

NEBNext UltraShear®

NEB #M7634S/L

24/96 reactions

Version 4.0_7/26

Table of Contents

Overview	2
Section 1	
NEBNext UltraShear Fragmentation Protocol.....	3
Section 2	
NEBNext UltraShear Fragmentation Coupled with NEBNext® Ultra™ II End Repair/dA-Tailing Module (NEB #E756S/L).....	5
Section 3	
NEBNext UltraShear Fragmentation Coupled with NEBNext Enzymatic Methyl-seq Kit v2 (NEB #E8015S/L)	7
Section 4	
NEBNext UltraShear Coupled with NEBNext Enzymatic 5hmC-seq™ Kit (NEB #E3350S/L).....	16
Section 5	
NEBNext UltraShear Fragmentation Coupled with NEBNext Enzymatic Methyl-seq Kit (NEB #E7120S/L)	24
Adaptor Trimming Sequences	35
Components	35
Revision History	36

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

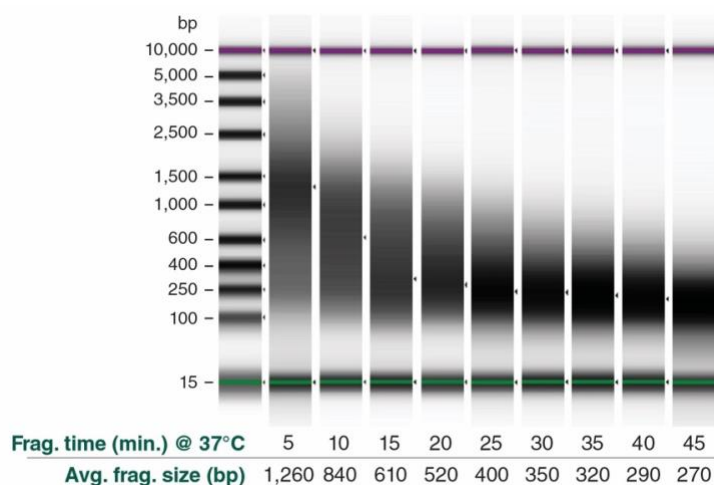
Where larger volumes, customized or bulk packaging are required, we encourage consultation with the Customized Solutions team at NEB. Please complete the NEB Custom Contact Form at www.neb.com/CustomContactForm to learn more.

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.



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 $\geq 75^{\circ}\text{C}$ 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.



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.).

Please read the “important guidelines about reagent handling and experimental setup” section at the end of the manual.

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	1:10

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) Diluted Control DNA Unmethylated Lambda (see Table 3.1)	1 µl
● (lilac) Diluted 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 $\geq 75^{\circ}\text{C}$ or on, and run the following program:
- 15 minutes at 20°C
 15 minutes at 65°C
 Hold at 4°C

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

Note: This step involves multiple tubes with yellow caps. Confirm tube identity throughout setup and use a clear, consistent organizational strategy to ensure correct reagent handling.

- 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 μ l (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 μ l 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 μ l of $\circ$ (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 μ l 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 $\geq 105^{\circ}\text{C}$ or on.
- 3.7A.2. Add 4 μ l Formamide to the 16 μ l 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 a pre-chilled metal cooling block on ice and allow the sample to fully cool (~ 2 minutes) before proceeding to Section 3.8. If a metal cooling block is not available, place on ice and ensure proper contact with the tube walls.

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 $\geq 105^{\circ}\text{C}$ or on.
- 3.7B.3. Add 4 μ l 0.05 N NaOH to the 16 μ l 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 in a pre-chilled metal cooling block on ice and allow the sample to fully cool (~2 minutes) before proceeding to Section 3.8. If a metal cooling block is not available, place on ice and ensure proper contact with the tube walls.

3.8. Deamination of Cytosines



Deamination Options A or B

Use **Option A (3.8.3A.)** for DNA inputs ≤ 100 ng.

Option B (3.8.3B.) is required for > 100 ng DNA inputs and can be used for all inputs (0.1 ng to 200 ng).

- 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 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.

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

Option A: (Inputs ≤ 100 ng)

- 3.8.3A. Place in a thermal cycler with the heated lid set to $\geq 85^{\circ}\text{C}$ or on, and run the following program:

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)

- 3.8.3B. Place in a thermal cycler with the heated lid set to $\geq 45^{\circ}\text{C}$ or on, and run the following program:

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 μ l
NEBNext LV UDI Primer Pair* * * *	5 μ l
• (blue) NEBNext Q5U Master Mix	45 μ l
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.

- 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.

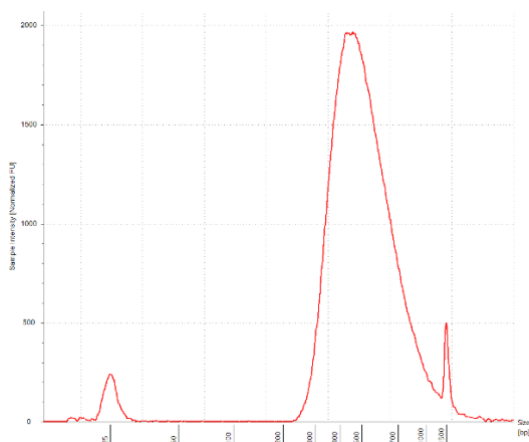


Figure 3.11. Representative TapeStation trace for an EM-seq v2 library prepared using 200 ng of NA12878 genomic DNA that was fragmented for 30 minutes at 37°C with NEBNext UltraShear. The library was run on a HS D1000 tape.

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.

Please read the [FAQ section](#) on NEB.com for additional information about this product.

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.

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 4.1. 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 4.1. 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 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) Diluted Control DNA Unmethylated Lambda (see Table 4.1)	1 µl
• (lilac) Diluted 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 $\geq 75^{\circ}\text{C}$ or on, and run the following program:
 15 minutes at 20°C
 15 minutes at 65°C
 Hold at 4°C

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 ≤ 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.

- 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 $\geq 45^{\circ}\text{C}$ 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 $\geq 105^{\circ}\text{C}$ 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 a pre-chilled metal cooling block on ice and allow the sample to fully cool (~2 minutes) before proceeding to Section 4.8. If a metal cooling block is not available, place on ice and ensure proper contact with the tube walls.

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 $\geq 105^{\circ}\text{C}$ 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 a pre-chilled metal cooling block on ice and allow the sample to fully cool (~2 minutes) before proceeding to Section 4.8. If a metal cooling block is not available, place on ice and ensure proper contact with the tube walls.

4.8. Deamination of 5mC and Cytosines



Deamination Options A or B

Use Option A (4.8.3A.) for DNA inputs ≤ 100 ng.

Option B (4.8.3B.) is required for > 100 ng DNA inputs and can be used for all inputs (0.1 ng to 200 ng).

- 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.

Option A: (Inputs ≤ 100 ng)

4.8.3A. Place in a thermal cycler with the heated lid set to ≥ 85°C or on, and run the following program:

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)

4.8.3B. Place in a thermal cycler with the heated lid set to ≥ 45°C or on, and run the following program:

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
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.

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: 5–6 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 μ l (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 μ l 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 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.
- 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 μ l 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.

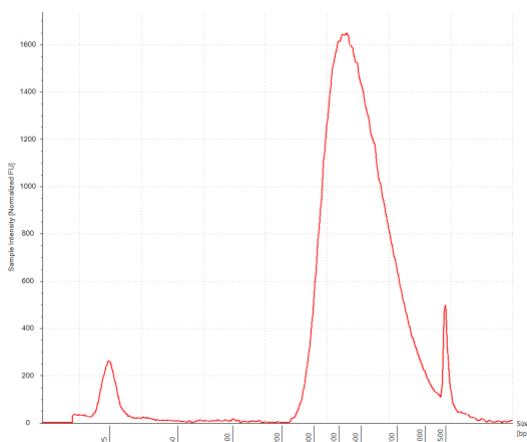


Figure 4.11. Representative TapeStation trace for an E5hmC-seq library prepared using 200 ng of human brain genomic DNA that was fragmented for 30 minutes at 37°C with NEBNext UltraShear. The library was run on a HS D1000 tape.

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.

Please read the [FAQ section](#) on NEB.com for additional information about this product.

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

Please read the section about Important guidelines about reagent handling and experimental setup

5.1. DNA Preparation & Fragmentation

5.1.1. 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 5.1. Dilutions of control DNAs for a range of genomic DNA inputs.

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

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 5.1 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 5.1 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) Diluted Control DNA Unmethylated Lambda (see Table 5.1)	1 µl
• (lilac) Diluted Control DNA CpG methylated pUC19 (see Table 5.1)	1 µl
Total Volume	26 µl

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 15 minutes at 65°C Hold at 4°C
B) 150–250 bp	15–25 minutes at 37°C 15 minutes at 65°C Hold at 4°C
C) 250–350 bp	10–20 minutes at 37°C 15 minutes at 65°C 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 $\geq 75^{\circ}\text{C}$ 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 . Do not premix the Ligation Master Mix, Ligation Enhancer and adaptor prior to use 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. Protection of 5-Methylcytosines and 5-Hydroxymethylcytosines

Note: This step involves multiple tubes with yellow caps. Confirm tube identity throughout setup and use a clear, consistent organizational strategy to ensure correct reagent handling.

- 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 by vortexing and/ or pipette mixing until the TET2 Reaction Buffer Supplement is completely in solution. Spin down before use. 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 by vortexing and/ or pipette mixing until the TET2 Reaction Buffer Supplement is completely in solution. Spin down before use. Write date on tube.

Critical: 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:

Note: For multiple reactions, a master mix can be prepared by combining and vortexing the components before addition to the sample DNA.

Note: Do not add Fe(II) to the master mix.

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. Mix well by vortexing.

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.

On ice, combine diluted • (yellow) Fe(II) Solution and Reaction Mixture with Oxidation Enzymes from step 5.5.2 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 for 1-2 seconds or by pipetting up and down at least 10 times and centrifuge briefly.

- 5.5.4. Place in a thermal cycler, and run the following program with the heated lid set to $\geq 45^{\circ}\text{C}$ 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
Protected DNA (Step 5.5.4.)	50 µl
• (yellow) Stop Reagent	1 µl
Total Volume	51 µl

Mix thoroughly by vortexing for 1-2 seconds 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 $\geq 45^{\circ}\text{C}$ 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 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.

- 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.

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.

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 $\geq 105^{\circ}\text{C}$ or on.
- 5.7A.2. Add 4 µl Formamide to the 16 µl of protected DNA (Step 5.6.11). Mix thoroughly by vortexing for 1–2 seconds 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 in a pre-chilled metal cooling block on ice and allow the sample to fully cool (~2 minutes) before proceeding to Section 5.8. If a metal cooling block is not available, place on ice and ensure proper contact with the tube walls.

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 $\geq 105^{\circ}\text{C}$ or on.
- 5.7B.3. Add 4 µl 0.1 N NaOH to the 16 µl of protected DNA (Step 5.6.11). Mix thoroughly by vortexing 1–2 seconds or by pipetting up and down at least 10 times and centrifuge briefly.
- 5.7B.4. Incubate at 50°C for 10 minutes in the pre-heated thermal cycler.

- 5.7B.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 5.8. If a metal cooling block is not available, place on ice and ensure proper contact with the tube walls.

5.8. Deamination of Cytosines

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

Note: For multiple reactions, a master mix can be prepared by combining and vortexing the components before addition 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

- 5.8.2. Mix thoroughly by vortexing 1-2 seconds or by pipetting up and down at least 10 times and centrifuge briefly.
- 5.8.3. Place in a thermal cycler and run the following program with the heated lid set to $\geq 45^{\circ}\text{C}$ 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. Process only as many samples that will allow you to complete the clean-up without drying out the beads. 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 60 seconds while the tubes are on the magnetic stand with the lid open.

Critical Step:

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. Please also see this FAQ: [Why, at some stages of the EM-seq protocol, do the NEBNext Sample Purification Beads behave differently when cleaning up the sample?](#)

- 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 from Step 5.9.11.:

COMPONENT	VOLUME
Deaminated DNA (Step 5.9.11.)	20 µl
NEBNext LV UDI Primer Pair* * * * *	5 µl
• (blue) NEBNext Q5U Master Mix	25 µl
Total Volume	50 µl

* Refer to the appropriate section 3 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. If using NEB #E7125S/L, please note that LV oligos and NEBNext Q5U® Master Mix (NEB #M0597S/L) are available separately.

*** The sample sheets can be located in Usage Guidelines for the respective NEBNext LV Unique Dual Index Primers Set 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 μ l of the supernatant to a new PCR tube.



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

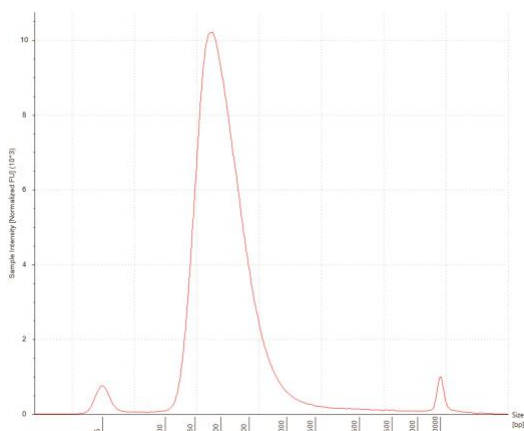
5.12. Library Quantification



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

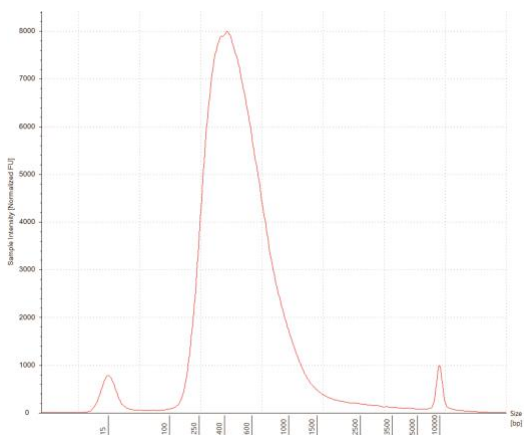
- 5.12.1. Use a TapeStation or Bioanalyzer to determine the size distribution and concentration of the libraries. A typical EM-seq library would have the following trace on a High Sensitivity D5000 ScreenTape on Agilent TapeStation for 200 ng of NA12878 genomic DNA for the following sequenced insert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

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



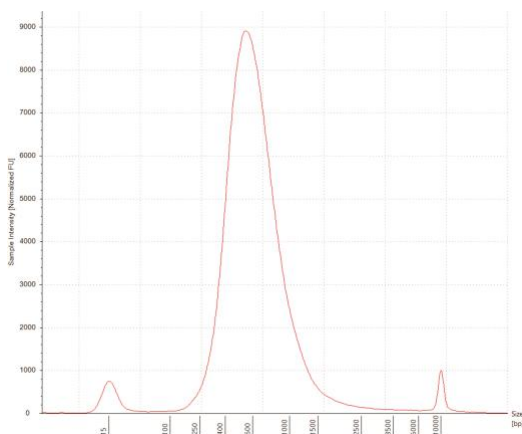
Sequence 2 x 76 base reads or 2 x 100 base reads using the preferred Illumina® platform.

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.

Please read the [FAQ section](#) on NEB.com for additional information about this product.

Important guidelines about reagent handling and experimental setup

- Remove only the kit reagents needed for a specific reaction in the EM-seq protocol. For example, for End Prep, remove the “Green” 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, for example ● (yellow) DTT and the ● (blue) NEBNext Q5U Master Mix. Precipitates should disappear once the tubes are at room temperature. For the precipitate to fully disappear it may be necessary to vortex the ● (yellow) DTT and ● (green) 500 mM DTT and ○ (white) NEBNext UltraShear Reaction Buffer or to gently flick the ● (blue) NEBNext Q5U Master Mix tube. All other 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. NEBNext UltraShear enzyme mix requires to be vortexed before and after adding to the sample. All tubes should be centrifuged briefly after mixing and stored on ice until used.
- Master mixes can be made for most steps in NEBNext UltraShear and EM-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 ● (red) NEBNext Ultra II Ligation Master Mix is viscous as are the Sample Purification Beads. It is difficult to accurately pipette these components. Please take care to transfer the correct volumes.
- The ● (orange) APOBEC Reaction Buffer and ○ (white) NEBNext UltraShear Reaction Buffer can form bubbles when mixed. Centrifuging these buffers 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 Oxidation 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 Oxidation, 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.

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. Also including new protocols: • NEBNext Enzymatic Methyl-seq v2 Kit • NEBNext Enzymatic 5hmC-seq Kit	12/24
3.0	Updated protocols for Sections 3, 4 and 5.	3/26
4.0	Update protocols for Sections 3, 4 and 5.	7/26

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