng | 1:500 | 1:1000 |
| 1 ng | 1:80 | 1:250 |
| 10 ng | 1:20 | 1:100 |
| 200 ng | No Dilution | 1:50 |

Regardless of sequencing depth, a minimum of 5,000 paired end reads with a read length of 76 bases for ● (lilac) Control DNA Unmethylated Lambda and 15,000 paired end reads with a read length of 76 bases for ● (lilac) Control DNA 5hmC T4 are needed to give enough coverage for accurate conversion estimates.

Different sequencing depths may be needed depending on the application, and therefore different strategies should be employed when deciding how much control DNA should be added. For example, some libraries may only need 2 million paired end reads whereas other may require 50 million paired end reads or even 1 billion paired end reads.

Additional considerations, regarding the amount of controls added, should be taken into account. For example, pre-sequencing libraries to a depth of 2–4 million paired end reads using the recommended dilution for the controls (Table 1.1), followed by deeper sequencing of these same libraries to a higher depth of 100–150 million paired reads per library would result in excess reads associated with the controls. This strategy is recommended for users who choose to check library conversion prior to deeper sequencing. Users who dilute controls based on pre-sequencing guidelines will have excess control reads, thus ensuring a higher confidence in library conversion.

Combine sample DNA (0.1–200 ng) with control DNAs as specified below. Sample DNA can be in any of the following buffers: 10 mM Tris-HCl pH 7.5 or 8.0, 1X TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA), or low TE (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA). **We do not recommend fragmenting input DNA in 0.1X TE (1 mM Tris-HCl, 0.1 mM EDTA) or water.**

| COMPONENT | VOLUME |
|---|---|
| Sample DNA | 48 µl |
| ● (lilac) Control DNA Unmethylated Lambda (see Table 1.1) | 1 µl |
| ● (lilac) Control DNA 5hmC T4 (see Table 1.1) | 1 µl |
| **Total Volume** | **50 µl** |

1.1.2. **Fragmenting DNA:** The combined 50 µl genomic DNA and control DNAs are fragmented to an average fragment size of ~350 bp (420–620 bp final Illumina library). Fragmentation can be done using a preferred fragmentation device such as a Covaris instrument.

Transfer the 50 µl of fragmented DNA to a new PCR tube for End Prep.

**NOTE: DNA does not need to be cleaned up or size selected before End Prep**

### 1.2. End Prep of Fragmented DNA

1.2.1. On ice, mix the following components in a sterile nuclease-free PCR tube:

| COMPONENT | VOLUME |
|---|---|
| Fragmented DNA (Step 1.1.2.) | 50 µl |
| • (green) NEBNext Ultra II End Prep Reaction Buffer | 7 µl |
| • (green) NEBNext Ultra II End Prep Enzyme Mix | 3 µl |
| **Total Volume** | **60 µl** |

**NOTE: NEBNext Ultra II End Prep Reaction Buffer and Enzyme Mix can be pre-mixed ahead of time as a master mix.**

1.2.2. Mix thoroughly by vortexing 1–2 seconds or by pipetting up and down at least 10 times and centrifuge briefly.

**Note: It is important to mix well. The presence of a small amount of bubbles will not interfere with the performance.**

1.2.3. Place in a thermal cycler with the heated lid set to ≥ 75°C or on, and run the following program:
15 minutes at 20°C
15 minutes at 65°C
Hold at 4°C

### 1.3. Ligation of E5hmC-seq Adaptor

1.3.1. On ice, add the following components directly to the End Prep reaction mixture and mix well:

| COMPONENT | VOLUME |
|---|---|
| End Prep reaction mixture (Step 1.2.3.) | 60 µl |
| • (red) NEBNext E5hmC-seq Adaptor | 2.5 µl |
| • (red) NEBNext Ligation Enhancer | 1 µl |
| • (red) NEBNext Ultra II Ligation Master Mix | 30 µl |
| **Total Volume** | **93.5 µl** |

**Note: The Ligation Enhancer and Ligation Master Mix can be mixed ahead of time and is stable for at least 8 hours at 4°C. Do not premix the Ligation Master Mix, Ligation Enhancer and E5hmC-seq Adaptor prior to use in the Adaptor Ligation Step. Premix adaptor and sample and then add the other ligation reagents.**

1.3.2. Mix thoroughly by vortexing 1–2 seconds or by pipetting up and down at least 10 times and centrifuge briefly.

**Caution: The Ligation Master Mix is viscous. Care should be taken to ensure adequate mixing of the ligation reaction, as incomplete mixing will result in reduced ligation efficiency. The presence of a small amount of bubbles will not interfere with performance.**

1.3.3. Place in a thermal cycler, and run the following program with the heated lid off:
15 minutes at 20°C
Hold at 4°C


**Safe Stopping Point: Samples can be stored overnight at –20°C.**

### 1.4. Clean-up of Adaptor Ligated DNA

The ratios recommended for NEBNext Sample Purification Beads in this manual have been experimentally optimized for every step; this is critical since buffer compositions differ between steps and across protocols e.g., post ligation recommendations will not apply to samples post PCR. Please adhere to these guidelines and not those recommended by other sources or for other kits.

1.4.1. Vortex Sample Purification Beads to resuspend.

1.4.2. Add 93 µl (1X ratio) of resuspended NEBNext Sample Purification Beads to each sample. Mix well by pipetting up and down at least 10 times. Be careful to expel all of the liquid out of the tip during the last mix.

1.4.3. Incubate samples on bench top for at least 5 minutes at room temperature.

1.4.4. Place the tubes against an appropriate magnetic stand to separate the beads from the supernatant.

1.4.5. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets **(Caution: do not discard the beads)**.

1.4.6. Add 200 µl of freshly prepared 80% ethanol to the tubes while on the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

1.4.7. Repeat the ethanol wash once for a total of two washes. Be sure to remove all visible liquid after the second wash using a p10 pipette tip.

1.4.8. Air-dry the beads for 1–2 minutes while the tubes are on the magnetic stand with the lid open.

**Caution: Do not over-dry the beads. This may result in lower recovery of DNA targets. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack, they are too dry.**

**Elution Options A or B**

**Option 1.4.9A: For > 10 ng DNA input**

1.4.9A.1. Remove the tubes from the magnetic stand. Elute the DNA targets from the beads by adding 29 µl of ○ (white) Elution Buffer.

1.4.9A.2. Mix well by pipetting up and down 10 times. Incubate for at least 1 minute at room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube before placing back on the magnetic stand.

1.4.9A.3. Place the tube on the magnetic stand. After 3 minutes (or whenever the solution is clear), transfer 28 µl of the supernatant to a new PCR tube.

**Option 1.4.9B: For ≤ 10ng DNA input**
**Note: Only to be added if Adaptor Ligation has occurred. Do not use with DNA that will not have adaptors ligated before conversion**

1.4.9B.1. Remove the tubes from the magnetic stand. Elute the DNA targets from the beads by adding 28 µl of ○ (white) Elution Buffer.

1.4.9B.2. Mix well by pipetting up and down 10 times. Incubate for at least 1 minute at room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube before placing back on the magnetic stand.

1.4.9B.3. Place the tube on the magnetic stand. After 3 minutes (or whenever the solution is clear), transfer 27 µl of the supernatant to a new PCR tube.

1.4.9B.4. Add 1 µl of the ● (red) NEBNext Carrier DNA to 27 µl of DNA from Step 1.4.9B.3.

**Safe Stopping Point: Samples can be stored overnight at –20°C**.

### 1.5. Glucosylation of 5-Hydroxymethylcytosines (5hmC)

1.5.1. On ice, add the following components directly to the E5hmC-seq adaptor ligated DNA:

| COMPONENT | VOLUME |
|---|---|
| E5hmC-seq adaptor ligated DNA (from Step 1.4.9A.3. or 1.4.9B.4.) | 28 µl |
| Nuclease-free water | 15 µl |
| ○ (yellow) NEBNext Glucosylation Reaction Buffer | 5 µl |
| ○ (yellow) UDP-Glucose | 1 µl |
| ○ (yellow) T4-BGT | 1 µl |
| **Total Volume** | **50 µl** |

Mix thoroughly by vortexing 1–2 seconds or by pipetting up and down at least 10 times and centrifuge briefly.
For multiple reactions, a master mix of the reaction components can be prepared before addition to the sample DNA.

1.5.2. Place in a thermal cycler, and run the following program with the heated lid set to ≥ 45°C or on:
1 hour at 37°C
Hold at 4°C

1.5.3. Transfer the samples to ice and add 1 µl of ○ (yellow) Stop Reagent.

| COMPONENT | VOLUME |
|---|---|
| Glucosylated DNA (from Step 1.5.2.) | 50 µl |
| ○ (yellow) Stop Reagent | 1 µl |
| **Total Volume** | **51 µl** |

Mix thoroughly by vortexing 1–2 seconds or by pipetting up and down at least 10 times and centrifuge briefly.

1.5.4. Place in a thermal cycler, and run the following program with the heated lid set to ≥ 45°C or on:
30 minutes at 37°C
Hold at 4°C


**Safe Stopping Point: Samples can be stored overnight at either 4°C in the thermal cycler or at –20°C in the freezer.**

### 1.6. Clean-up of Glucosylated DNA

The ratios recommended for NEBNext Sample Purification Beads in this manual have been experimentally optimized for every step; this is critical since buffer compositions differ between steps and across protocols e.g., post ligation recommendations will not apply to samples post PCR. Please adhere to these guidelines and not those recommended by other sources or for other kits.

1.6.1. Vortex Sample Purification Beads to resuspend.

1.6.2. Add 50 µl (1X ratio) of resuspended NEBNext Sample Purification Beads to each sample. Mix well by pipetting up and down at least 10 times. Be careful to expel all of the liquid out of the tip during the last mix.

1.6.3. Incubate samples on bench top for at least 5 minutes at room temperature.

1.6.4. Place the tubes against an appropriate magnetic stand to separate the beads from the supernatant.

1.6.5. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets **(Caution: do not discard the beads)**.

1.6.6. Add 200 µl of freshly prepared 80% ethanol to the tubes while on the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

1.6.7. Repeat the ethanol wash once for a total of two washes. Be sure to remove all visible liquid after the second wash using a p10 pipette tip.

1.6.8. Air-dry the beads for 30 seconds – 1 minute while the tubes are on the magnetic stand with the lid open.

**Caution: Do not over-dry the beads. This may result in lower recovery of DNA targets. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack, they are too dry.**

1.6.9. Remove the tubes from the magnetic stand. Elute the DNA targets from the beads by adding 17 µl of ○ (white) Elution Buffer.

1.6.10. Mix well by pipetting up and down 10 times. Incubate for at least 1 minute at room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube before placing back on the magnetic stand.

1.6.11. Place the tube on the magnetic stand. After 3 minutes (or whenever the solution is clear), transfer 16 µl of the supernatant to a new PCR tube.

**Caution: Carrying even a small amount of beads forward can lead to inefficient deamination.**


**Safe Stopping Point: Samples can be stored overnight at –20°C.**

### 1.7. Denaturation of DNA

**Note: All sample input ranges (0.1–200 ng) follow the same denaturation and deamination conditions.**

 **Denaturation Options A or B**

The DNA can be denatured using either Formamide or 0.1 N Sodium Hydroxide. Use Option A for denaturing with Formamide and Option B for denaturing with 0.1 N Sodium Hydroxide.

**Option 1.7A: Formamide (Recommended)**

1.7A.1. Pre-heat thermal cycler to 85°C with the heated lid set to ≥ 105°C or on.

1.7A.2. Add 4 µl Formamide to the 16 µl of glucosylated DNA (from Step 1.6.11.). Mix thoroughly by vortexing 1–2 seconds or by pipetting up and down at least 10 times and centrifuge briefly.

1.7A.3. Incubate at 85°C for 10 minutes in the pre-heated thermal cycler.

1.7A.4. Immediately place in cooling block on ice and allow the sample to fully cool (~2 minutes) before proceeding to Section 1.8.

**Option 1.7B: Sodium Hydroxide**

*Optional, See FAQ about preparing NaOH.*

1.7B.1. Prepare freshly diluted 0.1 N NaOH.

1.7B.2. Pre-heat thermal cycler to 85°C with the heated lid set to ≥ 105°C or on.

1.7B.3. Add 4 µl 0.1 N NaOH to the 16 µl of glucosylated DNA (from Step 1.6.11.). Mix thoroughly by vortexing 1–2 seconds or by pipetting up and down at least 10 times and centrifuge briefly.

1.7B.4. Incubate at 85°C for 10 minutes in the pre-heated thermal cycler.

1.7B.5. Immediately place in cooling block on ice and allow the sample to fully cool (~2 minutes) before proceeding to Section 1.8.

### 1.8. Deamination of 5mC and Cytosines

1.8.1. On ice, add the following components to the denatured DNA:

| COMPONENT | VOLUME |
|---|---|
| Denatured DNA (Step 1.7A.4. or 1.7B.5.) | 20 µl |
| Nuclease-free water | 14 µl |
| • (orange) Deamination Reaction Buffer | 4 µl |
| • (orange) Recombinant Albumin | 1 µl |
| • (orange) APOBEC | 1 µl |
| **Total Volume** | **40 µl** |

For multiple reactions, a master mix of the reaction components can be prepared before addition to the denatured DNA.

1.8.2. Mix thoroughly by vortexing 1–2 seconds or by pipetting up and down at least 10 times and centrifuge briefly.

1.8.3. Place in a thermal cycler, and run the following program with the heated lid set to ≥ 45°C or on:
3 hours at 37°C
Hold at 4°C

**Safe Stopping Point: Samples can be stored overnight at either 4°C in the thermal cycler or at –20°C in the freezer.**

**Note: The samples move directly into PCR with no bead clean-up.**

### 1.9. PCR Amplification

1.9.1. On ice, add the following components to the deaminated DNA from Step 1.8.3.:

| COMPONENT | VOLUME |
|---|---|
| Deaminated DNA (Step 1.8.3.) | 40 µl |
| UDI Primer Pair** | 5 µl |
| • (blue) NEBNext Q5U Master Mix | 45 µl |
| **Total Volume** | **90 µl** |

** NEBNext Primers for Epigenetics are supplied as a 24 Unique Dual Index Primer Pairs Plate in NEB #E3392S (Set 2B) or as a 96 Unique Dual Index Primer Pairs Plate in NEB #E3404S (Set 3). Sample sheets can be found on the NEB #E3392S and NEB #E3404S product pages (www.neb.com/E3392 ; www.neb.com/E3404).

1.9.2. Mix thoroughly by vortexing 1–2 seconds or by pipetting up and down at least 10 times and centrifuge briefly.

1.9.3. Place the tube in a thermal cycler with the heated lid set to 105°C and perform PCR amplification using the following cycling conditions:

| CYCLE STEP | TEMP | TIME | CYCLES |
|---|---|---|---|
| Initial Denaturation | 98°C | 30 seconds | 1 |
| Denaturation | 98°C | 10 seconds | 4–14* |
| Annealing | 62°C | 30 seconds | |
| Extension | 65°C | 60 seconds | |
| Final Extension | 65°C | 5 minutes | 1 |
| Hold | 4°C | ∞ | |

* Cycle Recommendations:

- 200 ng DNA input: 4 cycles
- 50 ng DNA input: 6 cycles
- 10 ng DNA input: 8 cycles
- 1 ng DNA input: 11 cycles
- 0.1 ng DNA input: 14 cycles

**Safe Stopping Point: Samples can be stored overnight at either 4°C in the thermal cycler or at –20°C in the freezer.**

### 1.10. Clean-up of Amplified Libraries

The ratios recommended for NEBNext Sample Purification Beads in this manual have been experimentally optimized for every step; this is critical since buffer compositions differ between steps and across protocols e.g., post ligation recommendations will not apply to samples post PCR. Please adhere to these guidelines and not those recommended by other sources or for other kits.

**Caution: The Sample Purification Beads behave differently during the post-PCR clean-up. After the bead washes, do not overdry the beads as they become very difficult to resuspend.**

1.10.1. Vortex Sample Purification Beads to resuspend.

1.10.2. Add 72 µl (0.8X ratio) of resuspended NEBNext Sample Purification Beads to each sample. Mix well by pipetting up and down at least 10 times. Be careful to expel all of the liquid out of the tip during the last mix.

1.10.3. Incubate samples on bench top for at least 5 minutes at room temperature.

1.10.4. Place the tubes against an appropriate magnetic stand to separate the beads from the supernatant.

1.10.5. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets **(Caution: do not discard the beads)**.

1.10.6. Add 200 µl of freshly prepared 80% ethanol to the tubes while on the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

1.10.7. Repeat the ethanol wash once for a total of two washes. Be sure to remove all visible liquid after the second wash using a p10 pipette tip.

1.10.8. Air-dry the beads for 1–2 minutes while the tubes are on the magnetic stand with the lid open.

**Caution: Do not over-dry the beads. This may result in lower recovery of DNA targets. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.**

1.10.9. Remove the tubes from the magnetic stand. Elute the DNA targets from the beads by adding 21 µl of ° (white) Elution Buffer. Optional: For long term storage of libraries, 21 µl of 1X TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) or Low TE (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA) can be used.

1.10.10. Mix well by pipetting up and down 10 times. Incubate for at least 1 minute at room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube before placing back on the magnetic stand.

1.10.11. Place the tube on the magnetic stand. After 3 minutes (or whenever the solution is clear), transfer 20 µl of the supernatant to a new PCR tube.

**Safe Stopping Point: Samples can be stored overnight at –20°C.**

### 1.11. Library Quantification and Sequencing

1.11.1. Use a Bioanalyzer or TapeStation to determine the size distribution and concentration of the libraries.

**Figure 3. E5hmC-seq library from 200 ng of human brain genomic DNA**

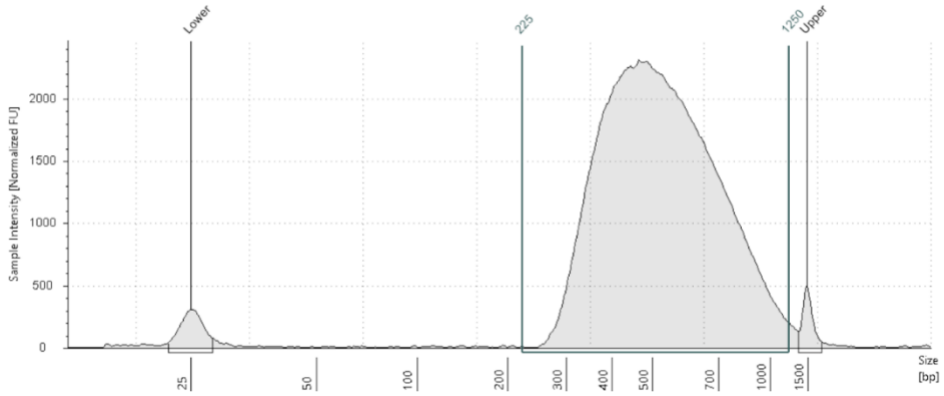


*Figure 3. Representative TapeStation trace for an E5hmC-seq library prepared using 200 ng of human brain genomic DNA. Library was run on a HS D1000 tape.*

E5hmC-seq libraries can be sequenced using the preferred Illumina platform, for example MiSeq, NextSeq or NovaSeq. The choice of sequencing read length is user dependent. Typical read lengths are 2 x 76, 2 x 100 or 2 x 150 base reads.

## Index Pooling Guidelines

For more detailed indexing information please refer to the manual for NEBNext Primers for Epigenetics (NEB #E3392S, #E3404S). For a link to download a sample sheet with the index sequences for use with the Illumina Experiment Manager (IEM) please go to our FAQ's tab on **www.neb.com/E3392** or **www.neb.com/E3404** – NEBNext Primers for Epigenetics (NEB #E3392S, #E3404S).

**Adaptor trimming sequences:**
The NEBNext libraries for Illumina resemble TruSeq® libraries and the adaptor sequences can be trimmed similar to TruSeq:

**Adaptor Read 1** AGATCGGAAGAGCACACGTCTGAACTCCAGTCA

**Adaptor Read 2** AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

## Checklist

**1.1 DNA Preparation**

1.1.1. Combine DNA and control DNA

*See Table 1.1 for recommended control dilutions*

[ _ ] 0.1–200 ng sample DNA

[ _ ] 1 µl of ● (lilac) Control DNA Unmethylated Lambda at recommended dilution for input amount

[ _ ] 1 µl of ● (lilac) Control DNA 5hmC T4 at recommended dilution for input amount

[ _ ] Add 10 mM Tris-HCl pH 7.5 or 8.0, 1X TE, or low TE pH 8.0 to bring the volume to 50 µl

1.1.2. Fragment DNA

[ _ ] Fragment to ~300 bp, using preferred instrument

[ _ ] Transfer the 50 µl of fragmented material directly to a PCR strip tube to begin library construction

**1.2. End Prep of Fragmented DNA**

Add End Prep Reagents to fragmented DNA (50 µl):

[ _ ] 7 µl ● (green) NEBNext Ultra II End Prep Reaction Buffer

[ _ ] 3 µl ● (green) NEBNext Ultra II End Prep Enzyme Mix

[ _ ] Vortex 1 – 2 seconds or pipette mix 10 times, centrifuge briefly

[ _ ] Incubate in thermal cycler (heated lid ≥ 75°C or on)

15 minutes at 20°C

15 minutes at 65°C

Hold at 4°C

**1.3. Ligation of E5hmC-seq Adaptor**

Add Ligation Reagents to 60 µl End Prepped DNA

[ _ ] 2.5 µl ● (red) NEBNext E5hmC-seq Adaptor

[ _ ] 1 µl ● (red) NEBNext Ligation Enhancer

[ _ ] 30 µl ● (red) NEBNext Ultra II Ligation Master Mix

[ _ ] Vortex 1 – 2 seconds or pipette mix 10 times, centrifuge briefly

[ _ ] Incubate in thermal cycler (heated lid off)

15 minutes at 20°C

Hold at 4°C

**1.4. Clean-up of Adaptor Ligated DNA**

[ _ ] Vortex beads

[ _ ] Add 93 µl of resuspended NEBNext Sample Purification Beads to each sample and mix by pipetting 10 times

[ _ ] Incubate 5 minutes

[ _ ] Place tubes on magnet for 5 minutes

[ _ ] Remove and discard the supernatant, while keeping the sample on the magnet

[ _ ] On magnet add 200 µl 80% ethanol, wait 30 seconds and then remove and discard the ethanol wash

[ _ ] Repeat the ethanol wash

[ _ ] Air-dry the beads for 1–2 minutes while on magnet

**A. Elution Option A for > 10 ng DNA input**

[ _ ] Remove the samples from the magnet and resuspend in 29 µl of ○ (white) Elution Buffer

[ _ ] Place back on the magnet, wait until the supernatant clears and transfer 28 µl of sample to fresh PCR tube

**B. Elution Option B for ≤ 10 ng DNA input**

[ _ ] Remove the samples from the magnet and resuspend in 28 µl of ○ (white) Elution Buffer

[ _ ] Place back on the magnet, wait until the supernatant clears and transfer 27 µl of sample to fresh PCR tube

[ _ ] Add 1 µl of the ● (red) NEBNext Carrier DNA to 27 µl of cleaned up Adaptor ligated DNA

**1.5. Glucosylation of 5-Hydroxymethylcytosines**

Add Glucosylation Reagents to 28 µl Adaptor Ligated DNA

[ _ ] 15 µl Nuclease-free water

[ _ ] 5 µl ● (yellow) NEBNext Glucosylation Reaction Buffer

[ _ ] 1 µl ● (yellow) UDP-Glucose

[ _ ] 1 µl ● (yellow) T4-BGT

[ _ ] Vortex 1–2 seconds or pipette mix 10 times, centrifuge briefly

[ _ ] Incubate in a thermal cycler (heated lid ≥ 45°C or on)

60 minutes at 37°C

Hold at 4°C

[ _ ] Add 1 µl ● (yellow) Stop Reagent

[ _ ] Vortex 1–2 seconds or pipette mix 10 times, centrifuge briefly

[ _ ] Incubate in a thermal cycler (heated lid ≥ 45°C or on)

30 minutes at 37°C

Hold at 4°C

**1.6. Clean-up of Glucosylated DNA**

[ _ ] Vortex beads

[ _ ] Add 50 µl of resuspended NEBNext Sample Purification Beads to each sample and mix by pipetting 10 times

[ _ ] Incubate 5 minutes

[ _ ] Place tubes on magnet for 5 minutes

[ _ ] Remove and discard the supernatant, while keeping the sample on the magnet

[ _ ] On magnet add 200 µl 80% ethanol, wait 30 seconds and then remove and discard the ethanol wash

[ _ ] Repeat the ethanol wash

[ _ ] Air-dry the beads for 30 seconds – 1 minute while on magnet

[ _ ] Remove the samples from the magnet and resuspend in 17 µl of ○ (white) Elution Buffer

[ _ ] Place back on the magnet, wait until the supernatant clears and transfer 16 µl of sample to fresh PCR tube

**1.7. Denaturation of DNA**

[ _ ] Use either Formamide (A) or Sodium Hydroxide (B)

**A. Formamide Denaturation (Recommended)**

[ _ ] Pre-heat thermal cycler to 85°C (heated lid ≥ 105°C or on)

[ _ ] Add 4 µl Formamide to the 16 µl glucosylated DNA

[ _ ] Vortex 1 – 2 seconds or pipette mix 10 times, centrifuge briefly

[ _ ] Incubate in pre-heated thermal cycler

85°C for 10 minutes

Immediately, place on ice until fully cooled (~2 minutes)

[ _ ] Proceed immediately to Section 1.8.

**B. Sodium Hydroxide Denaturation (Formamide is preferred)**

[ _ ] Prepare freshly diluted 0.1 N NaOH

[ _ ] Pre-heat thermal cycler to 85°C (heated lid ≥ 105°C or on)

[ _ ] Add 4 µl 0.1 N NaOH to the 16 µl glucosylated DNA

[ _ ] Vortex 1–2 seconds or pipette mix 10 times, centrifuge briefly

[ _ ] Incubate in pre-heated thermal cycler

85°C for 10 minutes

Immediately, place on ice until fully cooled (~2 minutes)

[ _ ] Proceed immediately to Section 1.8.

### 1.8. Deamination of 5mC and Cytosines

Add Deamination Reagents to 20 µl denatured DNA on ice

[ _ ] 14 µl Nuclease-free water

[ _ ] 4 µl ● (orange) Deamination Reaction Buffer

[ _ ] 1 µl ● (orange) Recombinant Albumin

[ _ ] 1 µl ● (orange) APOBEC

[ _ ] Vortex 1–2 seconds or pipette mix 10 times, centrifuge briefly

[ _ ] Incubate in a thermal cycler (heated lid ≥ 45°C or on)

3 hours at 37°C

Hold at 4°C

### 1.9. PCR Amplification

Add Amplification Reagents to 40 µl deaminated DNA

[ _ ] 5 µl UDI Primer Pair

[ _ ] 45 µl ● (blue) NEBNext Q5U Master Mix

[ _ ] Vortex 1–2 seconds or pipette mix 10 times, centrifuge briefly

[ _ ] Amplify in thermal cycler (heated lid ≥ 105°C or on)

| CYCLE STEP | TEMPERATURE | TIME | CYCLES |
|---|---|---|---|
| Initial Denaturation | 98°C | 30 s | 1 |
| Denaturation | 98°C | 10 s | 4–14* |
| Annealing | 62°C | 30 s | |
| Extension | 65°C | 60 s | |
| Final Extension | 65°C | 5 min | 1 |
| Hold | 4 | ∞ | |

* Cycle Recommendations:

- 200 ng DNA input: 4 cycles
- 50 ng DNA input: 6 cycles
- 10 ng DNA input: 8 cycles
- 1 ng DNA input: 11 cycles
- 0.1 ng DNA input: 14 cycles

### 1.10. Clean-up of Amplified Libraries

[ _ ] Vortex beads

[ _ ] Add 72 µl of resuspended NEBNext Sample Purification Beads to each sample and mix by pipetting 10 times

[ _ ] Incubate 5 minutes

[ _ ] Place tubes on magnet for 5 minutes

[ _ ] Remove and discard the supernatant, while keeping the sample on the magnet

[ _ ] On magnet add 200 µl 80% ethanol, wait 30 seconds and then remove and discard the ethanol wash

[ _ ] Repeat the ethanol wash

[ _ ] Air-dry the beads for 1–2 minutes while on magnet

[ _ ] Remove the samples from the magnet and resuspend in 21 µl of ○ (white) Elution Buffer. For longer term storage use 10 mM Tris-HCl pH 8.0, 0.1 mM EDTA.

[ _ ] Place back on the magnet, wait until the supernatant clears and transfer 20 µl of sample to fresh PCR tubes

### 1.11. Library Quantification and Sequencing

[ _ ] Use a Bioanalyzer or TapeStation to determine the size distribution and concentration of the libraries

[ _ ] Sequence using the preferred Illumina platform: 2 x 76 base, 2 x 100 base, or 2 x 150 base reads

## Kit Components

NEB #E3350S Table of Components

| NEB # | PRODUCT | VOLUME |
|---|---|---|
| E7123A | Control DNA Unmethylated Lambda | 0.024 ml |
| E3349A | Control DNA 5hmC T4 | 0.024 ml |
| E7647A | NEBNext Ultra II End Prep Reaction Buffer | 0.168 ml |
| E7646A | NEBNext Ultra II End Prep Enzyme Mix | 0.076 ml |
| E7648A | NEBNext Ultra II Ligation Master Mix | 0.72 ml |
| E7374A | NEBNext Ligation Enhancer | 0.024 ml |
| E3366A | NEBNext E5hmC-seq Adaptor | 0.06 ml |
| E3351A | NEBNext Carrier DNA | 0.024 ml |
| E3355S | NEBNext Sample Purification Beads | 5.16 ml |
| E7124A | Elution Buffer | 2.1 ml |
| E3352A | NEBNext Glucosylation Reaction Buffer | 0.12 ml |
| E3353A | UDP-Glucose | 0.024 ml |
| E3354A | T4-BGT | 0.024 ml |
| E7132A | Stop Reagent | 0.024 ml |
| E7133A | APOBEC | 0.024 ml |
| E3356A | Deamination Reaction Buffer | 0.096 ml |
| E3357A | Recombinant Albumin | 0.024 ml |
| E3369A | NEBNext Q5U Master Mix | 1.08 ml |

NEB #E3350L Table of Components

| NEB # | PRODUCT | VOLUME |
|---|---|---|
| E7123AA | Control DNA Unmethylated Lambda | 0.096 ml |
| E3349AA | Control DNA 5hmC T4 | 0.096 ml |
| E7647AA | NEBNext Ultra II End Prep Reaction Buffer | 0.672 ml |
| E7646AA | NEBNext Ultra II End Prep Enzyme Mix | 0.288 ml |
| E7648AA | NEBNext Ultra II Ligation Master Mix | 2.88 ml |
| E7374AA | NEBNext Ligation Enhancer | 0.096 ml |
| E3366AA | NEBNext E5hmC-seq Adaptor | 0.24 ml |
| E3351AA | NEBNext Carrier DNA | 0.096 ml |
| E3355L | NEBNext Sample Purification Beads | 20.6 ml |
| E7124AA | Elution Buffer | 8.6 ml |
| E3352AA | NEBNext Glucosylation Reaction Buffer | 0.480 ml |
| E3353AA | UDP-Glucose | 0.096 ml |
| E3354AA | T4-BGT | 0.096 ml |
| E7132AA | Stop Reagent | 0.096 ml |
| E7133AA | APOBEC | 0.096 ml |
| E3356AA | Deamination Reaction buffer | 0.384 ml |
| E3357AA | Recombinant Albumin | 0.096 ml |
| E3369AA | NEBNext Q5U Master Mix | 4.32 ml |

## Revision History

| REVISION # | DESCRIPTION | DATE |
|---|---|---|
| 1.0 | N/A | 12/23 |
| 1.1 | Added clarification to Section 1.1 (DNA Preparation). Also updated header and footer. | 2/24 |