

# INSTRUCTION MANUAL

## NEBNext® Enzymatic Methyl-seq Kit

NEB #E7120S/L

24/96 reactions

Version 9.0\_5/25

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### The Library Kit Includes

*The volumes provided are sufficient for preparation of up to 24 reactions (NEB #E7120S) or 96 reactions (NEB #E7120L). The NEBNext Sample Purification Beads should be stored at room temperature and all other reagents should be stored at –20°C. Colored bullets represent the color of the cap of the tube containing the reagent.*

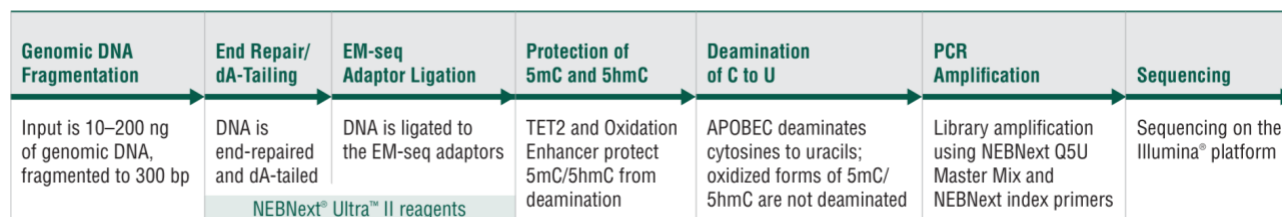
- (lilac) Control DNA CpG methylated pUC19
  - (lilac) Control DNA Unmethylated Lambda
  - (green) NEBNext Ultra™ II End Prep Reaction Buffer
  - (green) NEBNext Ultra II End Prep Enzyme Mix
  - (red) NEBNext Ultra II Ligation Master Mix
  - (red) NEBNext Ligation Enhancer
  - (red) NEBNext EM-seq™ Adaptor
  - (white) Elution Buffer
  - (yellow) TET2 Reaction Buffer
  - (yellow) TET2 Reaction Buffer Supplement
  - (yellow) Oxidation Supplement
  - (yellow) DTT
  - (yellow) Oxidation Enhancer
  - (yellow) TET2
  - (yellow) Fe(II) Solution
  - (yellow) Stop Reagent
  - (orange) APOBEC
  - (orange) APOBEC Reaction Buffer
  - (orange) BSA
  - (blue) NEBNext Q5U™ Master Mix
- NEBNext Sample Purification Beads

## Required Materials Not Included

- NEBNext UltraShear® (NEB #M7634) or Covaris® instrument and the required tubes or other fragmentation equipment
- [Any NEBNext LV Unique Dual Index Primer Set \(NEB #E3390, #E3392, #E3400, #E3402, #E3404, #E3406 and #E3408\)](#)
- PCR strip tubes or 96-well plates
- Hi-Di™ Formamide (Thermo Fisher Scientific® #4401457), Formamide (Sigma #F9037-100 ml), or 0.1 N NaOH. Formamide is preferred. If using NaOH, please see FAQ on NEB #E7120 FAQ page.
- 80% Ethanol
- 1X TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA), low TE (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA) or 10 mM Tris-HCl pH 7.5 or 8.0.
- Nuclease-free Water
- Magnetic rack/stand, such as NEBNext Magnetic Separation Rack (NEB #S1515S)
- Metal cooling block, such as Diversified Biotech® (#CHAM-1000)
- PCR machine
- Agilent® TapeStation®, Bioanalyzer® or other fragment analyzer and associated consumables

## Overview

**Figure 1. NEBNext Enzymatic Methyl-seq Kit Workflow.**



*The Enzymatic Methyl-seq kit (EM-seq) for Illumina contains all the components needed to make libraries that are enzymatically modified to detect 5-methylcytosines (5mC) and 5-hydroxymethylcytosines (5hmC).*

Figure 1 is an overview of the EM-seq workflow. Firstly, a library is made by ligating EM-seq adaptor to fragmented, end-repaired/dA-tailed genomic DNA. This is followed by two sets of enzymatic conversion steps to differentiate unmethylated cytosines from 5mC/5hmC. Finally, libraries are PCR amplified before sequencing.

**Figure 2. Overview of Sodium Bisulfite Conversion and EM-seq.**

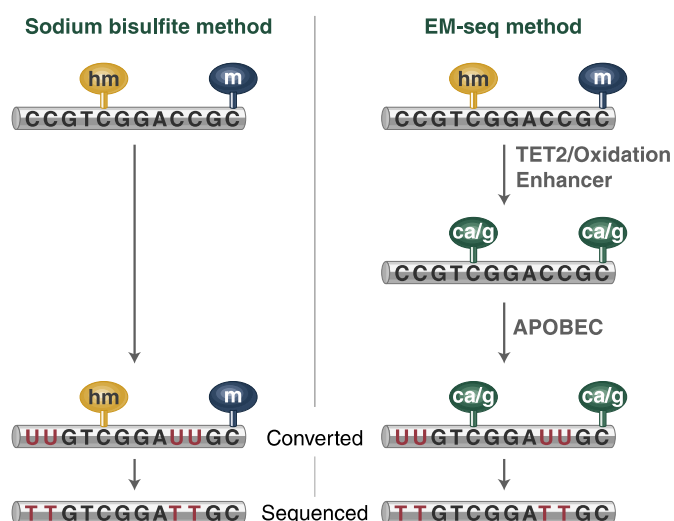


Figure 2 shows a comparison of the sodium bisulfite and EM-seq methods. Sodium bisulfite treatment of DNA results in the deamination of cytosines into uracils, however the modified forms of cytosine (5mC and 5hmC) are not deaminated. Therefore, the preference of bisulfite to chemically deaminate cytosines enables the methylation status of cytosines to be determined. When bisulfite treated DNA is PCR amplified, uracils are replaced by thymines and the 5mC/5hmC are replaced by cytosines. Once sequenced, unmethylated cytosines are represented by thymines and 5mC and 5hmC are represented by cytosines. By comparing sequences to non-converted genomes the appropriate methylation status can be assessed.

Enzymatic Methyl-seq is a two step enzymatic conversion process to detect modified cytosines. The first step uses TET2 and an oxidation enhancer to protect modified cytosines from downstream deamination. TET2 enzymatically oxidizes 5mC and 5hmC through a cascade reaction into 5-carboxycytosine [5-methylcytosine (5mC)  $\Rightarrow$  5-hydroxymethylcytosine (5hmC)  $\Rightarrow$  5-formylcytosine (5fC)  $\Rightarrow$  5-carboxycytosine (5caC)]. This protects 5mC and 5hmC from deamination. 5hmC can also be protected from deamination by glucosylation to form 5gmC using the oxidation enhancer. The second enzymatic step uses APOBEC to deaminate C but does not convert 5caC and 5gmC. The resulting converted sequence can be analyzed like bisulfite-treated DNA. Typical aligners used to analyze data include but are not limited to Bismark and BWAMeth.

The workflow described in the NEBNext Enzymatic Methyl-seq Kit is user-friendly and enables methylation detection from inputs ranging between 10–200 ng. EM-seq converted DNA is more intact than bisulfite-converted DNA, resulting in libraries with longer sequencing reads, reduced GC bias and more even genome coverage.

Please note that the bead volumes provided are sufficient for building standard size libraries described in Section 1, Protocol for use with Standard Size Libraries (370–420 bp). If following the Section 2 Protocol, for use with Large Size Libraries (470–520 bp), users need to supply additional beads due to the increased volumes needed for cleanups. We recommend using SPRIselect® Reagent Kit (Beckman Coulter, Inc. #B23317) or AMPure® XP Beads (Beckman Coulter, Inc. #A63881). If you are combining leftover beads from NEB #E7120 with the above products, we recommend combining with SPRIselect® Reagent Kit (Beckman Coulter, Inc. #B23317).

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

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

## Section 1

### Protocol for use with Standard Libraries (370–420 bp)

#### Symbols



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



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



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

#### Important Guidelines

##### Reagent Handling

Remove only the kit reagents needed for a specific reaction in the EM-seq protocol. For example, for 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, ● (green) NEBNext Ultra™ II End Prep Reaction Buffer, ● (yellow) DTT and the polymerase ● (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 ● (green) NEBNext Ultra II End Prep Reaction Buffer, ● (yellow) DTT 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. All tubes should be centrifuged briefly after mixing and stored on ice until used.

Master mixes can be made for most steps in 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 can form bubbles when mixed. Centrifuging the ● (orange) APOBEC Reaction Buffer can reduce their appearance making it easier to accurately dispense the buffer.

Once reactions are set up, mix either using a quick vortex or by pipetting up and down. Please note that if you choose to mix using a pipette, then we encourage you to use an appropriately sized pipette set to a volume that would ensure adequate mixing of the sample. For example, during the 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.

**Starting Material:** 10–200 ng DNA

### 1.1. DNA Preparation

- 1.1.1. **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 library construction to evaluate conversion efficiencies.

**Table 1.1. 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	1:50

The above dilutions are useful to perform a QC of conversion before deep sequencing using approximately 10 million paired-end 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 1.1. will provide sufficient coverage of controls for libraries sequenced to 10 million paired-end reads and above. Dilution of controls needs to be optimized by the user if sequencing lower than 10 million paired-end reads to obtain minimum coverage for ● (lilac) 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 1.1. 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 (10–200 ng) with control DNAs specified below.

**Sample DNA can be in any of the following buffers:** 10 mM Tris-HCl pH 7.5 or 8.0, 1X TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA), or low TE (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA). **Do not fragment input DNA in 0.1X TE (1 mM Tris-HCl, 0.1 mM EDTA) or water.**

COMPONENT	VOLUME
Sample DNA	48 µl
● (lilac) Control DNA Unmethylated Lambda (see Table 1.1.)	1 µl
● (lilac) Control DNA CpG methylated pUC19 (see Table 1.1.)	1 µl
<b>Total Volume</b>	<b>50 µl</b>

The sequences for pUC19 and lambda can also be found in our GitHub demo pipeline: [https://github.com/nebiolabs/EM-seq/blob/master/methylation\\_controls.fa](https://github.com/nebiolabs/EM-seq/blob/master/methylation_controls.fa)

### 1.1.2. DNA Fragmentation

The combined 50 µl genomic DNA and control DNAs are fragmented to an average fragment size of ~300 bp (370–420 bp final Illumina library). Fragmentation can be done using NEBNext UltraShear (NEB #M7634, follow the protocol provided in UltraShear manual) or a preferred fragmentation device such as a Covaris instrument.

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

Note: Enzymatic fragmentation is only recommended using NEBNext UltraShear. For instructions see the NEBNext UltraShear manual: <https://www.neb.com/en-us/-/media/nebus/files/manuals/manualm7634.pdf>

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

## 1.2. End Prep of Fragmented DNA

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

COMPONENT	VOLUME
Fragmented DNA (Step 1.1.2.)	50 µl
• (green) NEBNext Ultra II End Prep Reaction Buffer	7 µl
• (green) NEBNext Ultra II End Prep Enzyme Mix	3 µl
<b>Total Volume</b>	<b>60 µl</b>

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

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

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

- 1.2.3. Place in a thermal cycler with the heated lid set to  $\geq 75^{\circ}\text{C}$  or on, and run the following program:

30 minutes at  $20^{\circ}\text{C}$

30 minutes at  $65^{\circ}\text{C}$

Hold at  $4^{\circ}\text{C}$

## 1.3. Ligation of EM-seq Adaptor

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

COMPONENT	VOLUME
End Prep reaction mixture (Step 1.2.3.)	60 µl
• (red) NEBNext EM-seq Adaptor	2.5 µl
• (red) NEBNext Ligation Enhancer	1 µl
• (red) NEBNext Ultra II Ligation Master Mix	30 µl
<b>Total Volume</b>	<b>93.5 µl</b>

**Note:** Ligation Enhancer and Ligation Master Mix can be mixed ahead of time and is stable for at least 8 hours at  $4^{\circ}\text{C}$ .

**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.**

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

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

- 1.3.3. Place in a thermal cycler, and run the following program with the heated lid off:

15 minutes at  $20^{\circ}\text{C}$

Hold at  $4^{\circ}\text{C}$



**Safe Stopping Point:** Samples can be stored overnight at  $-20^{\circ}\text{C}$ .

## 1.4. 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.

- 1.4.1. Vortex the Sample Purification Beads to resuspend.

- 1.4.2. Add 110 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.

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

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

- 1.4.5. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets (**Caution: do not discard the beads**).
- 1.4.6. Add 200 µl of freshly prepared 80% ethanol to the tubes while on the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.
- 1.4.7. Repeat the ethanol wash once for a total of two washes.
- 1.4.8. Remove all visible liquid after the second wash using a p10 pipette tip.
- 1.4.9. 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.**
- 1.4.10. Remove the tubes from the magnetic stand. Elute the DNA target from the beads by adding 29 µl of ◦ (white) Elution Buffer.
- 1.4.11. Mix well by pipetting up and down 10 times. Incubate for at least 1 minute at room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube before placing back on the magnetic stand.
- 1.4.12. 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.**

## 1.5. Protection of 5-Methylcytosines and 5-Hydroxymethylcytosines

- 1.5.1. Prepare TET2 Buffer. Use Option A for #E7120S/#E7120G (24 reactions/G size) and Option B for #E7120L (96 reactions).

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

- 1.5.1A. 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.
- 1.5.1B. 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.**

- 1.5.2. 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 (from Step 1.4.12)	28 µl
◦ (yellow) TET2 Reaction Buffer (TET2 Reaction Buffer Supplement reconstituted in TET2 Reaction Buffer)	10 µl
◦ (yellow) Oxidation Supplement	1 µl
◦ (yellow) DTT	1 µl
◦ (yellow) Oxidation Enhancer	1 µl
◦ (yellow) TET2	4 µl
<b>Total Volume</b>	<b>45 µl</b>

Mix thoroughly by vortexing for 1–2 seconds or by pipetting up and down at least 10 times and centrifuge briefly. 5mC/5hmC oxidation is initiated by the addition of the Fe(II) Solution to the reaction in the next step.

- 1.5.3. Dilute the 500 mM ◐ (yellow) Fe(II) Solution by adding 1 µl to 1,249 µl of water. Mix well by vortexing.

**Note: The 500 mM ◐ (yellow) 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 DNA from Section 1.5.2. as described below:

COMPONENT	VOLUME
Reaction Mixture (Step 1.5.2.)	45 µl
Diluted ◐ (yellow) Fe(II) Solution (Step 1.5.3.)	5 µl
<b>Total Volume</b>	<b>50 µl</b>

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

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

1 hour at  $37^{\circ}\text{C}$

Hold at  $4^{\circ}\text{C}$

- 1.5.5. Transfer the samples to ice and add 1 µl of ◐ (yellow) Stop Reagent.

COMPONENT	VOLUME
Protected DNA (Step 1.5.4.)	50 µl
◐ (yellow) Stop Reagent	1 µl
<b>Total Volume</b>	<b>51 µl</b>

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

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

30 minutes at  $37^{\circ}\text{C}$

Hold at  $4^{\circ}\text{C}$



**Safe Stopping Point: Samples can be stored overnight at either  $4^{\circ}\text{C}$  in the thermal cycler or at  $-20^{\circ}\text{C}$  in the freezer.**

## 1.6. 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.**

- 1.6.1. Vortex Sample Purification Beads to resuspend.
- 1.6.2. Add 90 µl (1.8X) of resuspended NEBNext Sample Purification Beads to each sample. Mix well by pipetting up and down at least 10 times. Be careful to expel all of the liquid out of the tip during the last mix.
- 1.6.3. Incubate samples on bench top for at least 5 minutes at room temperature.
- 1.6.4. Place the tubes against an appropriate magnetic stand to separate the beads from the supernatant.
- 1.6.5. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets (**Caution: do not discard the beads**).
- 1.6.6. Add 200 µl of freshly prepared 80% ethanol to the tubes while on the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.
- 1.6.7. Repeat the wash once for a total of two washes.
- 1.6.8. Remove all visible liquid after the second wash using a p10 pipette tip.
- 1.6.9. 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.**
- 1.6.10. Remove the tubes from the magnetic stand. Elute the DNA target from the beads by adding 17 µl of ◐ (white) Elution Buffer.



- 1.6.11. Mix well by pipetting up and down 10 times. Incubate for at least 1 minute at room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube before placing back on the magnetic stand.
- 1.6.12. Place the tube on the magnetic stand. After 3 minutes (or whenever the solution is clear), transfer 16  $\mu$ 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.

## 1.7. Denaturation of DNA



The DNA can be denatured using either Formamide or 0.1 N Sodium Hydroxide.

Use **Option A** for denaturing using Formamide. Use **Option B** for denaturing using 0.1 N Sodium hydroxide.

### 1.7A. Formamide (Recommended)

- 1.7A.1. Pre-heat thermal cycler to 85°C with the heated lid set to  $\geq 105^\circ\text{C}$  or on.
- 1.7A.2. Add 4  $\mu$ l Formamide to the 16  $\mu$ l of protected DNA (Step 1.6.12). Mix thoroughly by vortexing for 1–2 seconds or by pipetting up and down at least 10 times, centrifuge briefly.
- 1.7A.3. Incubate at 85°C for 10 minutes in the pre-heated thermal cycler.
- 1.7A.4. **Critical Step:** Immediately place in cooling block on ice and allow the sample to fully cool (~ 2 minutes) before proceeding to Section 1.8.

### 1.7B. Sodium Hydroxide (Optional, See FAQ about preparing NaOH)

- 1.7B.1. Prepare freshly diluted 0.1 N NaOH.
- 1.7B.2. Pre-heat thermal cycler to 50°C with the heated lid set to  $\geq 105^\circ\text{C}$  or on.
- 1.7B.3. Add 4  $\mu$ l 0.1 N NaOH to the 16  $\mu$ l of protected DNA (Step 1.6.12). Mix thoroughly by vortexing 1–2 seconds or by pipetting up and down at least 10 times and centrifuge briefly.
- 1.7B.4. Incubate at 50°C for 10 minutes in the pre-heated thermal cycler.
- 1.7B.5. **Critical Step:** Immediately place in cooling block on ice and allow the sample to fully cool (~ 2 minutes) before proceeding to Section 1.8.

## 1.8. Deamination of Cytosines

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

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 1.7A.4. or 1.7B.5.)	20 $\mu$ l
Nuclease-free water	68 $\mu$ l
• (orange) APOBEC Reaction Buffer	10 $\mu$ l
• (orange) BSA	1 $\mu$ l
• (orange) APOBEC	1 $\mu$ l
<b>Total Volume</b>	<b>100 <math>\mu</math>l</b>

- 1.8.2. Mix thoroughly by vortexing for 1–2 seconds or by pipetting up and down at least 10 times and centrifuge briefly.
- 1.8.3. Place in a thermal cycler, and run the following program with the heated lid set to  $\geq 45^\circ\text{C}$  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.

## 1.9.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. Do not overdry the beads as they become very difficult to resuspend.

- 1.9.1. Vortex Sample Purification Beads to resuspend.
- 1.9.2. Add 100 µl (1.0X) of resuspended NEBNext Sample Purification Beads to each sample. Mix well by pipetting up and down at least 10 times. Be careful to expel all of the liquid out of the tip during the last mix.
- 1.9.3. Incubate samples on bench top for at least 5 minutes at room temperature.
- 1.9.4. Place the tubes against an appropriate magnetic stand to separate the beads from the supernatant.
- 1.9.5. 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**).
- 1.9.6. Add 200 µl of freshly prepared 80% ethanol to the tubes while on the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.
- 1.9.7. Repeat the wash once for a total of two washes.
- 1.9.8. Remove all visible liquid after the second wash using a p10 pipette tip.
- 1.9.9. 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: [Why, at some stages of the EM-seq protocol, do the NEBNext Sample Purification Beads behave differently when cleaning up the sample?](#)

- 1.9.10. Remove the tubes from the magnetic stand. Elute the DNA target from the beads by adding 21 µl of ◦ (white) Elution Buffer.
- 1.9.11. Mix well by pipetting up and down 10 times. Incubate for at least 1 minute at room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube before placing back on the magnetic stand.
- 1.9.12. Place the tube on the magnetic stand. After 3 minutes (or whenever the solution is clear), transfer 20 µl of the supernatant to a new PCR tube.



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

## 1.10.PCR Amplification

- 1.10.1. On ice, add the following components to the deaminated DNA from Step 1.9.12.:

COMPONENT	VOLUME
Deaminated DNA (Step 1.9.12.)	20 µl
UDI Index Primer Pair*	5 µl
• (blue) NEBNext Q5U Master Mix	25 µl
<b>Total Volume</b>	<b>50 µl</b>

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

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

- 1.10.3. 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.

### 1.11. 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.

- 1.11.1. Vortex Sample Purification Beads to resuspend.
- 1.11.2. Add 45 µl (0.9X) of resuspended NEBNext Sample Purification Beads to each sample. Mix well by pipetting up and down at least 10 times. Be careful to expel all of the liquid out of the tip during the last mix.
- 1.11.3. Incubate samples on bench top for at least 5 minutes at room temperature.
- 1.11.4. Place the tubes against an appropriate magnetic stand to separate the beads from the supernatant.
- 1.11.5. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets (**Caution: do not discard the beads**).
- 1.11.6. Add 200 µl of freshly prepared 80% ethanol to the tubes while on the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.
- 1.11.7. Repeat the wash once for a total of two washes.
- 1.11.8. Remove all visible liquid after the second wash using a p10 pipette tip.
- 1.11.9. 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.

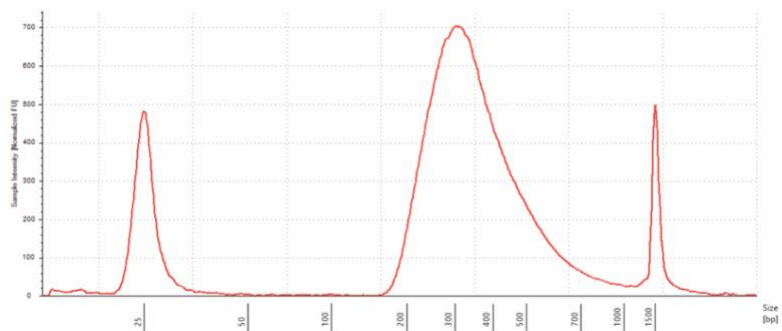
- 1.11.10. Remove the tubes from the magnetic stand. Elute the DNA target from the beads by adding 21 µl of ° (white) Elution Buffer. For long terms storage use, 21 µl of 1X TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA), Low TE (10 mM Tris, 0.1 mM EDTA, pH 8.0) or 0.1X TE (1mM Tris, 0.1 mM EDTA, pH 8.0).
- 1.11.11. Mix well by pipetting up and down 10 times. Incubate for at least 1 minute at room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube before placing back on the magnetic stand.
- 1.11.12. Place the tube on the magnetic stand. After 3 minutes (or whenever the solution is clear), transfer 20 µl of the supernatant to a new PCR tube.



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

## 1.12. Library Quantification

- 1.12.1. Use an Agilent TapeStation or Bioanalyzer to determine the size distribution and concentration of the libraries. A typical EM-seq library would have the following TapeStation trace.



*Figure 3: Representative TapeStation trace for an EM-seq library prepared using 50 ng of NA12878 genomic DNA. The library was run on a HS D1000 tape.*

EM-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 2

### Protocol for use with Large Size Libraries (470–520 bp)

#### Symbols



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



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



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

#### Important Guidelines

##### Reagent Handling

Remove only the kit reagents needed for a specific reaction in the EM-seq protocol. For example, for 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, ● (green) NEBNext Ultra™ II End Prep Reaction Buffer, ● (yellow) DTT and the polymerase ● (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 ● (green) NEBNext Ultra II End Prep Reaction Buffer, ● (yellow) DTT 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. All tubes should be centrifuged briefly after mixing and stored on ice until used.

Master mixes can be made for most steps in 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 can form bubbles when mixed. Centrifuging the ● (orange) APOBEC Reaction Buffer can reduce their appearance making it easier to accurately dispense the buffer.

Once reactions are set up, mix either using a quick vortex or by pipetting up and down. Please note that if you choose to mix using a pipette, then we encourage you to use an appropriately sized pipette set to a volume that would ensure adequate mixing of the sample. For example, during the 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.

**Starting Material:** 10–200 ng DNA.

## 2.1. DNA Preparation

- 2.1.1. **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 library construction to evaluate conversion efficiencies.

**Table 2.1.1. 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	1:50

The above dilutions are useful to perform a QC of conversion before deep sequencing using approximately 10 million paired-end 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 2.1.1. will provide sufficient coverage of controls for libraries sequenced to 10 million paired-end reads and above. Dilution of controls needs to be optimized by the user if sequencing lower than 10 million paired-end reads to obtain minimum coverage for • (lilac) 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 2.1.1. 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 (10–200 ng) with control DNAs specified below.

**Sample DNA can be in any of the following buffers:** 10 mM Tris-HCl pH 7.5 or 8.0, 1X TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA), or low TE (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA). **Do not fragment input DNA in 0.1X TE (1 mM Tris-HCl, 0.1 mM EDTA) or water.**

COMPONENT	VOLUME
Sample DNA	48 µl
• (lilac) Control DNA Unmethylated Lambda (see Table 2.1.1.)	1 µl
• (lilac) Control DNA CpG methylated pUC19 (see Table 2.1.1.)	1 µl
<b>Total Volume</b>	<b>50 µl</b>

The sequences for pUC19 and lambda can also be found in our GitHub demo pipeline: [https://github.com/nebiolabs/EM-seq/blob/master/methylation\\_controls.fa](https://github.com/nebiolabs/EM-seq/blob/master/methylation_controls.fa)

## 2.1.2. DNA Fragmentation

The combined 50 µl genomic DNA and control DNAs are fragmented to an average fragment size of ~500 bp (470–520 bp final Illumina library). Fragmentation can be done using NEBNext UltraShear (NEB #M7634, follow the protocol provided in UltraShear manual) or a preferred fragmentation device such as a Covaris instrument.

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

**Note: Enzymatic fragmentation is only recommended using NEBNext UltraShear. For instructions see the NEBNext UltraShear manual:** <https://www.neb.com/en-us/-/media/nebus/files/manuals/manualm7634.pdf>

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

## 2.2. End Prep of Fragmented DNA

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

COMPONENT	VOLUME
Fragmented DNA (Step 2.1.2.)	50 µl
• (green) NEBNext Ultra II End Prep Reaction Buffer	7 µl
• (green) NEBNext Ultra II End Prep Enzyme Mix	3 µl
<b>Total Volume</b>	<b>60 µl</b>

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

- 2.2.2. 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.**

- 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  $20^{\circ}\text{C}$

30 minutes at  $65^{\circ}\text{C}$

Hold at  $4^{\circ}\text{C}$

## 2.3. Ligation of EM-seq Adaptor

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

COMPONENT	VOLUME
End Prep reaction mixture (Step 2.2.3.)	60 µl
• (red) NEBNext EM-seq Adaptor	2.5 µl
• (red) NEBNext Ligation Enhancer	1 µl
• (red) NEBNext Ultra II Ligation Master Mix	30 µl
<b>Total Volume</b>	<b>93.5 µl</b>

**Note: Ligation Enhancer and Ligase Master Mix can be mixed ahead of time and is stable for at least 8 hours at  $4^{\circ}\text{C}$ . 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.**

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

**Caution: The Ligase 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.**

- 2.3.3. Place in a thermal cycler, and run the following program with the heated lid off:

15 minutes at  $20^{\circ}\text{C}$

Hold at  $4^{\circ}\text{C}$



**Safe Stopping Point: Samples can be stored overnight at  $-20^{\circ}\text{C}$ .**

## 2.4. 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.**

- 2.4.1. Vortex the Sample Purification Beads to resuspend.

- 2.4.2. Add 110 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.

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

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

- 2.4.5. 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**).
- 2.4.6. 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.
- 2.4.7. Repeat the ethanol wash once for a total of two washes.
- 2.4.8. Remove all visible liquid after the second wash using a p10 pipette tip.
- 2.4.9. 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.**

- 2.4.10. Remove the tubes from the magnetic stand. Elute the DNA target from the beads by adding 29 µl of ◦ (white) Elution Buffer.
- 2.4.11. 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.
- 2.4.12. 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.**

## 2.5. Protection of 5-Methylcytosines and 5-Hydroxymethylcytosines

- 2.5.1. Prepare TET2 Buffer. Use **Option A** for #E7120S/#E7120G (24 reactions/G size) and **Option B** for #E7120L (96 reactions).

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

- 2.5.1A. 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.
- 2.5.1B. 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.**

- 2.5.2. 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 2.4.12.)	28 µl
◦ (yellow) TET2 Reaction Buffer (TET2 Reaction Buffer Supplement reconstituted in TET2 Reaction Buffer)	10 µl
◦ (yellow) Oxidation Supplement	1 µl
◦ (yellow) DTT	1 µl
◦ (yellow) Oxidation Enhancer	1 µl
◦ (yellow) TET2	4 µl
<b>Total Volume</b>	<b>45 µl</b>

Mix thoroughly by vortexing for 1-2 seconds or by pipetting up and down at least 10 times and centrifuge briefly. 5mC/5hmC oxidation is initiated by the addition of the Fe(II) solution to the reaction in the next step.



- 2.5.3. Dilute the 500 mM • (yellow) Fe(II) Solution by adding 1 µl to 1,249 µl of water. Mix well by vortexing.

**Note: The 500 mM • (yellow) 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.**

One ice, combine diluted • (yellow) Fe(II) Solution and DNA with from Section 2.5.2 as described below:

COMPONENT	VOLUME
Reaction mixture (Step 2.5.2)	45 µl
Diluted • (yellow) Fe(II) Solution (Step 2.5.3)	5 µl
<b>Total Volume</b>	<b>50 µl</b>

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

- 2.5.4. 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  $37^{\circ}\text{C}$

Hold at  $4^{\circ}\text{C}$

- 2.5.5. Transfer the samples to ice and add • (yellow) Stop Reagent:

COMPONENT	VOLUME
Protected DNA (Step 2.5.4.)	50 µl
• (yellow) Stop Reagent	1 µl
<b>Total Volume</b>	<b>51 µl</b>

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

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

30 minutes at  $37^{\circ}\text{C}$

Hold at  $4^{\circ}\text{C}$



**Safe Stopping Point: Samples can be stored overnight at either  $4^{\circ}\text{C}$  in the thermal cycler or at  $-20^{\circ}\text{C}$  in the freezer.**

## 2.6. 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.**

- 2.6.1. Vortex Sample Purification Beads to resuspend.
- 2.6.2. Add 90 µl (1.8X) 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.
- 2.6.3. Incubate samples on bench top for at least 5 minutes at room temperature.
- 2.6.4. Place the tubes against an appropriate magnetic stand to separate the beads from the supernatant.
- 2.6.5. 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**).
- 2.6.6. 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.
- 2.6.7. Repeat the wash once for a total of two washes.
- 2.6.8. Remove all visible liquid after the second wash using a p10 pipette tip.
- 2.6.9. 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.**
- 2.6.10. Remove the tubes from the magnetic stand. Elute the DNA target from the beads by adding 17 µl of • (white) Elution Buffer.

- 2.6.11. 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.
- 2.6.12. 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.

## 2.7. Denaturation of DNA



The DNA can be denatured using either Formamide or 0.1 N Sodium Hydroxide.

Use **Option A** for denaturing using Formamide. Use **Option B** for denaturing using 0.1 N Sodium hydroxide.

### 2.7A. Formamide (Recommended)

- 2.7A.1. Pre-heat thermal cycler to 85°C with the heated lid set to  $\geq 105^\circ\text{C}$  or on.
- 2.7A.2. Add 4 µl Formamide to the 16 µl of protected DNA (Step 2.6.12). Mix thoroughly by vortexing for 1–2 seconds or by pipetting up and down at least 10 times, centrifuge briefly.
- 2.7A.3. Incubate at 85°C for 10 minutes in the pre-heated thermal cycler.
- 2.7A.4. **Critical Step:** Immediately place in cooling block on ice and allow the sample to fully cool (~ 2 minutes) before proceeding to Section 2.8

### 2.7B. Sodium Hydroxide (Optional, see FAQ about preparing NaOH)

- 2.7B.1. Prepare freshly diluted 0.1 N NaOH.
- 2.7B.2. Pre-heat thermal cycler to 50°C with the heated lid set to  $\geq 105^\circ\text{C}$  or on.
- 2.7B.3. Add 4 µl 0.1 N NaOH to the 16 µl of protected DNA (Step 2.6.12). Vortex to mix or by pipetting up and down at least 10 times, centrifuge briefly.
- 2.7B.4. Incubate at 50°C for 10 minutes in the pre-heated thermal cycler.
- 2.7B.5. **Critical Step:** Immediately place in cooling block on ice and allow the sample to fully cool (~ 2 minutes) before proceeding to Section 2.8.

## 2.8. Deamination of Cytosines

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

Note: For multiple reactions, a master mix of the reaction components can be prepared on ice. Vortex the master mix briefly before use.

COMPONENT	VOLUME
Denatured DNA (Step 2.7A.4. or 2.7B.5.)	20 µl
Nuclease-free water	68 µl
• (orange) APOBEC Reaction Buffer	10 µl
• (orange) BSA	1 µl
• (orange) APOBEC	1 µl
<b>Total Volume</b>	<b>100 µl</b>

- 2.8.2. Mix thoroughly by vortexing for 1–2 seconds or by pipetting up and down at least 10 times and centrifuge briefly.
- 2.8.3. 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.

## 2.9. 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. Do not overdry the beads as they become very difficult to resuspend.

- 2.9.1. Vortex Sample Purification Beads to resuspend.
- 2.9.2. Add 100 µl (1.0X) 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.
- 2.9.3. Incubate samples on bench top for at least 5 minutes at room temperature.
- 2.9.4. Place the tubes against an appropriate magnetic stand to separate the beads from the supernatant.
- 2.9.5. 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**).
- 2.9.6. 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.
- 2.9.7. Repeat the wash once for a total of two washes.
- 2.9.8. Remove all visible liquid after the second wash using a p10 pipette tip.
- 2.9.9. 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 [Why, at some stages of the EM-seq protocol, do the NEBNext Sample Purification Beads behave differently when cleaning up the sample? | NEB](#)

- 2.9.10. Remove the tubes from the magnetic stand. Elute the DNA target from the beads by adding 21 µl of ° (white) Elution Buffer.
- 2.9.11. 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.
- 2.9.12. 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.

## 2.10. PCR Amplification

- 2.10.1. On ice, add the following components to the deaminated DNA from Step 2.9.12:

COMPONENT	VOLUME
Deaminated DNA (Step 2.9.12.)	20 µl
UDI Index Primer Pair*	5 µl
• (blue) NEBNext Q5U Master Mix	25 µl
<b>Total Volume</b>	<b>50 µl</b>

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

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

- 2.10.3. 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.

## 2.11. 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.

- 2.11.1. Vortex Sample Purification Beads to resuspend.
- 2.11.2. Add 90 µl of water to each sample. Mix well by pipetting up and down at least 10 times.
- 2.11.3. Add 91 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.
- 2.11.4. Incubate samples on bench top for at least 5 minutes at room temperature.
- 2.11.5. Place the tubes against an appropriate magnetic stand to separate the beads from the supernatant.
- 2.11.6. 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**).
- 2.11.7. 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.
- 2.11.8. Repeat the ethanol wash once for a total of two washes.
- 2.11.9. Remove all visible liquid after the second wash using a p10 pipette tip.
- 2.11.10. 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.

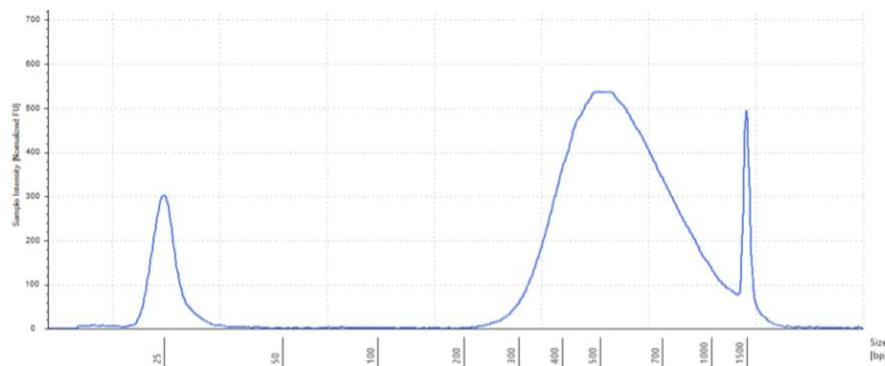
- 2.11.11. Remove the tubes from the magnetic stand. Elute the DNA target from the beads by adding 21 µl of ◦ (white) Elution Buffer. For long terms storage use, 21 µl of 1XTE (10 mM Tris, 1 mM EDTA, pH 8.0), Low TE (10 mM Tris, 0.1 mM EDTA, pH 8.0) or 0.1XTE (1mM Tris, 0.1 mM EDTA, pH 8.0).
- 2.11.12. 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.
- 2.11.13. 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.

## 2.12. Library Quantification

- 2.12.1. Use an Agilent TapeStation or Bioanalyzer to determine the size distribution and concentration of the libraries. A typical EM-seq library would have the following TapeStation trace.



*Representative TapeStation trace for an EM-seq library prepared using 50 ng of NA12878 genomic DNA. The library was run on a HS D1000 tape.*

EM-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.

## Kit Components

NEB #E7120S Table of Components

NEB #	PRODUCT	VOLUME
E7122A	Control DNA CpG methylated pUC19	0.024 ml
E7123A	Control DNA Unmethylated Lambda	0.024 ml
E7646A	NEBNext Ultra II End Prep Enzyme Mix	0.168 ml
E7647A	NEBNext Ultra II End Prep Reaction Buffer	0.078 ml
E7374A	NEBNext Ligation Enhancer	0.024 ml
E7648A	NEBNext Ultra II Ligation Master Mix	0.72 ml
E7137A	NEBNext Sample Purification Beads	8.6 ml
E7124A	Elution Buffer	2.1 ml
E7126A	TET2 Reaction Buffer	0.3 ml
E8013A	TET2 Reaction Buffer Supplement (x 3)	Lyophilized
E7128A	Oxidation Supplement	0.024 ml
E7139AA	DTT	0.5 ml
E7129A	Oxidation Enhancer	0.024 ml
E7130A	TET2	0.096 ml
E7131A	Fe(II) Solution	0.024 ml
E7132A	Stop Reagent	0.024 ml
E7133A	APOBEC	0.024 ml
E7134A	APOBEC Reaction Buffer	0.24 ml
E7135A	BSA	0.024 ml
E7136A	NEBNext Q5U Master Mix	0.6 ml
E7165A	NEBNext EM-seq Adaptor	0.06 ml

# NEB #E7120L Table of Components

NEB #	PRODUCT	VOLUME
E7122AA	Control DNA CpG methylated pUC19	0.096 ml
E7123AA	Control DNA Unmethylated Lambda	0.096 ml
E7646AA	NEBNext Ultra II End Prep Enzyme Mix	0.288 ml
E7647AA	NEBNext Ultra II End Prep Reaction Buffer	0.672 ml
E7374AA	NEBNext Ligation Enhancer	0.096 ml
E7648AA	NEBNext Ultra II Ligation Master Mix	2.88 ml
E7137AA	NEBNext Sample Purification Beads	34.6 ml
E7124AA	Elution Buffer	9 ml
E7126AA	TET2 Reaction Buffer	1.2 ml
E8013AA	TET2 Reaction Buffer Supplement (x 3)	Lyophilized
E7128AA	Oxidation Supplement	0.096 ml
E7139AA	DTT	0.5 ml
E7129AA	Oxidation Enhancer	0.096 ml
E7130AA	TET2	0.384 ml
E7131AA	Fe(II) Solution	0.096 ml
E7132AA	Stop Reagent	0.096 ml
E7133AA	APOBEC	0.096 ml
E7134AA	APOBEC Reaction Buffer	0.96 ml
E7135AA	BSA	0.096 ml
E7136AA	NEBNext Q5U Master Mix	2.4 ml
E7165AA	NEBNext EM-seq Adaptor	0.24 ml

## Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	4/19
2.0	Updated items in Checklist (Steps 1.5 and 1.10) also (Steps 2.5 and 2.10).	7/19
3.0	Correct step numbers in Section 2. Steps 2.7A and 2.7B.	8/19
4.0	Add DTT to kit and update protocol to include DTT.	2/20
5.0	Change Elution Buffer amount in Step 2.4.9 to 29 µl and in Step 2.4.11 change 29 µl supernatant to 28 µl. Step 1.5.2 and 2.5.2 updated the component description.	2/20
6.0	New format applied.	3/20
7.0	Protocol updates, updated table formatting, updated legal footer.	4/23
8.0	Protocol updates and update to include new NEB logo.	2/25
8.1	Reversed error on NaOH concentration in “Required Materials Not Included” section.	4/25
9.0	Updated to reflect changes to kit components: TET2 Reaction Buffer Supplement is now lyophilized and the EM-seq Index Primers have been removed	5/25

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