

NEBNext® Enzymatic 5hmC-seq Kit

NEB E3350S/L

24/96 reactions

Version 2.0_7/26

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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
- Any NEBNext LV Unique Dual Index Primer Set (NEB #E3390, #E3392, #E3400, #E3402, #E3404, #E3406 and #E3408)
- 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.

Where larger volumes, customized or bulk packaging are required, we encourage consultation with the Customized Solutions team at NEB. Please complete the NEB Custom Contact Form at www.neb.com/CustomContactForm to learn more.

Figure 1. NEBNext E5hmC-seq workflow

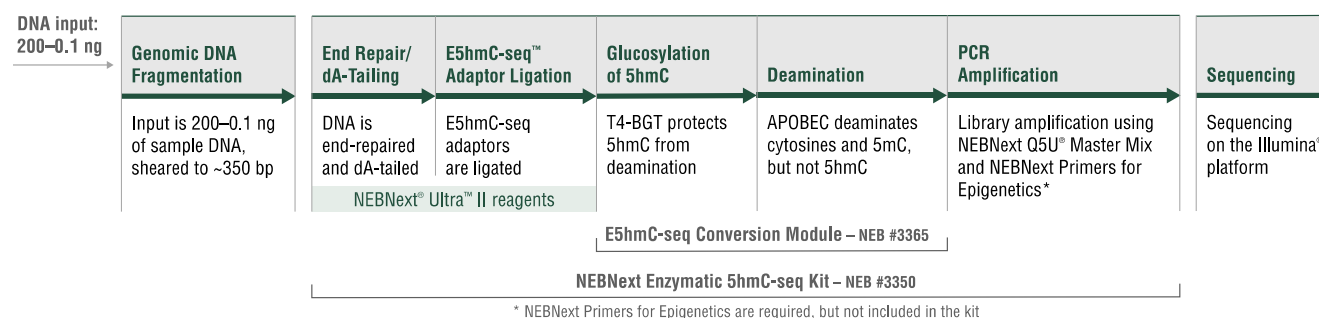


Figure 1 is an overview of the E5hmC-seq workflow. First, a library is made by ligating the E5hmC-seq adaptor to fragmented, end repaired/dA-tailed genomic DNA. This is followed by two sets of enzymatic conversion steps to differentiate 5hmC from cytosines and 5mC. Finally, libraries are PCR amplified before sequencing. PCR primers can be purchased separately as NEBNext LV Unique Dual Index Primers (NEB #E3390S, #E3392S – 24 reactions or NEB #E3400S, #E3402S, #E3404S, #E3406S, #E3408S – 96 reactions).

Figure 2. Overview of the E5hmC-seq conversion method

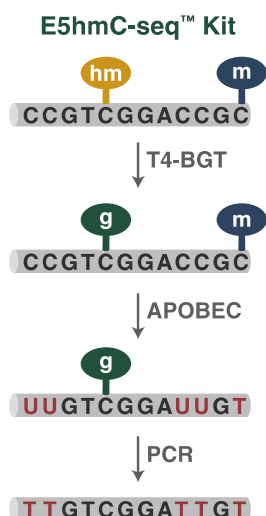


Figure 2 shows the two-step enzymatic conversion process used to detect 5hmC. The first step uses T4-BGT to glucosylate 5hmC. This protects 5hmC but not 5mC or cytosines from deamination by APOBEC. The DNA sequence shown in the final step of Figure 2 is generated after PCR amplification. PCR primers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

Important Guidelines

Reagent Handling

Remove only the kit reagents needed for a specific reaction in the EM-seq protocol. For example, for “Glucosylation of 5-Hydroxymethylcytosines (5hmC)” remove the yellow capped tubes. Frozen kit components can be thawed at room temperature. Once thawed, place immediately on ice.

Please note that some components may form a precipitate. Precipitates should disappear once the tubes are at room temperature. All components (stored at -20°C) should be stored on ice until used.

All components should be mixed before use. Typically, tubes containing enzymes can be mixed by flicking the tube and other buffers/reagents can be quickly vortexed. All tubes should be centrifuged briefly after mixing and stored on ice until used.

Master mixes can be made for most steps in E5hmC-seq unless otherwise stated in the protocol. If using a master mix, good practice is to add the component in the order specified in the protocol. Typically, enzymes are added last. Master mixes should be quickly vortexed then briefly centrifuged and stored on ice before use.

Care should be taken when handling some of the reagents to ensure the correct volumes are dispensed. For example, the Sample Purification Beads are viscous. It is difficult to accurately pipette these components. Please take care to transfer the correct volumes.

The ● (orange) Deamination Reaction Buffer can form bubbles when mixed. Centrifuging the ● (orange) Deamination Reaction Buffer can reduce their appearance making it easier to accurately dispense the buffer.

Once reactions are set up, mix either using a quick vortex or by pipetting up and down. Please note that if you choose to mix using a pipette, then we encourage you to use an appropriately sized pipette set to a volume that would ensure adequate mixing of the sample. For example, during the Glucosylation Reaction set up, 5 µl of diluted ● (yellow) Fe(II) Solution is added, use a 200 µl pipette set to 40 µl to mix – do not mix with a pipette set to 5 µl. Briefly centrifuge the mixed reactions before incubating the reactions.

Another example where adequate mixing is required is during Sample Purification Bead clean-ups. Here, please use a 200 µl pipette set to an appropriate volume to ensure mixing. For the bead clean up after Glucosylation, a pipette set to 80 µl would be sufficient whereas for the clean-up after APOBEC deamination a 160 µl pipette volume would be more appropriate.

After each NEBNext Sample Purification Bead clean-up please ensure that the samples are centrifuged to ensure that the sample is at the bottom of the tube prior to the next reaction in the protocol. If the samples were stored at -20°C, they can be thawed at room temp or on ice prior to centrifugation.

There are safe stop points in the protocol where reactions can be stored at -20°C. If a sample has been stored at -20°C then it should be thawed and briefly centrifuged before use in the next step in the protocol.

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.

Sample DNA Input Amount	Control DNA Dilution Recommendations
0.1 ng	1:1000
1 ng	1:250
10 ng	1:100
200 ng	1:10

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.

The dilutions recommended in Table 1.1. will provide sufficient coverage of controls for libraries sequenced to 10 million paired end reads and above. Dilution of controls needs to be optimized by the user if sequencing lower than 10 million paired-end reads to obtain minimum coverage for ● (lilac) Control DNA Unmethylated Lambda (5,000 paired end reads) and for ● (lilac) Control DNA 5hmC T4 (15,000 paired end reads). Users should be aware that deep sequencing using the dilutions recommended in Table 1.1. can result in more than the minimum required reads for the ● (lilac) Control DNA Unmethylated Lambda and the ● (lilac) Control DNA 5hmC T4. Ultimately, dilutions of the control DNAs should be optimized by the user.

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) Diluted Control DNA Unmethylated Lambda (see Table 1.1)	1 µl
● (lilac) Diluted 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 $\geq 75^{\circ}\text{C}$ 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.



- 1.4.9. **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 1.7.1A: Formamide (Recommended)

- 1.7A.1. Pre-heat thermal cycler to 85°C with the heated lid set to $\geq 105^{\circ}\text{C}$ 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 a pre-chilled metal cooling block on ice and allow the sample to fully cool (~2 minutes) before proceeding to Section 1.8. If a metal cooling block is not available, place on ice and ensure proper contact with the tube walls.

Option 1.7.1B: 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 $\geq 105^{\circ}\text{C}$ or on.

- 1.7B.3. Add 4 μ l 0.1 N NaOH to the 16 μ l 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 a pre-chilled metal cooling block on ice and allow the sample to fully cool (~2 minutes) before proceeding to Section 1.8. If a metal cooling block is not available, place on ice and ensure proper contact with the tube walls.

1.8. Deamination of 5mC and Cytosines



Deamination Options A or B

Use Option A (1.8.3A) for DNA inputs $\leq$ 100 ng

Option B (1.8.3B) is required for $>$ 100 ng DNA inputs and can be used for all inputs (0.1 ng to 200 ng).

- 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 μ l
Nuclease-free water	14 μ l
• (orange) Deamination Reaction Buffer	4 μ l
• (orange) Recombinant Albumin	1 μ l
• (orange) APOBEC	1 μ l
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.

Option A: (Inputs $\leq$ 100ng)

- 1.8.3A. Place in a thermal cycler, and run the following program with the heated lid set to $\geq$ 85°C or on:
 - 10 min at 37°C
 - 10 min at 42°C
 - 10 min at 50°C
 - 2 min at 75°C
 - Hold at 4°C

Option B: (Required for Inputs $>$ 100 ng and can be used for all inputs)

- 1.8.3B. Place in a thermal cycler, and run the following program with the heated lid set to $\geq$ 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
NEBNext LV UDI Primer Pair* * * * *	5 µl
• (blue) NEBNext Q5U Master Mix	45 µl
Total Volume	90 µl

* Refer to the appropriate section in the respective oligo manual for barcode pooling guidelines or visit: [NEBNext® Index Oligo Selector](#)

** NEBNext LV Unique Dual Index Primers must be purchased separately to the library prep kit. Refer to www.neb.com/oligos for additional information.

*** The sample sheets can be located in Usage Guidelines for the respective NEBNext LV Unique Dual Index Primers Set product page.

- 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: 4–5 cycles
- 50 ng DNA input: 5–6 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 μ l of $\circ$ (white) Elution Buffer. Optional: For long term storage of libraries, 21 μ l 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 μ l 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

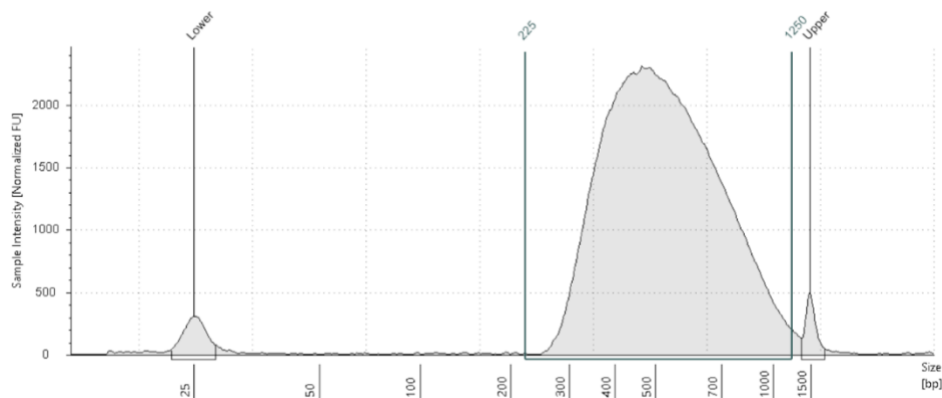


Figure 3. Representative TapeStation trace for an E5hmC-seq library prepared using 200 ng of human brain genomic DNA. Library was run on a HS D1000 tape.

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 LV Unique Dual Index Primers (NEB #E3390S, #E3392S, #E3400S, #E3402S, #E3404S, #E3406S, #E3408S). The sample sheets can be located in Usage Guidelines on the respective NEBNext LV Unique Dual Index Primers Set product pages.

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 $\geq$ 105°C or on)
- [_] Add 4 μ l 0.1 N NaOH to the 16 μ l 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 μ l denatured DNA on ice

- [_] 14 μ l Nuclease-free water
- [_] 4 μ l ● (orange) Deamination Reaction Buffer
- [_] 1 μ l ● (orange) Recombinant Albumin
- [_] 1 μ l ● (orange) APOBEC
- [_] Vortex 1–2 seconds or pipette mix 10 times, centrifuge briefly
- [_] Incubate in a thermal cycler (heated lid $\geq$ 45°C or on)
3 hours at 37°C
Hold at 4°C

1.9. PCR Amplification

Add Amplification Reagents to 40 μ l deaminated DNA

- [_] 5 μ l UDI Primer Pair
- [_] 45 μ l ● (blue) NEBNext Q5U Master Mix
- [_] Vortex 1–2 seconds or pipette mix 10 times, centrifuge briefly
- [_] Amplify in thermal cycler (heated lid $\geq$ 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 μ l 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 μ l 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 μ l of $\circ$ (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 μ l 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
2.0	Update protocol and add important guidelines.	7/26

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