# NEBNext UltraShear®

NEB #M7634S/L

24/96 reactions

Version 2.0_12/24

## Table of Contents



## The Product Includes

*The volumes provided are sufficient for preparation of up to 24 reactions (NEB #M7634S) or 96 reactions (NEB #M7634L). All reagents should be stored at –20°C. Colored bullets represent the color of the cap of the tube containing the reagent.*

- (white) NEBNext UltraShear
- (white) NEBNext UltraShear Reaction Buffer
- (green) 500 mM DTT

## Required Materials Not Included:

- 1X TE (10 mM Tris pH 8.0, 1 mM EDTA)
- 0.2 ml thin wall PCR tubes, for example TempAssure PCR flex-free 8-tube strops (USA Scientific #1402-4708)
- Magnetic rack/stand (NEB #S1515S; Alpaqua® #A001322 or equivalent)
- Thermal Cycler
- Vortex
- Microcentrifuge
- Agilent® TapeStation®, Bioanalyzer® or other fragment analyzer and associated consumables
- 80% Ethanol
- Metal cooling block, such as Diversified Biotech® (#CHAM-1000)

**NEBNext UltraShear fragmentation protocol (Section 1):**

- SPRIselect™ Reagent Kit (Beckman Coulter®, Inc. #B23317), AMPure® XP Beads (Beckman Coulter, Inc. #A63881) or Monarch® PCR & DNA Cleanup Kit (NEB# T1030S/L)

**For use with NEBNext Ultra II End Repair/dA-Tailing Module Protocol (Section 2):**

- NEBNext Ultra II End Repair/dA-Tailing Module (NEB #E7546S/L)
- Recommended Material Not Included: NEBNext Ultra II Ligation Module (NEB #E7595) and NEBNext Multiplex Oligos (www.neb.com/oligos)

**For use with NEBNext Enzymatic Methyl-seq v2 Protocol (Section 3):**

- NEBNext Enzymatic Methyl-seq v2 Kit (NEB #E8015S/L)
- Hi-Di™ Formamide (Thermo Fisher Scientific® #4401457), Formamide (Sigma #F9037-100 ml) or optional 0.05 N NaOH Formamide is preferred. If using NaOH, please see NEBNext Enzymatic Methyl-seq v2 Kit (NEB #E8015) FAQs
- Nuclease-free Water
- Any NEBNext LV Unique Dual Index Primer Set (NEB #E3390, #E3392, #E3400, #E3402, #E3404, #E3406 and #E3408)

**For use with NEBNext Enzymatic 5hmC-seq Protocol (Section 4):**

- NEBNext Enzymatic 5hmC-seq Kit (NEB #E3350S/L)
- Hi-Di Formamide (Thermo Fisher Scientific #4401457), Formamide (Sigma #F9037-100 ml) or optional 0.1 N NaOH Formamide is preferred. If using NaOH, please see the NEBNext Enzymatic 5hmC-seq Kit (NEB #E3350S) FAQ
- Nuclease-free Water
- Any NEBNext LV Unique Dual Index Primer Set (NEB #E3390, #E3392, #E3400, #E3402, #E3404, #E3406 and #E3408)

**For use with NEBNext Enzymatic Methyl-seq Protocol (Section 5):**

- NEBNext Enzymatic Methyl-seq Kit (NEB #E7120S/L)
- Hi-Di Formamide (Thermo Fisher Scientific #4401457), Formamide (Sigma #F9037-100 ml) or optional 0.1 N NaOH Formamide is preferred. If using NaOH, please see the NEBNext Enzymatic Methyl-seq Kit (NEB #E7120) FAQ
- Nuclease-free Water

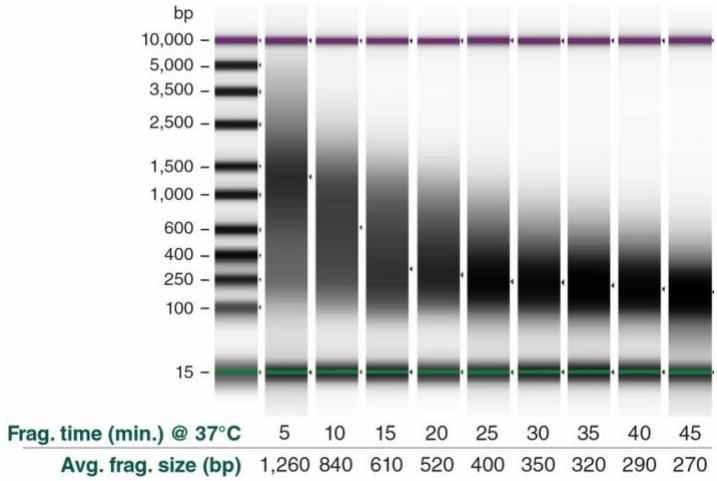
Where larger volumes, customized or bulk packaging are required, we encourage consultation with the Customized Solutions team at NEB. Please complete the NEB Custom Contact Form at www.neb.com/CustomContactForm to learn more.

**Please read the FAQ section on NEB.com for additional information about this product.**

## Overview

NEBNext UltraShear contains reagents to fragment genomic DNA upstream of library preparation for next-generation sequencing and is designed for use with challenging samples and workflows including methylated DNA and FFPE DNA. NEBNext UltraShear maintains methylation marks and can be used upstream of the NEBNext Enzymatic Methyl-seq v2 Kit (NEB #E8015S/L), NEBNext Enzymatic 5hmC-seq Kit (NEB #E3350S/L) and NEBNext Enzymatic Methyl-seq Kit (NEB #E7120S/L) in place of mechanical fragmentation (e.g., Covaris® shearing). The NEBNext UltraShear workflow is fast and user-friendly with minimal hands-on time.

**Figure 1. Example of DNA fragment size distribution for high-quality human DNA (NA12878) when fragmented with NEBNext UltraShear**




*50 ng of human DNA (NA12878) fragmented by NEBNext UltraShear for 5–45 minutes at 37˚C and cleaned-up using NEBNext Sample Purification Beads (2X) before running on Agilent TapeStation. The average fragmentation sizes and patterns (Agilent TapeStation D5000 HS) vary and are based on fragmentation time. The longer the fragmentation time the smaller the average size becomes with a tighter size distribution. Incubation time may need to be optimized for individual samples.*

## Section 1
## NEBNext UltraShear fragmentation protocol

**Symbols**

*This is a point where you can safely stop the protocol.*

*This caution sign signifies a step in the protocol that has multiple paths leading to the same end point but is dependent on a user variable, like the amount of input DNA.*

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

**Starting Material:** 5–250 ng purified, genomic DNA. We recommend that the DNA be in 1X TE (10 mM Tris pH 8.0, 1 mM EDTA). If the input DNA volume is less than 26 µl, add 1X TE to a final volume of 26 µl.

### 1.1. DNA Fragmentation

1.1.1. Ensure that the ○ (white) NEBNext UltraShear Reaction Buffer is completely thawed and quickly vortex to mix. Place on ice until use.

1.1.2. Vortex the ○ (white) NEBNext UltraShear for 5–10 seconds prior to use and place on ice.
**Note: It is important to vortex the enzyme mix prior to use for optimal performance.**

1.1.3. Add the following components to a 0.2 ml thin wall PCR tube on ice:

| COMPONENT | VOLUME PER LIBRARY |
|---|---|
| DNA | 26 µl |
| ○ (white) NEBNext UltraShear Reaction Buffer | 14 µl |
| ○ (white) NEBNext UltraShear | 4 µl |
| **Total Volume** | **44 µl** |

**Note: The ● (green) 500 mM DTT provided in the NEBNext UltraShear module is not for use with this protocol.**

**Note: A master mix can be prepared by combining the ○ (white) NEBNext UltraShear Reaction Buffer and ○ (white) NEBNext UltraShear on ice. Vortex the master mix 5-10 seconds and briefly spin in a microcentrifuge. Use master mix immediately.**

**Note: To fragment gDNA sample with NEBNext UltraShear in a buffer other than 1X TE (10 mM Tris pH 8.0, 1 mM EDTA) please see FAQs on the NEBNext UltraShear product page.**

1.1.4. Vortex the reaction for 5–10 seconds and briefly spin in a microcentrifuge.

1.1.5. In a thermal cycler, preheated and with the heated lid set to 75°C, run the following program:
5–45 minutes at 37°C
15 minutes at 65°C
Hold at 4°C

**Note: When working with fragmented/degraded DNA with low integrity and/or FFPE DNA, we suggest fragmenting with NEBNext UltraShear for less time (5–15 minutes at 37°C; optimizations may be needed).**

**Note: If you are not achieving the desired fragmentation profile with incubation at 37 ˚C for 30 mins or more, please follow additional recommendations in the FAQ to speed up fragmentation.**

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

*The fragmented DNA can be cleaned-up using various methods.*

1.1.6. Clean-up fragmented DNA using SPRIselect, AMPure XP beads or Monarch PCR & DNA Cleanup Kit following manufacturer's guidelines.

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

*The cleaned-up DNA can be analyzed by various fragment analyzers to determine size distribution.*

1.1.7. Use a Tapestation, Bioanalyzer or other fragment analyzer to determine the size distribution and proceed with downstream application of interest.

## Section 2
## NEBNext UltraShear fragmentation coupled with NEBNext Ultra II End Repair/dA-Tailing Module (NEB #E7546S/L)

**Symbols**

*This is a point where you can safely stop the protocol.*

●

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

**Note: NEBNext Ultra II End Repair/dA-Tailing Module (NEB #E756S/L) is required and must be purchased separately.**

**Starting Material:** 5–250 ng purified, genomic DNA. We recommend that the DNA be in 1X TE (10 mM Tris pH 8.0, 1 mM EDTA). If the input DNA volume is less than 26 µl, add 1X TE to a final volume of 26 µl.

### 2.1. DNA Fragmentation

2.1.1. Ensure that the ○ (white) NEBNext UltraShear Reaction Buffer is completely thawed and quickly vortex to mix. Place on ice until use.

2.1.2. Vortex the ○ (white) NEBNext UltraShear for 5–10 seconds prior to use and place on ice.

**Note: It is important to vortex the enzyme mix prior to use for optimal performance.**

2.1.3. Add the following components to a 0.2 ml thin wall PCR tube on ice:

| COMPONENT | VOLUME PER LIBRARY |
|---|---|
| DNA | 26 µl |
| ○ (white) NEBNext UltraShear Reaction Buffer | 14 µl |
| ○ (white) NEBNext UltraShear | 4 µl |
| **Total Volume** | **44 µl** |

**Note: A master mix can be prepared by combining the ○ (white) NEBNext UltraShear Reaction Buffer and ○ (white) NEBNext UltraShear on ice. Vortex the master mix 5–10 seconds and briefly spin in a microcentrifuge. Use master mix immediately.**

**Note: To fragment gDNA sample with NEBNext UltraShear in a buffer other than 1X TE (10 mM Tris pH 8.0, 1 mM EDTA) please see FAQs on the NEBNext UltraShear product page.**

2.1.4. Vortex the reaction for 5–10 seconds and briefly spin in a microcentrifuge.

2.1.5. In a thermal cycler, preheated and with the heated lid set to 75°C, run the following program.
5–45 minutes at 37°C
15 minutes at 65°C
Hold at 4°C

**Note: When working with fragmented/degraded DNA with low integrity and/or FFPE DNA, we suggest fragmenting with NEBNext UltraShear for less time (5–15 minutes at 37°C; optimizations may be needed).**

**Note: If you are not achieving the desired fragmentation profile with incubation at 37 °C for 30 mins or more, please follow additional recommendations in the FAQ to speed up fragmentation.**

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

**2.2. End Prep of Fragmented DNA**

**Note: NEBNext Ultra II End Repair/dA-Tailing Module (NEB #E7546) is required for this protocol. Only the • (green) NEBNext Ultra II End Prep Enzyme Mix is used in this protocol, not the • (green) NEBNext End Prep Reaction Buffer.**

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

| COMPONENT | VOLUME |
|---|---|
| Fragmented DNA (Step 2.1.5.) | 44 µl |
| • (green) 500 mM DTT | 2 µl |
| • (green) NEBNext Ultra II End Prep Enzyme Mix | 3 µl |
| **Total Volume** | **49 µl** |

**Note: A master mix can be prepared by combining the • (green) 500 mM DTT and • (green) NEBNext Ultra II End Prep Enzyme Mix on ice, then pipette mix the master mix and briefly spin in a microcentrifuge. Use master mix immediately.**

2.2.2. Set a 100 µl or 200 µl pipette to 40 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

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

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


**If necessary, samples can be stored at -20°C; however, a slight loss in yield may be observed. We recommend immediately continuing with adaptor ligation using NEBNext Ultra II Ligation Module (NEB #E7595) and separately purchasing NEBNext or customer supplied adaptor and primers.**

**Note: NEBNext oligo kits are supplied with Adaptors and Primers and are available separately; review the available options at www.neb.com/oligos.**

**Note: If using adaptors and primers from another vendor, please review this FAQ: https://www.neb.com/faqs/2019/03/08/can-i-use-this-nebnext-kit-with-adaptors-and-primers-from-other-vendors-than-neb.**

## Section 3
## NEBNext UltraShear fragmentation coupled with NEBNext Enzymatic Methyl-seq v2 Kit (NEB #E8015S/L)

**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.*

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

**Note: NEBNext Enzymatic Methyl-seq v2 Kit (NEB #E8015S/L) and any of the NEBNext LV Unique Dual Index Primers (NEB #E3390S, #E3392S, #E3400S, #E3402S, #E3404S, #E3406S, #E3408S) are required and must be purchased separately.**

**Note: The protocol used for NEBNext UltraShear (NEB #M7634S/L) combined with NEBNext Enzymatic Methyl-seq v2 Kit (NEB #E8015S/L) differs from the standard NEBNext Enzymatic Methyl-seq v2 Kit protocol. Be aware that the instructions are not interchangeable. The key difference is that Covaris DNA fragmentation is not needed. Please note additional changes in the following section of this protocol: End Prep of Fragmented DNA (3.2.1.).**

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

### 3.1. DNA Preparation & Fragmentation

3.1.1. Sample DNA and Control DNA: The following table is a guide for the amount of • (lilac) Control DNA Unmethylated Lambda and • (lilac) Control DNA CpG methylated pUC19 to be added to samples prior to EM-seq v2 library construction to evaluate conversion efficiencies.

**Table 3.1 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 | 1:50 |

The above dilutions are useful to perform a QC of conversion before deep sequencing using approximately 10 million paired reads. This read depth is sufficient to achieve a minimum of 5,000 paired end reads mapping to • (lilac) unmethylated Lambda DNA and 500 paired end reads mapping to • (lilac) CpG methylated pUC19. This level of coverage is needed 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 applications may only need 2 million paired end reads whereas others may require 50 million paired end reads or even 500 million paired end reads.

The dilutions recommended in Table 3.1 will provide sufficient coverage of controls for libraries sequenced to 10 million paired reads and above. Dilution of controls needs to be optimized by the user if sequencing lower than 10 million paired reads to obtain minimum coverage for • (lilac) unmethylated lambda (5,000 paired end reads) and • (lilac) CpG methylated pUC19 (500 paired end reads). Number of reads mapping to • (lilac) unmethylated lambda and • (lilac) CpG methylated pUC19 will be in the range of 0.5 to 1% with the suggested dilutions. Users should be aware that deep sequencing using the dilutions recommended in Table 4.1 can result in more than the minimum required • (lilac) unmethylated lambda and • (lilac) CpG methylated pUC19 reads. Ultimately, dilutions of the control DNAs should be optimized by the user.

Combine sample DNA (0.1–200 ng) with control DNAs specified below and make up the volume to 26 µl with 1X TE (10 mM Tris pH 8.0, 1 mM EDTA).

| COMPONENT | VOLUME |
|---|---|
| Sample DNA | 24 µl |
| • (lilac) Control DNA Unmethylated Lambda (see Table 3.1) | 1 µl |
| • (lilac) Control DNA CpG methylated pUC19 (see Table 3.1) | 1 µl |
| **Total Volume** | **26 µl** |

3.1.2. Ensure that the ◦ (white) NEBNext UltraShear Reaction Buffer is completely thawed and quickly vortex to mix. Place on ice until use.

3.1.3. Vortex the ◦ (white) NEBNext UltraShear for 5–10 seconds prior to use and place on ice.

3.1.4. Add the following components to a 0.2 ml thin wall PCR tube on ice:

| COMPONENT | VOLUME PER LIBRARY |
|---|---|
| gDNA combined with control DNA (Step 3.1.1.) | 26 µl |
| ◦ (white) NEBNext UltraShear Reaction Buffer | 14 µl |
| ◦ (white) NEBNext UltraShear | 4 µl |
| **Total Volume** | **44 µl** |

**Note: A master mix can be prepared by combining the ◦ (white) NEBNext UltraShear Reaction Buffer and ◦ (white) NEBNext UltraShear on ice. Vortex the master mix 5-10 seconds and briefly spin in a microcentrifuge. Use master mix immediately.**

**Note: To fragment gDNA sample with NEBNext UltraShear in a buffer other than 1X TE (10 mM Tris pH 8.0, 1 mM EDTA) please see FAQs on the NEBNext UltraShear product page.**

3.1.5. Vortex the reaction for 5–10 seconds and briefly spin in a microcentrifuge.

3.1.6. In a thermal cycler, preheated and with the heated lid set to 75°C, run the following program.

25–35 minutes at 37°C
15 minutes at 65°C
Hold at 4°C

**Note: When working with fragmented/degraded DNA with low integrity and/or FFPE DNA, we suggest fragmenting with NEBNext UltraShear for less time (5–15 minutes at 37°C; optimizations may be needed).**


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

### 3.2. End Prep of Fragmented DNA

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

| COMPONENT | VOLUME |
|---|---|
| Fragmented DNA (Step 3.1.6.) | 44 µl |
| • (green) 500 mM DTT | 2 µl |
| • (green) NEBNext Ultra II End Prep Enzyme Mix | 3 µl |
| **Total Volume** | **49 µl** |

**Note: The • (green) NEBNext Ultra II End Prep Reaction Buffer from the EM-seq v2 kit is not used for this protocol.**

**Note: The • (green) 500 mM DTT from M7634 is used in 3.2.1 and not the • (yellow) DTT from the EM-seq v2 Kit.**

**Note: A master mix can be prepared by combining the • (green) 500 mM DTT and • (green) NEBNext Ultra II End Prep Enzyme Mix on ice, then pipette mix the master mix and briefly spin in a microcentrifuge. Use master mix immediately.**

3.2.2 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.**

3.2.3. 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

### 3.3. Ligation of EM-seq Adaptor

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

| COMPONENT | VOLUME |
|---|---|
| End Prep reaction mixture (from Step 3.2.3.) | 49 µl |
| • (red) NEBNext EM-seq Adaptor | 2.5 µl |
| • (red) NEBNext Ligation Enhancer | 1 µl |
| • (red) NEBNext Ultra II Ligation Master Mix | 30 µl |
| **Total Volume** | **82.5 µl** |

**Note: The • (red) Ligation Enhancer and • (red) 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 EM-seq adaptor prior to use in the Adaptor Ligation Step. Premix adaptor and sample and then add the other ligation reagents.**

3.3.2. 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**.

3.3.3. 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.**

### 3.4. Clean-Up of Adaptor Ligated DNA

**Note: 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.**

3.4.1. Vortex Sample Purification Beads to resuspend.

3.4.2. Add 93 µl (1.1X 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.

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

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

3.4.5. 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)**.

3.4.6. 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.

3.4.7. 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.

3.4.8. 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.**

3.4.9. **Elution Options A or B**

**Option 3.4.9A: For > 10 ng DNA input**

3.4.9A.1 Remove the tubes from the magnetic stand. Elute the DNA targets from the beads by adding 29 µl of ○ (white) Elution Buffer.

3.4.9A.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.

3.4.9A.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 3.4.9B: For ≤ 10 ng 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.**

3.4.9B.1 Remove the tubes from the magnetic stand. Elute the DNA targets from the beads by adding 28 µl of ○ (white) Elution Buffer.

3.4.9B.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.

3.4.9B.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.

3.4.9B.4 Add 1 µl of the ● (red) NEBNext Carrier DNA to 27 µl of DNA from Step 3.4.9B.3.

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

**3.5. Protection of 5-Methylcytosines and 5-Hydroxymethylcytosines**

3.5.1. Prepare TET2 Buffer. Use Option A if you have #E8015S/#E8015G (24 reactions/G size) and Option B if you have #E8015L (96 reactions).

**Note: The TET2 Reaction Buffer Supplement is lyophilized. Centrifuge before use to ensure it is at the bottom of the tube**.

3.5.1A. Add 100 µl of ○ (yellow) TET2 Reaction Buffer to one tube of ○ (yellow) TET2 Reaction Buffer Supplement and mix well (for the 24-reaction/G size kit). Write date on tube.

3.5.1B. Add 400 µl of ○ (yellow) TET2 Reaction Buffer to one tube of ○ (yellow) TET2 Reaction Buffer Supplement and mix well (for the 96-reaction kit). Write date on tube.

**Note: The reconstituted buffer should be stored at -20°C and discarded after 4 months.**

3.5.2. Prepare Diluted ○ (yellow) T4-BGT.

**Only for ≤ 10 ng DNA input:** Dilute the ○ (yellow) T4-BGT 1:10 using the ○ (yellow) T4-BGT Diluent.

For example, add 9 µl of ○ (yellow) T4-BGT Diluent to 1 µl of ○ (yellow) T4-BGT and mix by vortexing for 1–2 seconds. Briefly centrifuge before use.

**Note: The diluted T4-BGT should be used immediately and discarded after use.**

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

**Note: Undiluted T4-BGT is used for samples > 10 ng**

**Diluted T4-BGT is used for samples ≤ 10 ng**

| COMPONENT | VOLUME |
|---|---|
| EM-seq adaptor ligated DNA (from Step 3.4.9A.3. or 3.4.9B.4.) | 28 µl |
| ◦ (yellow) TET2 Reaction Buffer (TET2 Reaction Buffer Supplement reconstituted in TET2 Reaction Buffer) | 10 µl |
| ◦ (yellow) UDP-Glucose | 1 µl |
| ◦ (yellow) DTT | 1 µl |
| ◦ (yellow) T4-BGT or Diluted T4-BGT | 1 µl |
| ◦ (yellow) TET2 | 4 µl |
| **Total Volume** | **45 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. 5mC/5hmC oxidation is initiated by the addition of the Fe(II) solution to the reaction in the next step.

3.5.4. Dilute the ◦ (yellow) 500 mM Fe(II) Solution by adding 1 µl to 1249 µl of water.

**Note: The ◦ (yellow) 500 mM Fe(II) solution color can vary between colorless to yellow, this is normal. Use the diluted solution immediately, do not store it. Discard after use.**

Combine diluted Fe(II) Solution and reaction mixture (from Step 3.5.3.) as described below:

| COMPONENT | VOLUME |
|---|---|
| Reaction mixture (from Step 3.5.3.) | 45 µl |
| Diluted Fe(II) Solution (from Step 3.5.4.) | 5 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.

3.5.5. 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

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

| COMPONENT | VOLUME |
|---|---|
| Protected DNA (Step 3.5.5.) | 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.

3.5.7. 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.**

### 3.6. Clean-Up of Protected DNA

**Note: 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.**

3.6.1. Vortex NEBNext Sample Purification Beads to resuspend.

3.6.2. 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.

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

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

3.6.5. 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)**.

3.6.6. 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.

3.6.7. 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.

3.6.8. 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.**

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

3.6.10. 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.

3.6.11. 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.**

### 3.7. 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 0.05 N Sodium Hydroxide. Use Option A for denaturing using Formamide and Option B for denaturing using 0.05 N Sodium Hydroxide.

**Option 3.7A: Formamide (Recommended)**

3.7A.1. Pre-heat thermal cycler to 85°C with the heated lid set to ≥ 105°C or on.

3.7A.2. Add 4 µl Formamide to the 16 µl of protected DNA (from Step 3.6.11.). Mix thoroughly by vortexing 1–2 seconds or by pipetting up and down at least 10 times and centrifuge briefly.

3.7A.3. Incubate at 85°C for 10 minutes in the pre-heated thermal cycler.

3.7A.4. Immediately place in cooling block on ice and allow the sample to fully cool (~ 2 minutes) before proceeding to Section 3.8.

**Option 3.7B: Sodium Hydroxide**

*Optional, See FAQ about preparing NaOH.*

3.7B.1. Prepare freshly diluted 0.05 N NaOH.

3.7B.2. Pre-heat thermal cycler to 85°C with the heated lid set to ≥ 105°C or on.

3.7B.3. Add 4 µl 0.05 N NaOH to the 16 µl of protected DNA (from Step 3.6.11.). Mix thoroughly by vortexing 1–2 seconds or by pipetting up and down at least 10 times and centrifuge briefly.

3.7B.4. Incubate at 85°C for 10 minutes in the pre-heated thermal cycler.

3.7B.5. Immediately place on ice and allow the sample to fully cool (~ 2 minutes) before proceeding to Section 3.8.

### 3.8. Deamination of Cytosines

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

| COMPONENT | VOLUME |
|---|---|
| Denatured DNA (from Step 3.7A.4. or 3.7B.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.

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

3.8.3. 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.**

### 3.9. PCR Amplification

3.9.1. On ice, add the following components to the deaminated DNA from Step 3.8.3.:

| COMPONENT | VOLUME |
|---|---|
| Deaminated DNA (from Step 3.8.3.) | 40 µl |
| UDI Primer Pair* | 5 µl |
| • (blue) NEBNext Q5U Master Mix | 45 µl |
| **Total Volume** | 90 µl |

* NEBNext LV Unique Dual Index Primers must be purchased separately from the library prep kit. Refer to the corresponding NEBNext LV Unique Dual Index Primers manual for determining valid barcode combinations.

3.9.2. Mix thoroughly by vortexing or by pipetting up and down at least 10 times, centrifuge briefly.

3.9.3. 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 | ∞ | |

| DNA INPUT | PCR CYCLES |
|---|---|
| 200 ng | 4–5 |
| 50 ng | 5–6 |
| 10 ng | 8 |
| 1 ng | 11 |
| 0.1 ng | 14 |

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

### 3.10. Clean-Up of Amplified Libraries

**Note: 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 over dry the beads as they become very difficult to resuspend.**

3.10.1. Vortex NEBNext Sample Purification Beads to resuspend.

3.10.2. 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.

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

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

3.10.5. 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)**.

3.10.6. 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.

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

3.10.8. 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.**

3.10.9. 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.

3.10.10. 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.

3.10.11. 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.**

### 3.11. Library Quantification and Sequencing

3.11.1. Use an Agilent TapeStation or Bioanalyzer to determine the size distribution and concentration of the libraries.

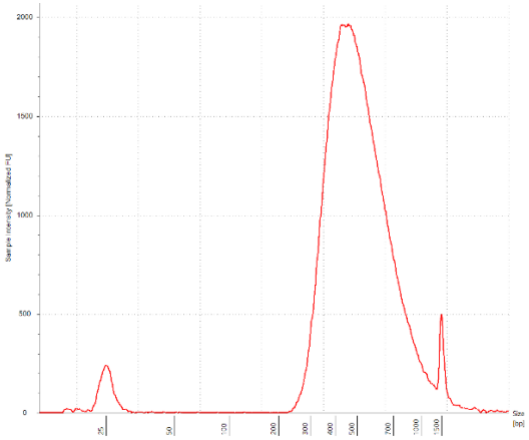



*Figure 3.11. Representative TapeStation trace for an EM-seq v2 library prepared using 200 ng of NA12878 genomic DNA that was fragmented for 30 minutes at 37°C with NEBNext UltraShear. The library was run on a HS D1000 tape.*

EM-seq v2 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.

## Section 4
## NEBNext UltraShear fragmentation coupled with NEBNext Enzymatic 5hmC-seq Kit (E5hmC-seq) (NEB #E3350S/L)

**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.*

**Note: NEBNext Enzymatic 5hmC-seq Kit (NEB #E3350S/L) and any of the NEBNext LV Unique Dual Index Primers (NEB #E3390S, #E3392S, #E3400S, #E3402S, #E3404S, #E3406S, #E3408S) are required and must be purchased separately.**

**Note: The protocol used for NEBNext UltraShear (NEB #M7634S/L) combined with NEBNext Enzymatic 5hmC-seq Kit (NEB #E3350S/L) differs from the standard NEBNext Enzymatic 5hmC-seq Kit protocol. Be aware that the instructions are not interchangeable. The key difference is that Covaris DNA fragmentation is not needed. Please note additional changes in the following section of this protocol: End Prep of Fragmented DNA (4.2.1.).**

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

### 4.1. DNA Preparation & Fragmentation

4.1.1. 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 4.1 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 4.1), 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 and make up the volume to 26 µl with 1X TE (10 mM Tris pH 8.0, 1 mM EDTA).

| COMPONENT | VOLUME |
|---|---|
| Sample DNA | 24 µl |
| • (lilac) Control DNA Unmethylated Lambda (see Table 4.1) | 1 µl |
| • (lilac) Control DNA 5hmC T4 (see Table 4.1) | 1 µl |
| **Total Volume** | **26 µl** |

4.1.2. Ensure that the ∘ (white) NEBNext UltraShear Reaction Buffer is completely thawed and quickly vortex to mix. Place on ice until use.

4.1.3. Vortex the ∘ (white) NEBNext UltraShear for 5–10 seconds prior to use and place on ice.

4.1.4. Add the following components to a 0.2 ml thin wall PCR tube on ice:

| COMPONENT | VOLUME PER LIBRARY |
|---|---|
| gDNA combined with control DNA (Step 4.1.1.) | 26 µl |
| ∘ (white) NEBNext UltraShear Reaction Buffer | 14 µl |
| ∘ (white) NEBNext UltraShear | 4 µl |
| **Total Volume** | **44 µl** |

**Note: A master mix can be prepared by combining the ∘ (white) NEBNext UltraShear Reaction Buffer and ∘ (white) NEBNext UltraShear on ice. Vortex the master mix 5-10 seconds and briefly spin in a microcentrifuge. Use master mix immediately.**

**Note: To fragment gDNA sample with NEBNext UltraShear in a buffer other than 1X TE (10 mM Tris pH 8.0, 1 mM EDTA) please see FAQs on the NEBNext UltraShear product page.**

4.1.5. Vortex the reaction for 5-10 seconds and briefly spin in a microcentrifuge.

4.1.6. In a thermal cycler, preheated and with the heated lid set to 75°C, run the following program.
25–35 minutes at 37°C
15 minutes at 65°C
Hold at 4°C

**Note: When working with fragmented/degraded DNA with low integrity and/or FFPE DNA, we suggest fragmenting with NEBNext UltraShear for less time (5–15 minutes at 37°C; optimizations may be needed).**


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

### 4.2. End Prep of Fragmented DNA

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

| COMPONENT | VOLUME |
|---|---|
| Fragmented DNA (Step 4.1.6.) | 44 µl |
| • (green) 500 mM DTT | 2 µl |
| • (green) NEBNext Ultra II End Prep Enzyme Mix | 3 µl |
| **Total Volume** | **49 µl** |

**Note: The • (green) NEBNext Ultra II End Prep Reaction Buffer from the E5hmC-seq Kit is not used for this protocol.**

**Note: A master mix can be prepared by combining the • (green) 500 mM DTT and • (green) NEBNext Ultra II End Prep Enzyme Mix on ice then pipette mix the master mix and briefly spin in a microcentrifuge. Use master mix immediately.**

4.2.2. 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.**

4.2.3. 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

### 4.3. Ligation of E5hmC-seq Adaptor

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

| COMPONENT | VOLUME |
|---|---|
| End Prep reaction mixture (Step 4.2.3.) | 49 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** | **82.5 µl** |

**Note: The • (red) Ligation Enhancer and • (red) 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.**

4.3.2. 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.**

4.3.3. 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.**

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

**Note: 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.**

4.4.1. Vortex NEBNext Sample Purification Beads to resuspend.

4.4.2. Add 93 µl (1.1X 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.

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

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

4.4.5. 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)**.

4.4.6. 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.

4.4.7. 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.

4.4.8. 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.**

4.4.9. **Elution Options A or B**

**Option 4.4.9A: For > 10 ng DNA input**

4.4.9A.1. Remove the tubes from the magnetic stand. Elute the DNA targets from the beads by adding 29 µl of ○ (white) Elution Buffer.

4.4.9A.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.

4.4.9A.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 4.4.9B: 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**.

4.4.9B.1. Remove the tubes from the magnetic stand. Elute the DNA targets from the beads by adding 28 µl of ○ (white) Elution Buffer.

4.4.9B.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.

4.4.9B.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.

4.4.9B.4. Add 1 µl of the ● (red) NEBNext Carrier DNA to 27 µl of DNA from Step 4.4.9B.3.


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

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

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

| COMPONENT | VOLUME |
|---|---|
| E5hmC-seq adaptor ligated DNA (from Step 4.4.9A.3. or 4.4.9B.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.

4.5.2. 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

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

| COMPONENT | VOLUME |
|---|---|
| Glucosylated DNA (from Step 4.5.2.) | 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.

4.5.4. 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.**

### 4.6. Clean-up of Glucosylated DNA

**Note: 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.**

4.6.1. Vortex NEBNext Sample Purification Beads to resuspend.

4.6.2. 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.

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

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

4.6.5. 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)**.

4.6.6. 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.

4.6.7. 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.

4.6.8. 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.**

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

4.6.10. 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.

4.6.11. 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.**

### 4.7. 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 4.7A: Formamide (Recommended)**

4.7A.1. Pre-heat thermal cycler to 85°C with the heated lid set to ≥ 105°C or on.

4.7A.2. Add 4 µl Formamide to the 16 µl of glucosylated DNA (from Step 4.6.11.). Mix thoroughly by vortexing 1–2 seconds or by pipetting up and down at least 10 times and centrifuge briefly.

4.7A.3. Incubate at 85°C for 10 minutes in the pre-heated thermal cycler.

4.7A.4. Immediately place in cooling block on ice and allow the sample to fully cool (~2 minutes) before proceeding to Section 4.8.

**Option 4.7B: Sodium Hydroxide**

*Optional, See FAQ about preparing NaOH.*

4.7B.1. Prepare freshly diluted 0.1 N NaOH.

4.7B.2. Pre-heat thermal cycler to 85°C with the heated lid set to ≥ 105°C or on.

4.7B.3. Add 4 µl 0.1 N NaOH to the 16 µl of glucosylated DNA (from Step 4.6.11.). Mix thoroughly by vortexing 1–2 seconds or by pipetting up and down at least 10 times and centrifuge briefly.

4.7B.4. Incubate at 85°C for 10 minutes in the pre-heated thermal cycler.

4.7B.5. Immediately place in cooling block on ice and allow the sample to fully cool (~2 minutes) before proceeding to Section 4.8.

### 4.8. Deamination of 5mC and Cytosines

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

| COMPONENT | VOLUME |
|---|---|
| Denatured DNA (Step 4.7A.4. or 4.7B.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.

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

4.8.3. 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.**

### 4.9. PCR Amplification

4.9.1. On ice, add the following components to the deaminated DNA from Step 4.8.3.:

| COMPONENT | VOLUME |
|---|---|
| Deaminated DNA (Step 4.8.3.) | 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).

**Note: E5hmC-seq is compatible with both NEBNext LV Unique Dual Index Primers and NEBNext Primers for Epigenetics and must be purchased separately from the library prep kit. The NEBNext LV Unique Dual Index Primers Set 2B has identical primer pairs to those found in NEBNext Primers for Epigenetics (Unique Dual Index Set 2B) and cannot be combined. The NEBNext LV Unique Dual Index Primers Set 3 has identical primer pairs to those found in NEBNext Primers for Epigenetics (Unique Dual Index Set 3) and cannot be combined. Refer to the corresponding NEBNext LV Unique Dual Index Primers or NEBNext Primers for Epigenetics manual and FAQs for more information.**

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

4.9.3. 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: 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.**

### 4.10. Clean-up of Amplified Libraries

**Note: 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.**

4.10.1. Vortex NEBNext Sample Purification Beads to resuspend.

4.10.2. 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.

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

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

4.10.5. 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)**.

4.10.6. 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.

4.10.7. 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.

4.10.8. 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.**

4.10.9. 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.

4.10.10. 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.

4.10.11. 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.**

### 4.11. Library Quantification and Sequencing

4.11.1. 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**

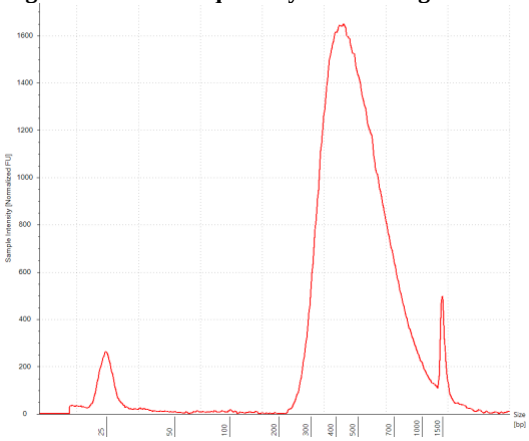



*Figure 4.11. Representative TapeStation trace for an E5hmC-seq library prepared using 200 ng of human brain genomic DNA that was fragmented for 30 minutes at 37°C with NEBNext UltraShear. The library was run on a HS D1000 tape.*

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.

## Section 5
## NEBNext UltraShear fragmentation coupled with NEBNext Enzymatic Methyl-seq Kit (NEB #E7120S/L)

**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.*

**Note: NEBNext Enzymatic Methyl-seq (NEB #E7120S/L) is required and must be purchased separately.**

**Note: The protocol used for NEBNext UltraShear (NEB #M7634S/L) combined with NEBNext Enzymatic Methyl-seq differs from the standard NEBNext Enzymatic Methyl-seq protocol(s) (NEB #E7120S/L). Be aware that the instructions are not interchangeable. The key difference is that Covaris DNA fragmentation is not needed. Please note additional changes in the following sections of this protocol: End Prep of Fragmented DNA (5.2.1.), Clean-up of Deaminated DNA (5.9.), and Clean-up of Amplified Libraries (5.11.).**

**Starting Material:** 10–200 ng double stranded DNA

### 5.1. DNA Preparation and Fragmentation of sample

5.1.1. Combine genomic DNA (10–200 ng) with control DNAs specified below and make up the volume to 26 µl with 1X TE (10 mM Tris pH 8.0, 1 mM EDTA).

| COMPONENT | VOLUME |
|---|---|
| gDNA | 24 µl |
| • (lilac) Control DNA Unmethylated Lambda (see Table 5.1) | 1 µl |
| • (lilac) Control DNA CpG methylated pUC19 (see Table 5.1) | 1 µl |
| **Total Volume** | **26 µl** |

The following table is a guide for the amount of • (lilac) Control DNA Unmethylated Lambda DNA and • (lilac) Control DNA CpG methylated pUC19 to be added to samples prior to EM-seq library construction. Expected read numbers along with read length should be considered to ensure that sufficient controls are included for the users individual sequencing goals.

**Table 5.1 Dilutions of control DNAs for a range of genomic DNA inputs. These are suitable for shallow/pre-sequencing (approx. 2–4 million paired reads) prior to deep sequencing (approx. 100–150 million paired reads) on NovaSeq, HiSeq or NextSeq.**

| DILUTION OF • (LILAC) CONTROL DNA UNMETHYLATED LAMBDA AND • (LILAC) CONTROL DNA CpG METHYLATED pUC19 | | |
|---|---|---|
| DNA Input Amount | Pre-sequencing 2–4 Million Paired Reads | Deep Sequencing 100–150 Million Paired Reads |
| 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 unmethylated Lambda DNA, and 500 paired end reads with a read length of 76 bases for CpG methylated pUC19, are needed to give enough coverage for accurate conversion estimates.

Different applications may require different sequencing depths 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 others may require 50 million paired end reads or even 300 million 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 5.1), 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.

5.1.2. Ensure that the ○ (white) NEBNext UltraShear Reaction Buffer is completely thawed and quickly vortex to mix. Place on ice until use.

5.1.3. Vortex the ○ (white) NEBNext UltraShear for 5–10 seconds prior to use and place on ice.

**Note: It is important to vortex the enzyme mix prior to use for optimal performance.**

5.1.4. Add the following components to a 0.2 ml thin wall PCR tube on ice:

| COMPONENT | VOLUME PER LIBRARY |
|---|---|
| gDNA combined with control DNA (Step 5.1.1.) | 26 µl |
| ○ (white) NEBNext UltraShear Reaction Buffer | 14 µl |
| ○ (white) NEBNext UltraShear | 4 µl |
| **Total Volume** | **44 µl** |

**Note: A master mix can be prepared by combining the ○ (white) NEBNext UltraShear Reaction Buffer and ○ (white) NEBNext UltraShear on ice. Vortex the master mix 5-10 seconds and briefly spin in a microcentrifuge. Use master mix immediately.**

**Note: To fragment gDNA sample with NEBNext UltraShear in a buffer other than 1X TE (10 mM Tris pH 8.0, 1 mM EDTA) please see FAQs on the NEBNext UltraShear product page.**

5.1.5. Vortex the reaction for 5–10 seconds and briefly spin in a microcentrifuge.

*Use the chart below to determine the incubation time and temperature required to generate the desired average sequenced insert size. Incubation time may need to be optimized for individual samples.*

5.1.6. In a thermal cycler, preheated and with the heated lid set to 75°C, run the following program:

| AVERAGE SEQUENCED INSERT SIZE | FRAGMENTATION TIME AND TEMPERATURE |
|---|---|
| A) 120–150 bp | 15–25 minutes at 45°C<br>15 minutes at 65°C<br>Hold at 4°C |
| B) 150–250 bp | 15–25 minutes at 37°C<br>15 minutes at 65°C<br>Hold at 4°C |
| C) 250–350 bp | 10–20 minutes at 37°C<br>15 minutes at 65°C<br>Hold at 4°C |

**Note: Fragmentation time selection, from table above, will be coupled with clean-up recommendations. Each fragmentation group (A, B, or C) in Section 5.1.6. has specific sample purification bead clean-up recommendations in Section 5.9.2. and 5.11.3. Do not deviate from the paired recommendations as loss of sample may occur.**

**Note: When working with fragmented/degraded DNA with low integrity and/or FFPE DNA, we suggest fragmenting with NEBNext UltraShear for less time (5–15 minutes at 37°C; optimizations may be needed), please see FAQs on the product page.**

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

### 5.2. End Prep of Fragmented DNA

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

| COMPONENT | VOLUME |
|---|---|
| Fragmented DNA (Step 5.1.6.) | 44 µl |
| • (green) 500 mM DTT | 2 µl |
| • (green) NEBNext Ultra II End Prep Enzyme Mix | 3 µl |
| **Total Volume** | **49 µl** |

**Note: The • (green) NEBNext Ultra II End Prep Reaction Buffer from the Enzymatic Methyl-seq kit (NEB #E7120S/L) is not used for this protocol.**

**Note: The • (green) 500 mM DTT from M7634 is used in 5.2.1 and not the ○ (yellow) DTT from the Enzymatic Methyl-seq Kit (NEB #E7120S/L).**

**Note: A master mix can be prepared by combining the • (green) 500 mM DTT and • (green) NEBNext Ultra II End Prep Enzyme Mix on ice, then pipette mix the master mix and briefly spin in a microcentrifuge. Use master mix immediately.**

5.2.2. Set a 100 µl or 200 µl pipette to 40 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

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

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

### 5.3. Ligation of EM-seq Adaptor

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

| COMPONENT | VOLUME |
|---|---|
| End Prep reaction mixture (Step 5.2.3.) | 49 µl |
| • (red) NEBNext EM-seq Adaptor | 2.5 µl |
| • (red) NEBNext Ligation Enhancer | 1 µl |
| • (red) NEBNext Ultra II Ligation Master Mix | 30 µl |
| **Total Volume** | **82.5 µl** |

**Note: • (red) Ligation Enhancer and • (red) Ligation Master Mix can be mixed ahead of time and is stable for at least 8 hours at 4°C. We do not recommend adding adaptor to a premix in the adaptor ligation step. Premix adaptor and sample and then add the other ligation reagents.**

5.3.2. Set a 100 µl or 200 µl pipette to 70 µl and then pipette the entire volume up and down 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

**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.**

5.3.3. 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.**

### 5.4. Clean-Up of Adaptor Ligated DNA

**Note: 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.**

5.4.1. Vortex Sample Purification Beads to resuspend.

5.4.2. Add 110 µl 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.

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

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

5.4.5. 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)**.

5.4.6. Add 200 µl of 80% freshly prepared ethanol to the tubes while in 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.

5.4.7. 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.

5.4.8. Air dry the beads for up to 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 target. 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.**

5.4.9. Remove the tubes from the magnetic stand. Elute the DNA target from the beads by adding 29 µl of Elution Buffer ○ (white).

5.4.10. 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.

5.4.11. 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.


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

### 5.5. Oxidation of 5-Methylcytosines and 5-Hydroxymethylcytosines

5.5.1. Prepare TET2 Buffer. Use option A if you have E7120S/E7120G (24 reactions/G size) and option B if you have E7120L (96 reactions).

**Note: The ○ (yellow) TET2 Reaction Buffer Supplement is a powder. Centrifuge before use to ensure it is at the bottom of the tube.**

5.5.1A. Add 100 µl of ○ (yellow) TET2 Reaction Buffer to one tube of ○ (yellow) TET2 Reaction Buffer Supplement and mix well. Write date on tube.

5.5.1B. Add 400 µl of ○ (yellow) TET2 Reaction Buffer to one tube of ○ (yellow) TET2 Reaction Buffer Supplement and mix well. Write date on tube.

**Note: The reconstituted buffer should be stored at -20°C and discarded after 4 months.**

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

| COMPONENT | VOLUME |
|---|---|
| EM-seq adaptor ligated DNA (Step 5.4.11.) | 28 µl |
| ∘ (yellow) TET2 Reaction Buffer (TET2 Reaction Buffer plus reconstituted TET2 Reaction Buffer Supplement; Step 5.5.1.) | 10 µl |
| ∘ (yellow) Oxidation Supplement | 1 µl |
| ∘ (yellow) DTT | 1 µl |
| ∘ (yellow) Oxidation Enhancer | 1 µl |
| ∘ (yellow) TET2 | 4 µl |
| **Total Volume** | **45 µl** |

Mix thoroughly by vortexing, centrifuge briefly. For multiple reactions, a master mix of the above reaction components can be prepared before addition to the sample DNA. 5mC/5hmC oxidation is initiated by the addition of the Fe(II) solution to the reaction in the next step.

5.5.3. Dilute the ∘ (yellow) 500 mM Fe(II) Solution by adding 1 µl to 1,249 µl of water.

**Note: The ∘ (yellow) 500 mM Fe(II) solution color can vary between colorless to yellow, this is normal. Use the diluted solution immediately, do not store it. Discard after use.**

Combine diluted ∘ (yellow) Fe(II) Solution and Reaction Mixture with Oxidation Enzymes as described below:

| COMPONENT | VOLUME |
|---|---|
| Reaction Mixture (Step 5.5.2.) | 45 µl |
| Diluted Fe(II) Solution (Step 5.5.3.) | 5 µl |
| **Total Volume** | **50 µl** |

Mix thoroughly by vortexing or by pipetting up and down at least 10 times, centrifuge briefly.

5.5.4 Place in a thermal cycler, and run the following program with the heated lid set to [3] 45°C or on:
1 hour at 37°C
Hold at 4°C

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

| COMPONENT | VOLUME |
|---|---|
| Oxidized DNA (Step 5.5.4.) | 50 µl |
| ∘ (yellow) Stop Reagent | 1 µl |
| **Total Volume** | **51 µl** |

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

5.5.6. 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.**

### 5.6. Clean-Up of TET2 Oxidized DNA

**Note: 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.**

5.6.1. Vortex Sample Purification Beads to resuspend.

5.6.2. Add 90 µl 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.

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

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

5.6.5. 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)**.

5.6.6. Add 200 µl of 80% freshly prepared ethanol to the tubes while in 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.

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

5.6.8. Air dry the beads for up to 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 target. 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.**

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

5.6.10. 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.

5.6.11. 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.


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

### 5.7. Denaturation of DNA

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

#### 5.7A: Formamide (Recommended)

5.7A.1. Pre-heat thermal cycler to 85°C with the heated lid set to ≥ 105°C or on.

5.7A.2. Add 4 µl Formamide to the 16 µl of oxidized DNA (Step 5.6.11). Vortex to mix or by pipetting up and down at least 10 times, centrifuge briefly.

5.7A.3. Incubate at 85°C for 10 minutes in the pre-heated thermal cycler with the heated lid on.

5.7A.4. Immediately place on ice and allow the sample to fully cool (~2 minutes) before proceeding to Section 5.8.

#### 5.7B: Sodium Hydroxide (Optional, See FAQ about preparing NaOH)

5.7B.1. Prepare freshly diluted 0.1 N NaOH.

5.7B.2. Pre-heat thermal cycler to 50°C with the heated lid set to ≥ 105°C or on.

5.7B.3. Add 4 µl 0.1 N NaOH to the 16 µl of oxidized DNA (Step 5.6.11). Vortex to mix or by pipetting up and down at least 10 times, centrifuge briefly.

5.7B.4. Incubate at 50°C for 10 minutes in the pre-heated thermal cycler.

5.7B.5. Immediately place on ice and allow the sample to fully cool (~2 minutes) before proceeding to Section 5.8.

**5.8. Deamination of Cytosines**

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

| COMPONENT | VOLUME |
|---|---|
| Denatured DNA (Step 5.7A.4. or 5.7B.5.) | 20 µl |
| Nuclease-free water | 68 µl |
| • (orange) APOBEC Reaction Buffer | 10 µl |
| • (orange) BSA | 1 µl |
| • (orange) APOBEC | 1 µl |
| **Total Volume** | **100 µl** |

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

5.8.2. Mix thoroughly by vortexing or by pipetting up and down at least 10 times, centrifuge briefly.

5.8.3. 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.**

**5.9. Clean-Up of Deaminated DNA**

**Note: 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 APOBEC clean-up. After the bead washes, do not overdry the beads as they become very difficult to resuspend.**

5.9.1. Vortex Sample Purification Beads to resuspend.

 *Use the chart below to determine the amount of NEBNext Sample Purification Beads required to generate the desired average sequenced insert size.*

**Note: The clean-up of deaminated DNA step using the NEBNext UltraShear with NEBNext Enzymatic Methyl-seq protocol differs from the standard NEBNext Enzymatic Methyl-seq protocol (NEB #E7120S/L).**

| AVERAGE SEQUENCED INSERT SIZE | CLEAN-UP OF DEAMINATED DNA |
|---|---|
| A) 120–150 bp | Add 80 µl of resuspended NEBNext Sample Purification Beads to each sample. |
| B) 150–250 bp | Add 80 µl of resuspended NEBNext Sample Purification Beads to each sample. |
| C) 250–350 bp | Add 65 µl of resuspended NEBNext Sample Purification Beads. to each sample. |

**Note: Clean-up recommendations, from the table above, are coupled with fragmentation time recommendations in step 5.1.6. Each fragmentation group (A, B, or C) in step 5.1.6. has specific sample purification bead clean-up recommendations in step 5.9.2. and 5.11.3. Do not deviate from the paired recommendations as loss of sample may occur.**

**Note: When working with fragmented/degraded DNA with low integrity and/or FFPE DNA, we suggest performing 0.9X sample purification bead cleanups after Deamination of Cytosines (Step 5.9.2.), please see FAQs on the NEBNext UltraShear product page.**

5.9.2. Add the appropriate volume of resuspended NEBNext Sample Purification Beads determined by the chart above 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. Vortexing for 3–5 seconds on high can also be used. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

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

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

5.9.5. 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)**.

5.9.6. Add 200 µl of 80% freshly prepared ethanol to the tubes while in 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.

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

5.9.8. Air dry the beads for up to 90 seconds 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 target. 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.**

5.9.9. Remove the tubes from the magnetic stand. Elute the DNA target from the beads by adding 21 µl of ∘ (white) Elution Buffer.

5.9.10. 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.

5.9.11. 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.**

### 5.10. PCR Amplification

5.10.1. On ice, add the following components to the deaminated DNA:

| COMPONENT | VOLUME |
|---|---|
| Deaminated DNA (Step 5.9.11.) | 20 µl |
| EM-seq Index Primer*, ** | 5 µl |
| • (blue) NEBNext Q5U Master Mix | 25 µl |
| **Total Volume** | **50 µl** |

* Refer to Section 3 in NEB #E7120S/L manual for barcode pooling guidelines or visit NEBNext® Index Oligo Selector.

** EM-seq primers are supplied in tubes in NEB #E7120S (24 reactions) or as a 96 Unique Dual Index Primers Pairs Plate in NEB #E7120L (96 reactions). If using NEB #E7125S/L, please note that oligos (EM-seq adaptors and primers; NEB #E7140S/L) and NEBNext Q5U® Master Mix (NEB #M0597S/L) are available separately.

**Note: EM-seq indexing primers are fully listed in the EM-seq manual (NEB #E7120) and sample sheets can be located in Usage Guidelines for EM-seq (NEB #E7120) product page.**

5.10.2. Mix thoroughly by vortexing or by pipetting up and down at least 10 times, centrifuge briefly.

5.10.3. 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 | 4–8* |
| Annealing | 62°C | 30 seconds | |
| Extension | 65°C | 60 seconds | |
| Final Extension | 65°C | 5 minutes | 1 |
| Hold | 4°C | ∞ | |

* Cycle Recommendations:
- 10 ng DNA input: 8 cycles
- 50 ng DNA input: 5–6 cycles
- 200 ng DNA input: 4–5 cycles


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

### 5.11. Clean-Up of Amplified Libraries

**Note: 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.**

5.11.1. Vortex Sample Purification Beads to resuspend.

5.11.2. Add 50 µl of water to each sample. Mix well by pipetting up and down at least 10 times.

 *Use the chart below to determine the amount of NEBNext Sample Purification Beads required to generate the desired average sequenced insert size.*

**Note: The NEBNext UltraShear with NEBNext Enzymatic Methyl-seq protocol for clean-up of amplified library differs from the standard NEBNext Enzymatic Methyl-seq protocol (NEB #E7120S/L).**

| AVERAGE SEQUENCED INSERT SIZE | CLEAN-UP OF AMPLIFIED LIBRARY |
|---|---|
| A) 120–150 bp | Add 80 µl of resuspended NEBNext Sample Purification Beads to each sample. |
| B) 150–250 bp | Add 80 µl of resuspended NEBNext Sample Purification Beads to each sample. |
| C) 250–350 bp | Add 65 µl of resuspended NEBNext Sample Purification Beads to each sample. |

**Note: Clean-up recommendations, from the table above, are coupled with fragmentation time recommendations in step 5.1.6. Each fragmentation group (A, B, or C) in step 5.1.6. has specific sample purification bead clean-up recommendations in step 5.9.2. and 5.11.3. Do not deviate from the paired recommendations as loss of sample may occur.**

**Note: When working with fragmented/degraded DNA with low integrity and/or FFPE DNA, we suggest performing 0.9X sample purification bead cleanups after PCR Amplification (Step 5.11.3.), please see FAQs on the NEBNext UltraShear product page.**

5.11.3. Add the appropriate volume of resuspended NEBNext Sample Purification Beads determined by the chart above 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. Vortexing for 3–5 seconds on high can also be used. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out. Be careful to expel all of the liquid out of the tip during the last mix.

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

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

5.11.6. 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)**.

5.11.7. Add 200 µl of 80% freshly prepared ethanol to the tubes while in 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.

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

5.11.9. Air dry the beads for up to 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 target. 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.**

5.11.10. Remove the tubes from the magnetic stand. Elute the DNA target from the beads by adding 21 µl of Elution Buffer ○ (white). For long term storage, 21 µl of 1X TE (10 mM Tris, 1 mM EDTA, pH 8.0), 21 µl of Low TE (10 mM Tris, 0.1 mM EDTA, pH 8.0), or 21 µl of 0.1X TE (1 mM Tris, 0.1 mM EDTA, pH 8.0).

5.11.10. 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.

5.11.11. 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.

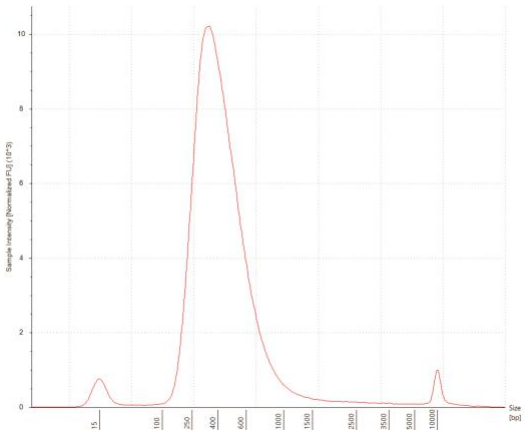

**Safe Stopping Point: Samples can be stored at -20°C.**

### 5.12. Library Quantification

*The library can be analyzed by various fragment analyzers to determine library size.*

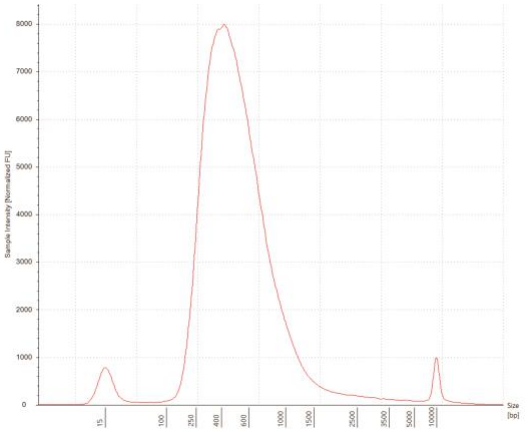
5.12.1. Use a TapeStation or Bioanalyzer to determine the size distribution and concentration of the libraries. A typical EM-seq library would have the following trace on a High Sensitivity D5000 ScreenTape on Agilent TapeStation for 200 ng of NA12878 genomic DNA for the following sequenced insert sizes.

**Library Profile for Average Sequenced Insert Size (120–150 bp)**



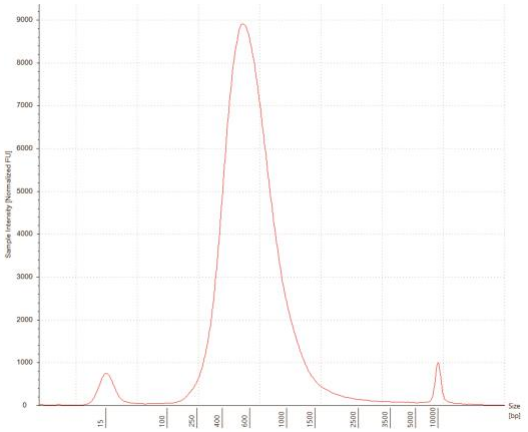
*Sequence 2 x 76 base reads or 2 x 100 base reads using the preferred Illumina® platform.*

**Library Profile for Average Sequenced Insert Size (150–250 bp)**



*Sequence 2 x 76 base reads or 2 x 100 base reads using the preferred Illumina platform.*

**Library Profile for Average Sequenced Insert Size (250–350 bp)**



*Sequence 2 x 100 base reads, or 2 x 150 base reads using the preferred Illumina platform.*

## Adaptor Trimming Sequences
## NEBNext Enzymatic Methyl-seq Kit v2 (NEB #E8015S/L, NEBNext Enzymatic 5hmC-seq Kit (NEB #E3350S/L) and NEBNext Enzymatic Methyl-seq Kit (E7120S/L)

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

## Components

NEB #M7634S Table of Components

| NEB # | PRODUCT | VOLUME |
|---|---|---|
| M7634S | NEBNext UltraShear | 0.096 ml |
| B9042S | NEBNext UltraShear Reaction Buffer | 0.336 ml |
| B1079S | 500 mM DTT | 0.048 ml |

NEB #M7634L Table of Components

| NEB # | PRODUCT | VOLUME |
|---|---|---|
| M7634L | NEBNext UltraShear | 0.384 ml |
| B9042L | NEBNext UltraShear Reaction Buffer | 2 x 0.672 ml |
| B1079L | 500 mM DTT | 0.192 ml |

## Revision History

| REVISION # | DESCRIPTION | DATE |
|---|---|---|
| 1.0 | N/A | 6/23 |
| 2.0 | Updated protocols for Sections 1, 2 and 5.<br>Also including new protocols:<br>• NEBNext Enzymatic Methyl-seq v2 Kit<br>• NEBNext Enzymatic 5hmC-seq Kit | 12/24 |