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# INSTRUCTION MANUAL

# NEBNext UltraShear™

NEB #M7634S/L

24/96 reactions

Version 1.0_6/23

## Table of Contents


Overview ........ 2

Section 1
NEBNext UltraShear Protocol ........ 3

Section 2
NEBNext UltraShear with NEBNext® Ultra™ II End Repair/dA-Tailing Module Protocol ........ 5

Section 3
NEBNext UltraShear with NEBNext Enzymatic Methyl-seq Kit Protocol ........ 7

Components ........ 17

Revision History ........ 17


## The Product Includes

*The volumes provided are sufficient for preparation of up to 24 reactions (NEB #M7634S) and 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
- Magnetic rack/stand (NEB #S1515S; Alpaqua® #A001322 or equivalent)
- PCR machine
- Vortex
- Microcentrifuge
- Bioanalyzer®, TapeStation® or other fragment analyzer and associated consumables
- 80% Ethanol

**For use with NEBNext UltraShear Protocol:**

- SPRIselect™ Reagent Kit (Beckman Coulter, Inc. #B23317), AMPure® XP Beads (Beckman Coulter, Inc. #A63881) or Monarch® PCR & DNA Cleanup Kit (NEB# T1030S/L) are recommended for Section 1.

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

- NEBNext Ultra II End Repair/dA-Tailing Module (NEB #E7546S/L) for Section 2.
- Recommended Material Not Included: NEBNext Ultra II Ligation Module (NEB #E7595) and NEBNext Multiplex Oligos (www.neb.com/oligos).

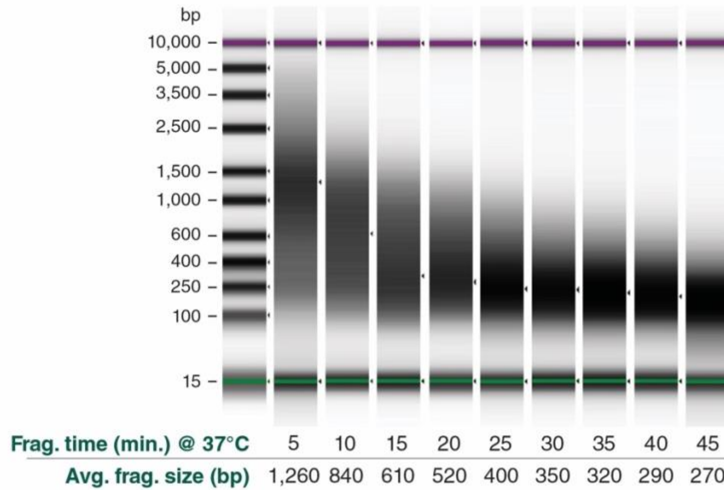
**For use with NEBNext Enzymatic Methyl-seq Protocol:**

- NEBNext Enzymatic Methyl-seq Kit (NEB #E7120S/L) for Section 3.
- Formamide (Sigma #F9037-100 ml) or optional 0.1 N NaOH. Formamide is preferred. If using NaOH, please see NEBNext Enzymatic Methyl-seq Kit (NEB #E7120) FAQs (https://www.neb.com/faqs/2020/02/07/can-i-use-naoh-sodium-hydroxide-instead-of-formamide-to-denature-my-dna-prior-to-the-deamination-reaction)
- Nuclease-free Water

## Overview

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

**Figure 1. Example of size distribution of high-quality human DNA (NA12878) following fragmentation (5–45 minutes at 37°C) with NEBNext UltraShear.**




*50 ng human DNA (NA12878) fragmented by NEBNext UltraShear for 5–45 minutes at 37°C. 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 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** |

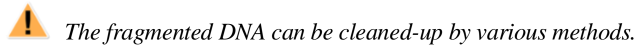
**Note: The ● (green) 500 mM DTT provided in the NEBNext UltraShear module is not for use with this protocol.**

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: If working with already fragmented, 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.**



*The fragmented DNA can be cleaned-up by 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 Bioanalyzer, TapeStation, or other fragment analyzer to determine the size distribution.

## Section 2

## NEBNext UltraShear with NEBNext Ultra II End Repair/dA-Tailing Module Protocol (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.*

**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** |

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: If working with already fragmented, 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.**

**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 NEBNext Ultra II End Prep Enzyme Mix is used in this protocol, but not the 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** |

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

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

2.2.3. Place in a thermal cycler with the heated lid set to ≥ 75°C or on, and run the following program:

30 minutes at 20°C

30 minutes at 65°C

Hold at 4°C

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

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

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

# Section 3

## NEBNext UltraShear with NEBNext Enzymatic Methyl-seq Protocol (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 for this section.**

**Note: The NEBNext UltraShear with NEBNext Enzymatic Methyl-seq (NEB #M7634S/L) protocol differs from the standard NEBNext Enzymatic Methyl-seq protocol(s) (NEB #E7120S/L) and the instructions are not interchangeable. The key difference is that Covaris DNA fragmentation is not needed; additionally, please note changes in the following sections of this protocol: End Prep of Fragmented DNA (3.2.1.), Clean-up of Deaminated DNA (3.9.), and Clean-up of Amplified Libraries (3.11.).**

**Starting Material:** 10–200 ng DNA

### 3.1. DNA Preparation and Fragmentation

DNA and Control DNA

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

| COMPONENT | VOLUME |
|---|---|
| gDNA | 24 µl |
| • (lilac) Control DNA Unmethylated Lambda (see Table 1.1) | 1 µl |
| • (lilac) Control DNA CpG methylated pUC19 (see Table 1.1) | 1 µl |
| **Total Volume** | **26 µl** |

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

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

| DILUTION OF • (LILAC) CONTROL DNA UNMETHYLATED LAMBDA AND • (LILAC) CONTROL DNA CpG METHYLATED pUC19 | | |
|---|---|---|
| **DNA Input Amount** | **Pre-sequencing 2–4 Million Paired Reads** | **Deep Sequencing 100–150 Million Paired Reads** |
| 10 ng | 1:20 | 1:100 |
| 200 ng | No Dilution | 1:50 |

Regardless of sequencing depth, a minimum of 5,000 paired end reads with a read length of 76 bases for unmethylated Lambda DNA, and 500 paired end reads with a read length of 76 bases for CpG methylated pUC19, are needed to give enough coverage for accurate conversion estimates.

Different applications may require different sequencing depths and therefore different strategies should be employed when deciding how much control DNA should be added. For example, some libraries may only need 2 million paired end reads whereas others may require 50 million paired end reads or even 300 million paired end reads.

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

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

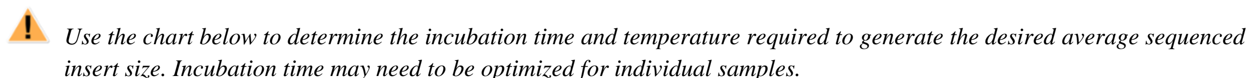
3.1.3. Vortex the ○ (white) NEBNext UltraShear for 5–10 seconds prior to use and place on ice.

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

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** |

3.1.5. 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.*

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

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

**Note: Fragmentation time selection, from table above, will be coupled with clean-up recommendations. Each fragmentation group (A, B, or C) in section 3.1.6. has specific sample purification bead clean-up recommendations in section 3.9.2. and 3.11.3. Do not deviate from the paired recommendations as loss of sample may occur.**

**Note: See FAQ if input DNA is already fragmented, low integrity and/or FFPE DNA.**

**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 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 is used in 3.2.1 and not the ○ (yellow) DTT from the Enzymatic Methyl-seq Kit (NEB #E7120S/L).**

3.2.2. 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.**

3.2.3. Place in a thermal cycler with the heated lid set to ≥ 75°C or on, and run the following program:

30 minutes at 20°C

30 minutes at 65°C

Hold at 4°C

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

3.3.2. 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.**

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**

3.4.1. Vortex Sample Purification Beads to resuspend.

3.4.2. 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.

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 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.

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 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.**

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

3.4.10. 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.

3.4.11. 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.**

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

3.5.1. 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.**

3.5.1A. Add 100 µl of ○ (yellow) TET2 Reaction Buffer to one tube of ○ (yellow) TET2 Reaction Buffer Supplement and mix well. Write date on tube.

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

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

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

| COMPONENT | VOLUME |
|---|---|
| EM-seq adaptor ligated DNA (Step 3.4.11.) | 28 µl |
| ○ (yellow) TET2 Reaction Buffer (TET2 Reaction Buffer plus reconstituted TET2 Reaction Buffer Supplement; Step 3.5.1.) | 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.

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

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

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

| COMPONENT | VOLUME |
|---|---|
| Reaction Mixture (Step 3.5.2.) | 45 µl |
| Diluted Fe(II) Solution (Step 3.5.3.) | 5 µl |
| **Total Volume** | **50 µl** |

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

3.5.4 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

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

| COMPONENT | VOLUME |
|---|---|
| Oxidized DNA (Step 3.5.4.) | 50 µl |
| ○ (yellow) Stop Reagent | 1 µl |
| **Total Volume** | **51 µl** |

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

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

30 minutes at 37°C

Hold at 4°C

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

**3.6. Clean-Up of TET2 Oxidized DNA**

3.6.1. Vortex Sample Purification Beads to resuspend.

3.6.2. 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.

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

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

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

3.6.6. Add 200 µl of 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.

3.6.7. 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.

3.6.8. 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.**

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

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

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

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

### 3.7. 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.*

#### 3.7A: Formamide (Recommended)

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

3.7A.2. Add 4 µl Formamide to the 16 µl of oxidized DNA (Step 3.6.11). Vortex to mix or by pipetting up and down at least 10 times, centrifuge briefly.

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

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

#### 3.7B: Sodium Hydroxide (Optional, See FAQ about preparing NaOH)

3.7B.1. Prepare freshly diluted 0.1 N NaOH.

3.7B.2. Pre-heat thermal cycler to 50°C with the heated lid set to ≥ 105°C or on.

3.7B.3. Add 4 µl 0.1 N NaOH to the 16 µl of oxidized DNA (Step 3.6.11). Vortex to mix or by pipetting up and down at least 10 times, centrifuge briefly.

3.7B.4. Incubate at 50°C for 10 minutes in the pre-heated thermal cycler.

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

### 3.8. Deamination of Cytosines

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

| COMPONENT | VOLUME |
|---|---|
| Denatured DNA (Step 3.7A.4. or 3.7B.5.) | 20 µl |
| Nuclease-free water | 68 µl |
| ● (orange) APOBEC Reaction Buffer | 10 µl |
| ● (orange) BSA | 1 µl |
| ● (orange) APOBEC | 1 µl |
| **Total Volume** | **100 µl** |

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

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

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

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

### 3.9. Clean-Up of Deaminated DNA

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

3.9.1. 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 NEBNext UltraShear with NEBNext Enzymatic Methyl-seq protocol for clean-up of deaminated DNA 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, will be coupled with fragmentation time recommendations. Each fragmentation group (A, B, or C) in section 3.1.6. has specific sample purification bead clean-up recommendations in section 3.9.2. and 3.11.3. Do not deviate from the paired recommendations as loss of sample may occur.**

**Note: See FAQ if input DNA is already fragmented, low integrity and/or FFPE DNA.**

3.9.2. 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.

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

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

3.9.5. 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**).

3.9.6. 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.

3.9.7. 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.

3.9.8. Air dry the beads for up to 90 seconds while the tubes are on the magnetic stand with the lid open.

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

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

3.9.10. 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.

3.9.11. 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.**

### 3.10. PCR Amplification

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

| COMPONENT | VOLUME |
| --- | --- |
| Deaminated DNA (Step 3.9.11.) | 20 µl |
| EM-seq Index Primer*, ** | 5 µl |
| ● (blue) NEBNext Q5U Master Mix | 25 µl |
| **Total Volume** | **50 µl** |

* Refer to Section 3 in NEB #E7120S/L manual for barcode pooling guidelines.

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

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

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

3.10.3. 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 cycles

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

### 3.11. Clean-Up of Amplified Libraries

3.11.1. Vortex Sample Purification Beads to resuspend.

3.11.2. 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, will be coupled with fragmentation time recommendations. Each fragmentation group (A, B, or C) in section 3.1.6. has specific sample purification bead clean-up recommendations in section 3.9.2. and 3.11.3. Do not deviate from the paired recommendations as loss of sample may occur.**

**Note: See FAQ if input DNA is already fragmented, low integrity and/or FFPE DNA.**

3.11.3. 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.

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

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

3.11.6. 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)**.

3.11.7. 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.

3.11.8. 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.

3.11.9. 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.**

3.11.10. 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).

3.11.10. 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.

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

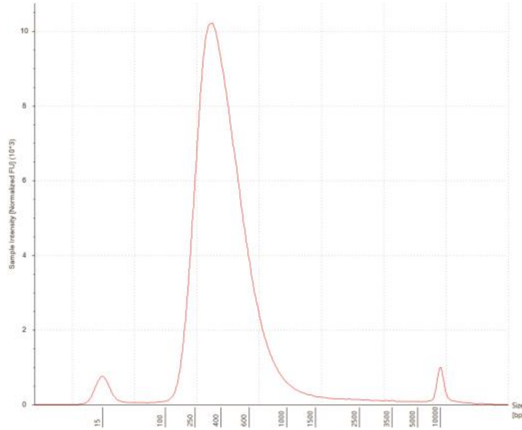
**Safe Stopping Point: Samples can be stored at -20°C.**

### 3.12. Library Quantification

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

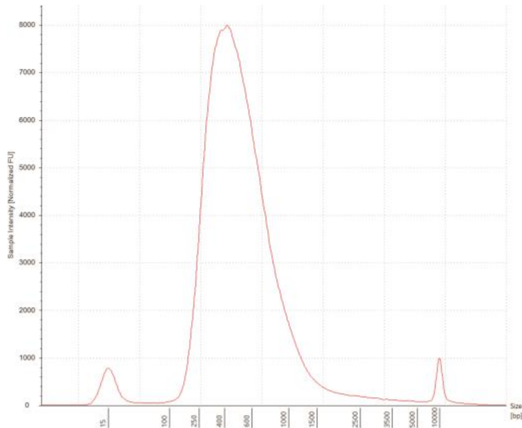
3.12.1. Use a Bioanalyzer or TapeStation to determine the size distribution and concentration of the libraries. A typical EM-seq library would have the following trace on a High Sensitivity D5000 ScreenTape on Agilent TapeStation for 200 ng of NA12878 genomic DNA for the following sequenced insert sizes.

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



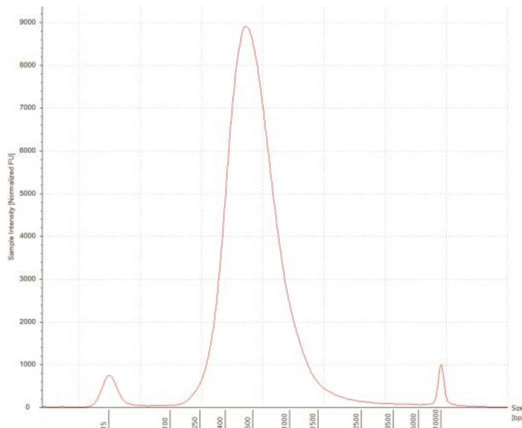

*Sequence 2 x 76 base reads or 2 x 100 base reads using the preferred Illumina® platform.*

**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.*

## 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 |

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New England Biolabs, Inc., 240 County Road, Ipswich, MA 01938-2723 Telephone: (978) 927-5054 Toll Free: (USA Orders) 1-800-632-5227 (USA Tech) 1-800-632-7799 Fax: (978) 921-1350 e-mail: info@neb.com