NEBNext® Enzymatic Methyl-seq Kit

NEB #E7120S/L

24/96 reactions

Version 7.0_4/23

Table of Contents

The Library Kit Includes

The volumes provided are sufficient for preparation of up to 24 reactions (NEB #E7120S) and 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 **(Please Note: This buffer may be clear or yellow in color.)**
- (yellow) Stop Reagent
- (orange) APOBEC
- (orange) APOBEC Reaction Buffer
- (orange) BSA
- (blue) NEBNext Q5U™ Master Mix

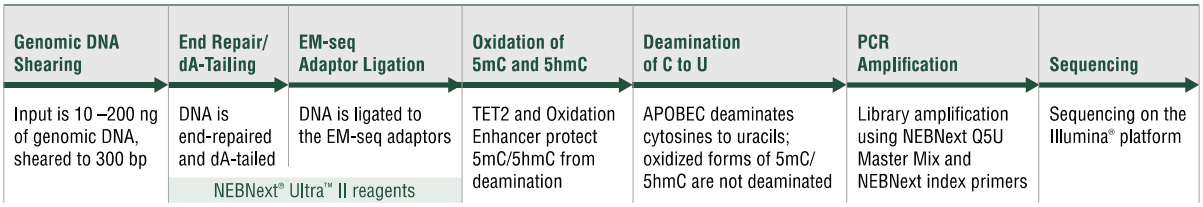
NEBNext Multiplex Oligos for Enzymatic Methyl-seq (24 Unique Dual Index Primer Pairs) or (96 Unique Dual Index Primer Pairs)
NEBNext Sample Purification Beads

Required Materials Not Included

- Covaris® S2 instrument or other fragmentation equipment
- PCR strip tubes
- Recommended: Formamide (Sigma #F9037-100 ml) or optional 0.1 N NaOH. Formamide is preferred. If using NaOH, please see FAQ on NEB #E7120 FAQ page.
- 80% Ethanol
- 1XTE (10 mM Tris, 1 mM EDTA, pH 8.0), 10 mM Tris 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).
- Nuclease-free Water
- Magnetic rack/stand, such as NEBNext Magnetic Separation Rack (NEB #S1515S)
- PCR machine
- Bioanalyzer®, TapeStation® and associated consumables or other fragment analyzer

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 sheared 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.

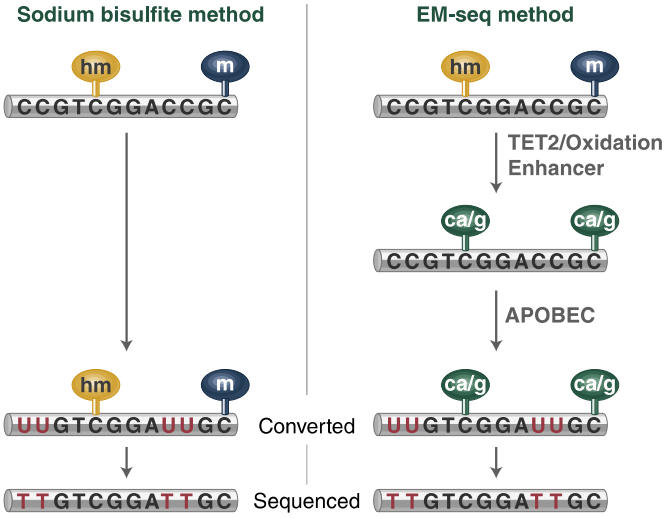


Figure 2 shows a comparison of the sodium bisulfite and EM-seq methods. Sodium bisulfite treatment of DNA results in the deamination of cytosines into uracils, however the modified forms of cytosine (5mC and 5hmC) are not deaminated. Therefore, the preference of bisulfite to chemically deaminate cytosines 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) ⇒5-hydroxymethylcytosine (5hmC) ⇒ 5-formylcytosine (5fC) ⇒ 5-carboxycytosine (5caC)]. This protects 5mC and 5hmC from deamination. 5hmC can also be protected from deamination by glucosylation to form 5ghmc using the oxidation enhancer. The second enzymatic step uses APOBEC to deaminate C but does not convert 5caC and 5ghmC. 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 ng–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 E7120 with the above products, we recommend combing 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.

For larger volume requirements, customized and bulk packaging is available by purchasing through the OEM & Custom Solutions department at NEB. Please contact custom@neb.com for further information.

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.

Starting Material: 10 ng–200 ng DNA

1.1.DNA Preparation

1.1.1.DNA and Control DNA

DNA and Control DNA

Combine genomic DNA (10–200 ng) with control DNAs specified below. Genomic DNA can be in any of the following buffers: 10 mM Tris pH 8.0, 1X TE (10mM Tris pH 8.0, 1mM EDTA), or low TE (10 mM Tris pH 8.0, 0.1 mM EDTA).

COMPONENT	VOLUME
gDNA	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
Total Volume	**50 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) on a MiSeq prior to deep sequencing (approx. 100-150 million paired reads) on NovaSeq, HiSeq or NextSeq.

DILUTION OF • (LILAC) LAMBDA CONTROL AND • (LILAC) pUC19 CONTROL		
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 other 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 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.

1.1.2.Shearing DNA

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 a preferred fragmentation device such as a Covaris instrument. Enzymatic fragmentation is not recommended as this may result in the removal of methylation marks.

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

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

1.2.End Prep of Sheared DNA

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

COMPONENT	VOLUME
Sheared DNA (Step 1.1.2.)	50 µl
• (green) NEBNext Ultra II End Prep Reaction Buffer	7 µl
• (green) NEBNext Ultra II End Prep Enzyme Mix	3 µl
Total Volume	**60 µl**

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

1.2.2. Set a 100 µl or 200 µl pipette to 50 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.

1.2.3. 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

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
Total Volume	**93.5 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. 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. Set a 100 µl or 200 µl pipette to 80 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.

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

15 minutes at 20°C

Hold at 4°C

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

1.4.Clean-Up of Adaptor Ligated DNA

1.4.1. Vortex Sample Purification Beads to resuspend.

1.4.2. Add 110 µl (~1.1X) 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 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.

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

1.4.8. Air dry the beads for 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.

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

1.4.10. 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.

1.4.11. 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.

1.5.Oxidation of 5-Methylcytosines and 5-Hydroxymethylcytosines

1.5.1. 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.

1.5.1A. Add 100 µl of ○(yellow) TET2 Reaction Buffer to one tube of ○(yellow) TET2 Reaction Buffer Supplement and mix well. Write date on tube.

1.5.1B. 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.

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

COMPONENT	VOLUME
EM-seq adaptor ligated DNA (from Step 1.4.11)	28 µl
○ (yellow) TET2 Reaction Buffer (TET2 Reaction Buffer plus reconstituted TET2 Reaction Buffer Supplement)	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.

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

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.

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

COMPONENT	VOLUME
Reaction Mixture (Step 1.5.2)	45 µl
Diluted ⚬(yellow) Fe(II) Solution (Step 1.5.3)	5 µl
Total Volume	**50 µl**

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

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

1 hour at 37°C

Hold at 4°C

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

COMPONENT	VOLUME
Oxidized DNA (Step 1.5.4.)	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.

1.5.6. 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.

1.6. Clean-Up of TET2 Oxidized DNA

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 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.

1.6.7. 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.

1.6.8. 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.

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

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

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

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 and 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 ≥ 105°C or on.

1.7A.2. Add 4 µl Formamide to the 16 µl of oxidized DNA (Step 1.6.11). Vortex to mix 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. Immediately place on ice and allow the sample to fully cool (~2 min) 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 ≥ 105°C or on.

1.7B.3. Add 4 µl 0.1 N NaOH to the 16 µl of oxidized DNA (Step 1.6.11). Vortex to mix or by pipetting up and down at least 10 times, centrifuge briefly.

1.7B.4. Incubate at 50°C for 10 minutes in the pre-heated thermal cycler.

1.7B.5. Immediately place on ice and allow the sample to fully cool (~2 min) before proceeding to Section 1.8.

1.8. Deamination of Cytosines

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

COMPONENT	VOLUME
Denatured DNA (Step 1.7A.4. or 1.7B.5.)	20 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.

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

1.8.3. 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.

1.9. 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.

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 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.

1.9.7. 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.

1.9.8. 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.

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

1.9.10. 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.

1.9.11. 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.

1.10.PCR Amplification

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

COMPONENT	VOLUME
Deaminated DNA (Step 1.9.11.)	20 µl
EM-seq Index Primer*, **	5 µl
• (blue) NEBNext Q5U Master Mix	25 µl
Total Volume	**50 µl**

*Refer to Section 3 for barcode pooling guidelines.

** EM-seq primers are supplied in tubes in NEB #E7120S or as a 96 Unique Dual Index Primers Pairs Plate in NEB #E7120L.

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 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

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 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.

1.11.7. 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.

1.11.8. 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.

1.11.9. 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).

1.11.10. 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.

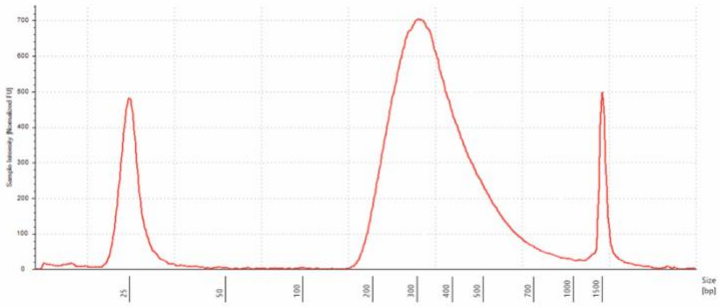
1.11.11. Place the tube on the magnetic stand. After 3 minutes (or whenever the solution is clear), transfer 20 µl of the supernatant to a new PCR tube.

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

1.12.Library Quantification

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

50 ng of NA12878 genomic DNA



Sequence using the preferred Illumina platform. 2 x 76 base reads or 2 x 100 base reads for standard sized libraries.

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.

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

Starting Material: 10 ng–200 ng DNA.

2.1.DNA Preparation

2.1.1.

DNA and Control DNA

Combine genomic DNA (10–200 ng) with control DNAs specified below. Genomic DNA can be in any of the following buffers: 10 mM Tris pH 8.0, 1X TE (10mM Tris pH 8.0, 1mM EDTA), or low TE (10 mM Tris pH 8.0, 0.1 mM EDTA).

COMPONENT	VOLUME
gDNA	48 µl
• (lilac) Control DNA Unmethylated Lambda (see Table 2.1)	1 µl
• (lilac) Control DNA CpG methylated pUC19 (see Table 2.1)	1 µl
Total Volume	**50 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 2.1 Dilutions of control DNAs for a range of genomic DNA inputs. These are suitable for shallow/pre-sequencing (approx. 2 - 4 million paired reads) on a MiSeq prior to deep sequencing (approx. 100-150 million paired reads) on NovaSeq, HiSeq or NextSeq.

DILUTION OF • (LILAC) LAMBDA CONTROLAND • (LILAC) pUC19 CONTROL		
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 other 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 2.1), 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 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.

2.1.2. Shearing DNA

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 a preferred fragmentation device such as a Covaris instrument. Enzymatic fragmentation is not recommended as this may result in the removal of methylation marks.

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

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

2.2. End Prep of Sheared DNA

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

COMPONENT	VOLUME
Fragmented DNA	50 µl
• (green) NEBNext Ultra II End Prep Reaction Buffer	7 µl
• (green) NEBNext Ultra II End Prep Enzyme Mix	3 µl
Total Volume	**60 µl**

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

2.2.2 Set a 100 µl or 200 µl pipette to 50 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

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
Total Volume	**93.5 µl**

Note: Ligation Enhancer and Ligase 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.

2.3.2. Set a 100 µl or 200 µl pipette to 80 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 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°C

Hold at 4°C

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

2.4. Clean-Up of Adaptor Ligated DNA

2.4.1. Vortex Sample Purification Beads to resuspend.

2.4.2. Add 110 µl (~1.1X) 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 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.

2.4.7. 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.

2.4.8. 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.

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

2.4.10. 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.

2.4.11. 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.

2.5. Oxidation of 5-Methylcytosines and 5-Hydroxymethylcytosines

2.5.1. Prepare TET2 Buffer. Use option A if you are using E7120S/E7120G (24 reactions/G size) and option B if you are using 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.

2.5.1A. Add 100 µl of ○ (yellow) TET2 Reaction Buffer to one tube of ○ (yellow) TET2 Reaction Buffer Supplement and mix well. Write date on tube.

2.5.1B. 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.

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

COMPONENT	VOLUME
Em-Seq Adaptor ligated DNA (Step 2.4.11.)	28 µl
○ (yellow) TET2 Reaction Buffer (TET2 Reaction Buffer plus reconstituted TET2 Reaction Buffer Supplement)	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.

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

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.

Combine diluted ○ (yellow) Fe(II) Solution and EM-seq DNA with Oxidation Enzymes (from Step 2.5.2).

COMPONENT	VOLUME
Reaction mixture (Step 2.5.2)	45 µl
Diluted ⚬ (yellow) Fe(II) Solution (Step 2.5.3)	5 µl
Total Volume	**50 µl**

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

2.5.4. 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

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

COMPONENT	VOLUME
Oxidized DNA (Step 2.5.4.)	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.

2.5.6. 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.

2.6. Clean-Up of TET2 Oxidized DNA

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 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.

2.6.7. 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.

2.6.8. 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.

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

2.6.10. 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.

2.6.11. 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.

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 and 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 ≥ 105°C or on.

2.7A.2. Add 4 µl Formamide to the 16 µl of oxidized DNA (Step 2.6.11). Vortex to mix 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. Immediately place on ice and allow the sample to fully cool (~2 min) 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 ≥ 105°C or on.

2.7B.3. Add 4 µl 0.1 N NaOH to the 16 µl of oxidized DNA (Step 2.6.11). 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. Immediately place on ice and allow the sample to fully cool (~2 min) before proceeding to Section 2.8.

2.8. Deamination of Cytosines

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

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
Total Volume	**100 µl**

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

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

2.8.3. 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.

2.9. 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.

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 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.

2.9.7. 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.

2.9.8. 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.

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

2.9.10. 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.

2.9.11. 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.

2.10.PCR Amplification

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

COMPONENT	VOLUME
Deaminated DNA (Step 2.9.11.)	20 µl
EM-seq Index Primer *,**	5 µl
• (blue) NEBNext Q5U Master Mix	25 µl
Total Volume	**50 µl**

*Refer to Section 3 for barcode pooling guidelines.

** EM-seq primers are supplied in tubes in NEB #E7120S or as a 96 Unique Dual Index Primers Pairs Plate in NEB #E7120L.

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 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

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 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.

2.11.8. 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.

2.11.9. 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.

2.11.10. 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 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).

2.11.11. 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.

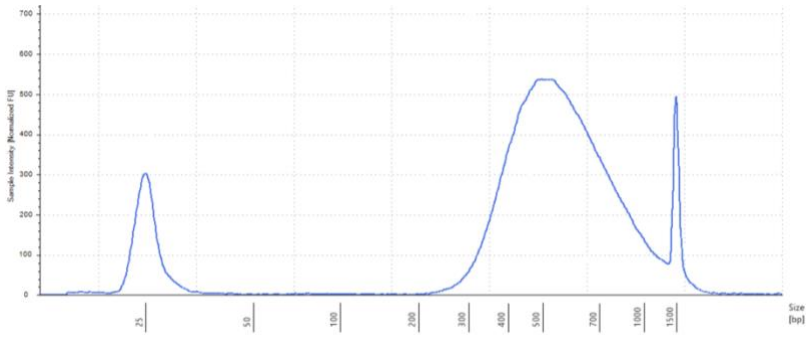
2.11.12. Place the tube on the magnetic stand. After 3 minutes (or whenever the solution is clear), transfer 20 µl of the supernatant to a new PCR tube.

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

2.12.Library Quantification

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

50 ng of NA12878 genomic DNA.



Sequence using the preferred Illumina platform. 2 x 100 base reads or 2 x 150 base reads for large insert libraries.

Section 3
Index Pooling Guidelines

For more detailed indexing information please refer to the manual for NEBNext Multiplex Oligos for Enzymatic Methyl-seq (Unique Dual Index Primer Pairs), NEB #E7140.

For a link to download a sample sheet with the index sequences for use with the Illumina Experiment Manager (IEM) please go to our FAQ's tab on www.neb.com/E7140 – NEBNext Multiplex Oligos for Enzymatic Methyl-seq (Unique Dual Index Primer Pairs), NEB #E7140.

Adaptor trimming sequences:

The NEBNext libraries for Illumina resemble TruSeq libraries and can be trimmed similar to TruSeq:

Adaptor Read 1 AGATCGGAAGAGCACACGTCTGAACTCCAGTCA
Adaptor Read 2 AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

24 Reaction Kit (NEB #E7120S)

For multiplexing < 24 samples use Table 3.1 for some valid index combinations.

Table 3.1.

PLEX	TUBE NUMBER
2	1 and 2*
	3 and 4
	5 and 6
	7 and 8
≥ 3	Any 2 plex plus any other index

* Use only one primer tube per library

*** Forward Strand Workflow** for the following instruments: NovaSeq 6000 with v1.0 reagents kits, MiniSeq with rapid reagent kits, MiSeq®, HiSeq® 2000/2500 (pair-end flow cell), HiSeq 3000/4000 (single-read flow cell).

*** Reverse Complement Workflow** for the following instruments: iSeq 100, MiniSeq with standard reagent kits, NextSeq Systems, NovaSeq 6000 with v1.5 reagent kits, HiSeq 2000/5000 (single-read flow cell), HiSeq 3000/4000 (paired-end flow cell).

Note: Please see Illumina Document "Indexed Sequencing Overview" document number 15057455

The index primer sequences, for different Illumina sequencer input sheets are indicated in Table 3.2.

Table 3.2 Index Sequences (Color coded based on HiSeq/MiSeq guidelines)

TUBE NUMBER	EXPECTED i7 INDEX READ	EXPECTED i5 INDEX READ	
		Forward Strand Workflow*	Reverse Complement Workflow*
1	CACTGTAG	AAGCGACT	AGTCGCTT
2	GTGCACGA	TGATAGGC	GCCTATCA
3	AAGCGACT	ACGAATCC	GGATTCGT
4	TGATAGGC	GTCTGAGT	ACTCAGAC
5	ACGAATCC	ATTACCCA	TGGGTAAT
6	GTCTGAGT	GACTTGTG	CACAAGTC
7	ATTACCCA	CACTGTAG	CTACAGTG
8	GACTTGTG	GTGCACGA	TCGTGCAC
9	TTCAATAG	TCCCACGA	TCGTGGGA
10	GTTTGCTC	ACCAACAG	CTGTTGGT
11	ACCGGAGT	AAGGAAGG	CCTTCCTT
12	CTTGACGA	GCACACAA	TTGTGTGC
13	TGTTCGCC	AGGTAGGA	TCCTACCT
14	ACAAGGCA	TCGCGCAA	TTGCGCGA
15	CCTGTCAA	ATGGCTGT	ACAGCCAT
16	CCATCCGC	AAGGCGTA	TACGCCTT
17	ATGGCTGT	CCTGTCAA	TTGACAGG
18	AAGGCGTA	CCATCCGC	GCGGATGG
19	AGGTAGGA	TGTTCGCC	GGCGAACA
20	TCGCGCAA	ACAAGGCA	TGCCTTGT
21	AAGGAAGG	ACCGGAGT	ACTCCGGT
22	GCACACAA	CTTGACGA	TCGTCAAG
23	TCCCACGA	TTCAATAG	CTATTGAA
24	ACCAACAG	GTTTGCTC	GAGCAAAC

96 Reaction Kit (NEB #E7120L)

For multiplexing < 96 samples use Table 3.3 for some valid index combinations.

Table 3.3.

PLEX	WELL POSITION
< 4	Not recommended
4	A6, B6, C6, and D6
	A12, B12, C12, and D12
	B6, C6, D6, and E6
	B12, C12, D12, and E12
	C1, D1, E1, and F1
	C7, D7, E7, and F7
	E4, F4, G4, and H4
	E10, F10, G10, H10
5	A1, B1, C1, D1, E1
	A6, B6, C6, D6, E6
	A7, B7, C7, D7, E7
	A12, B12, C12, D12, E12
	B1, C1, D1, E1, F1
	B6, C6, D6, E6, F6
	B7, C7, D7, E7, F7
	B12, C12, D12, E12, F12
	C1, D1, E1, F1, G1
	C2, D2, E2, F2, G2
	C4, D4, E4, F4, G4
	C7, D7, E7, F7, G7
	C8, D8, E8, F8, G8
	C10, D10, E10, F10, G10
	D4, E4, F4, G4, H4
	D10, E10, F10, G10, H10
6-7	Any 5 plex plus 1-2 adjacent wells from the same column
8	Any column

*** Forward Strand Workflow** for the following instruments: NovaSeq 6000 with v1.0 reagents kits, MiniSeq with rapid reagent kits, MiSeq®, HiSeq® 2000/2500 (pair-end flow cell), HiSeq 3000/4000 (single-read flow cell).

*** Reverse Complement Workflow** for the following instruments: iSeq 100, MiniSeq with standard reagent kits, NextSeq Systems, NovaSeq 6000 with v1.5 reagent kits, HiSeq 2000/5000 (single-read flow cell), HiSeq 3000/4000 (paired-end flow cell).

Note: Please see Illumina Document "Indexed Sequencing Overview" document number 15057455

The index primer sequences, for different Illumina sequencer input sheets are indicated in Table 3.4.

Table 3.4 Index Sequences (Color coded based on HiSeq/MiSeq guidelines)

WELL POSITION	EXPECTED i7 INDEX READ	EXPECTED i5 INDEX READ	
		Forward Strand Workflow*	Reverse Complement Workflow*
A1	TTACCGAC	CGAATACG	CGTATTCG
B1	TCGTCTGA	GTCCTTGA	TCAAGGAC
C1	TTCCAGGT	CAGTGCTT	AAGCACTG
D1	TACGGTCT	TCCATTGC	GCAATGGA
E1	AAGACCGT	GTCGATTG	CAATCGAC
F1	CAGGTTCA	ATAACGCC	GGCGTTAT
G1	TAGGAGCT	GCCTTAAC	GTTAAGGC
H1	TACTCCAG	GGTATAGG	CCTATACC
A2	AGTGACCT	TCTAGGAG	CTCCTAGA
B2	AGCCTATC	TGCGTAAC	GTTACGCA
C2	TCATCTCC	CTTGCTAG	CTAGCAAG
D2	CCAGTATC	AGCGAGAT	ATCTCGCT
E2	TTGCGAGA	TATGGCAC	GTGCCATA
F2	GAACGAAG	GAATCACC	GGTGATTC
G2	CGAATTGC	GTAAGGTG	CACCTTAC
H2	GGAAGAGA	CGAGAGAA	TTCTCTCG
A3	TCGGATTC	CGCAACTA	TAGTTGCG
B3	CTGTACCA	CACAGACT	AGTCTGTG
C3	GAGAGTAC	TGGAAGCA	TGCTTCCA
D3	TCTACGCA	CAATAGCC	GGCTATTG
E3	GCAATTCC	CTCGAACA	TGTTCGAG
F3	CTCAGAAG	GGCAAGTT	AACTTGCC
G3	GTCCTAAG	AGCTACCA	TGGTAGCT
H3	GCGTTAGA	CAGCATAC	GTATGCTG
A4	CAAGGTAC	CGTATCTC	GAGATACG
B4	AGACCTTG	TTACGTGC	GCACGTAA
C4	GTCGTTAC	AGCTAAGC	GCTTAGCT
D4	GTAACCGA	AAGACACC	GGTGTCTT
E4	GAATCCGT	CAACTCCA	TGGAGTTG
F4	CATGAGCA	GATCTTGC	GCAAGATC
G4	CTTAGGAC	CTTCACTG	CAGTGAAG
H4	ATCTGACC	CTCGACTT	AAGTCGAG
A5	TCCTCATG	GTACACCT	AGGTGTAC
B5	AGGATAGC	CCAAGGTT	AACCTTGG
C5	GGAGGAAT	GAACGGTT	AACCGTTC
D5	GACGTCAT	CCAGTTGA	TCAACTGG
E5	CCGCTTAA	GTCATCGT	ACGATGAC
F5	GACGAACT	CAATGCGA	TCGCATTG
G5	TCCACGTT	GGTTGAAC	GTTCAACC
H5	AACCAGAG	CTTCGGTT	AACCGAAG

WELL POSITION	EXPECTED i7 INDEX READ	EXPECTED i5 INDEX READ	
		Forward Strand Workflow*	Reverse Complement Workflow*
A6	GTCAGTCA	CGGCATTA	TAATGCCG
B6	CCTTCCAT	CACGCAAT	ATTGCGTG
C6	AGGAACAC	GGAATGTC	GACATTCC
D6	CTTACAGC	TGGTGAAG	CTTCACCA
E6	TACCTGCA	GGACATCA	TGATGTCC
F6	AGACGCTA	GGTGTACA	TGTACACC
G6	CAACACAG	GATAGCCA	TGGCTATC
H6	GTACCACA	CCACAACA	TGTTGTGG
A7	CGAATACG	TTACCGAC	GTCGGTAA
B7	GTCCTTGA	TCGTCTGA	TCAGACGA
C7	CAGTGCTT	TTCCAGGT	ACCTGGAA
D7	TCCATTGC	TACGGTCT	AGACCGTA
E7	GTCGATTG	AAGACCGT	ACGGTCTT
F7	ATAACGCC	CAGGTTCA	TGAACCTG
G7	GCCTTAAC	TAGGAGCT	AGCTCCTA
H7	GGTATAGG	TACTCCAG	CTGGAGTA
A8	TCTAGGAG	AGTGACCT	AGGTCACT
B8	TGCGTAAC	AGCCTATC	GATAGGCT
C8	CTTGCTAG	TCATCTCC	GGAGATGA
D8	AGCGAGAT	CCAGTATC	GATACTGG
E8	TATGGCAC	TTGCGAGA	TCTCGCAA
F8	GAATCACC	GAACGAAG	CTTCGTTC
G8	GTAAGGTG	CGAATTGC	GCAATTCG
H8	CGAGAGAA	GGAAGAGA	TCTCTTCC
A9	CGCAACTA	TCGGATTC	GAATCCGA
B9	CACAGACT	CTGTACCA	TGGTACAG
C9	TGGAAGCA	GAGAGTAC	GTACTCTC
D9	CAATAGCC	TCTACGCA	TGCGTAGA
E9	CTCGAACA	GCAATTCC	GGAATTGC
F9	GGCAAGTT	CTCAGAAG	CTTCTGAG
G9	AGCTACCA	GTCCTAAG	CTTAGGAC
H9	CAGCATAC	GCGTTAGA	TCTAACGC
A10	CGTATCTC	CAAGGTAC	GTACCTTG
B10	TTACGTGC	AGACCTTG	CAAGGTCT
C10	AGCTAAGC	GTCGTTAC	GTAACGAC
D10	AAGACACC	GTAACCGA	TCGGTTAC
E10	CAACTCCA	GAATCCGT	ACGGATTC
F10	GATCTTGC	CATGAGCA	TGCTCATG
G10	CTTCACTG	CTTAGGAC	GTCCTAAG
H10	CTCGACTT	ATCTGACC	GGTCAGAT

WELL POSITION	EXPECTED i7 INDEX READ	EXPECTED i5 INDEX READ	
		Forward Strand Workflow*	Reverse Complement Workflow*
A11	GTACACCT	TCCTCATG	CATGAGGA
B11	CCAAGGTT	AGGATAGC	GCTATCCT
C11	GAACGGTT	GGAGGAAT	ATTCCTCC
D11	CCAGTTGA	GACGTCAT	ATGACGTC
E11	GTCATCGT	CCGCTTAA	TTAAGCGG
F11	CAATGCGA	GACGAACT	AGTTCGTC
G11	GGTTGAAC	TCCACGTT	AACGTGGA
H11	CTTCGGTT	AACCAGAG	CTCTGGTT
A12	CGGCATTA	GTCAGTCA	TGACTGAC
B12	CACGCAAT	CCTTCCAT	ATGGAAGG
C12	GGAATGTC	AGGAACAC	GTGTTCCT
D12	TGGTGAAG	CTTACAGC	GCTGTAAG
E12	GGACATCA	TACCTGCA	TGCAGGTA
F12	GGTGTACA	AGACGCTA	TAGCGTCT
G12	GATAGCCA	CAACACAG	CTGTGTTG
H12	CCACAACA	GTACCACA	TGTGGTAC

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
E7127A	TET2 Reaction Buffer Supplement (x 3)	powder
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
E7141A	EM-seq Index Primer 1	0.005 ml
E7142A	EM-seq Index Primer 2	0.005 ml
E7143A	EM-seq Index Primer 3	0.005 ml
E7144A	EM-seq Index Primer 4	0.005 ml
E7145A	EM-seq Index Primer 5	0.005 ml

E7146A	EM-seq Index Primer 6	0.005 ml
E7147A	EM-seq Index Primer 7	0.005 ml
E7148A	EM-seq Index Primer 8	0.005 ml
E7149A	EM-seq Index Primer 9	0.005 ml
E7150A	EM-seq Index Primer 10	0.005 ml
E7151A	EM-seq Index Primer 11	0.005 ml
E7152A	EM-seq Index Primer 12	0.005 ml
E7153A	EM-seq Index Primer 13	0.005 ml
E7154A	EM-seq Index Primer 14	0.005 ml
E7155A	EM-seq Index Primer 15	0.005 ml
E7156A	EM-seq Index Primer 16	0.005 ml
E7157A	EM-seq Index Primer 17	0.005 ml
E7158A	EM-seq Index Primer 18	0.005 ml
E7159A	EM-seq Index Primer 19	0.005 ml
E7160A	EM-seq Index Primer 20	0.005 ml
E7161A	EM-seq Index Primer 21	0.005 ml
E7162A	EM-seq Index Primer 22	0.005 ml
E7163A	EM-seq Index Primer 23	0.005 ml
E7164A	EM-seq Index Primer 24	0.005 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
E7127AA	TET2 Reaction Buffer Supplement (x 3)	powder
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
E7166A	NEBNext 96 Unique Dual Index Primer Pairs Plate	0.005 ml x 96

CheckList (Section 1)

1.1 DNA Preparation

1.1.1. Combine DNA and control DNA

Sequencing on MiSeq before NovaSeq, HiSeq or NextSeq (Check 2-4 M paired end reads per EM-seq Library)

[_] 10–200 ng sample DNA

[_] 1 µl of 0.1 ng/µl pUC19 control DNA • (lilac)

[_] 1 µl of 2 ng/µl unmethylated lambda DNA • (lilac)

[_] Add 0.1X TE pH 8.0 to 50 µl

For direct sequencing on NovaSeq, HiSeq or NextSeq

[_] 10–200 ng sample DNA

[_] 1 µl of pUC19 DNA • (lilac) diluted 100X to 0.001 ng/µl pUC19

[_] 1 µl of unmethylated lambda DNA • (lilac) diluted 100X to 0.02 ng/µl

[_] Add 0.1X TE pH 8.0 to 50 µl

1.1.2. Shear DNA

[_] Shear to 240–290 bp, use preferred instrument.

[_] Transfer the 50 µl of sheared material directly to a PCR strip tube to begin library construction.

1.2. End Prep of Sheared DNA

Add End Prep Reagents to sample (50 µl):

[_] 7 µl NEBNext Ultra II End Prep Reaction Buffer • (green)

[_] 3 µl NEBNext Ultra II End Prep Enzyme Mix • (green)

[_] Vortex or pipette mix 10 times with pipette, quick spin

[_] Incubate in thermal cycler

30 minutes 20°C

30 minutes 65°C

Hold at 4°C

1.3. Ligation of EM-seq Adaptor

Add Ligation Reagents to End Repaired DNA

[_] 2.5 µl NEBNext EM-seq adaptor • (red)

[_] 1 µl NEBNext Ligation Enhancer • (red)

[_] 30 µl NEBNext Ultra II Ligation Master Mix • (red)

[_] Vortex or pipette mix 10 times with pipette, quick spin

[_] Incubate in thermal cycler

15 minutes 20°C (heated lid off)

Hold at 4°C

1.4. Clean-Up of Adaptor Ligated DNA

[_] Vortex beads

[_] Add 110 µl of resuspended NEBNext Sample Purification Beads to each sample and mix by pipetting 10 times

[_] Incubate 5 min

[_] Place tubes on magnet for 5 min

[_] Remove and discard the supernatant, while keeping the sample on the magnet

[_] On magnet add 200 µl 80% ethanol, wait 30 seconds and then remove and discard the ethanol wash

[_] Repeat the 80% ethanol wash

[_] Airdry the beads for up to 2 min while on magnet

[_] Remove the samples from the magnet and resuspend in 29 µl of Elution Buffer ◦ (white)

[_] Place back on the magnet, wait until the supernatant clears and transfer 28 µl of sample to fresh PCR tubes

1.5. Oxidation of 5-Methylcytosines and 5-Hydroxymethylcytosines

[_] Reconstitute the TET2 Reaction Buffer Supplement ◦ (yellow) using TET2 Reaction Buffer ◦ (yellow)

24 Reaction kit: 1 tube TET2 Reaction Buffer Supplement ◦ (yellow) & 100 µl TET2 Reaction Buffer ◦ (yellow)

96 Reaction kit: 1 tube TET2 Reaction Buffer Supplement ◦ (yellow) & 400 µl TET2 Reaction Buffer ◦ (yellow)

Once reconstituted the Tet2 Reaction Buffer should be used within 4 months

[_] Add Oxidation Reagents to 28 µl Adaptor Ligated DNA

[_] 10 µl Reconstituted TET2 Reaction Buffer ◦ (yellow)

[_]1 µl Oxidation Supplement ◦ (yellow)

[_] 1 µl DTT ◦ (yellow)

[_] 1 µl Oxidation Enhancer ◦ (yellow)

[_] 4 µl TET2 ◦ (yellow)

[_] Mix by vortexing or pipette mix 10 times, centrifuge briefly

[_] Make diluted Fe(II) Solution.

Add 1 µl 500 mM Fe(II) Solution ◦ (yellow) to 1,249 µl of water. Use immediately, do not store.

[_] Add 5 µl Diluted Fe(II) Solution to sample

[_] Mix by vortexing or pipette mix 10 times, centrifuge briefly, then incubate in a thermal cycler

60 minutes 37°C

Hold at 4°C

[_]Add 1 µl Stop Reagent ◦ (yellow)

[_] Mix by vortexing or pipette mix 10 times, centrifuge briefly

[_]Incubate in a thermal cycler

30 minutes 37°C

Hold at 4°C

1.6. Clean-Up of TET2 converted DNA

[_] Vortex beads

[_] Add 90 µl of resuspended NEBNext Sample Purification Beads to each sample and mix by pipetting 10 times

[_] Incubate 5 min

[_] Place tubes on magnet for 5 min

[_] Remove and discard the supernatant, while keeping the sample on the magnet

[_] On magnet add 200 µl 80% ethanol, wait 30 seconds and then remove and discard the ethanol wash

[_] Repeat the 80% ethanol wash

[_] Airdry the beads for up to 2 min while on magnet

[_] Remove the samples from the magnet and resuspend in 17 µl of Elution Buffer ◦ (white)

[_] Place back on the magnet, wait until the supernatant clears and transfer 16 µl of sample to fresh PCR tubes

1.7. Denaturation of DNA

Use either Formamide (A) or Sodium Hydroxide (B)

A. Formamide Denaturation (Recommended)

[_] Pre-heat thermal cycler to 85°C

[_] Add 4 µl Formamide to the 16 µl oxidized DNA

[_] Mix by vortexing or pipette mix 10 times, centrifuge briefly

[_] Incubate in a thermal cycler

85°C for 10 min

Immediately, place on ice

[_] Proceed immediately into Section 1.8

B. Sodium Hydroxide Denaturation (Optional: Formamide is preferred)

[_] Prepare freshly diluted 0.1 N NaOH

[_] Pre-heat thermal cycler to 50°C

[_] Add 4 µl 0.1 N NaOH to the 16 µl oxidized DNA

[_] Mix by vortexing or pipette mix 10 times, centrifuge briefly

[_] Incubate in a thermal cycler

50°C for 10 min

Immediately, place on ice

[_] Proceed immediately into Section 1.8

1.8. Deamination of Cytosines

Add Deamination Reagents to 20 µl denatured DNA on ice

[_] 68 µl Nuclease-free water

[_] 10 µl APOBEC Reaction Buffer • (orange)

[_] 1 µl BSA • (orange)

[_] 1 µl APOBEC • (orange)

[_] Mix by vortexing or pipette mix 10 times, centrifuge briefly

[_] Incubate in a thermal cycler

37°C for 3 hours

Hold at 4°C

1.9. Clean-Up of Deaminated DNA

[_] Vortex beads

[_] Add 100 µl of resuspended NEBNext Sample Purification Beads to each sample and mix by pipetting 10 times

[_] Incubate 5 min

[_] Place tubes on magnet for 5 min

[_] Remove and discard the supernatant, while keeping the sample on the magnet

[_] On magnet add 200 µl 80% ethanol, wait 30 seconds and then remove and discard the ethanol wash

[_] Repeat the 80% ethanol wash

[_] Airdry the beads for up to 90 sec while on magnet. Do not over-dry as beads become difficult to resuspend

[_] Remove the samples from the magnet and resuspend in 21 µl of Elution Buffer ◦ (white)

[_] Place back on the magnet, wait until the supernatant clears and transfer 20 µl of sample to fresh PCR tubes

1.10 PCR Amplification

Add Amplification Reagents to 20 µl deaminated DNA

[_] 5 µl NEBNext Unique Dual Index Primer Pairs

[_] 25 µl NEBNext Q5U Master Mix • (blue)

[_] Mix by vortexing or pipette mix 10 times, centrifuge briefly

[_] Amplify in thermal cycler

CYCLE STEP	TEMPERATURE	TIME	CYCLES
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	10 s	4–8*
Annealing	62°C	30 s	
Extension	65°C	60 s	
Final Extension	65°C	5 min	1
Hold	4	∞	

Cycle recommendation:

10 ng DNA: 8 cycles

50 ng DNA: 5–6 cycles

200 ng DNA: 4 cycles

1.11. Clean-Up of Amplified Libraries

Vortex beads

[_] Add 45 µl of resuspended NEBNext Sample Purification Beads to each sample and mix by pipetting 10 times

[_] Incubate 5 min

[_] Place tubes on magnet for 5 min

[_] Remove and discard the supernatant, while keeping the sample on the magnet

[_] On magnet add 200 µl 80% ethanol, wait 30 seconds and then remove and discard the ethanol wash

[_] Repeat the 80% ethanol wash

[_] Airdry the beads for up to 2 min while on magnet

[_] Remove the samples from the magnet and resuspend in 21 µl of Elution Buffer ∘ (white) or 10 mM Tris, 0.1 mM EDTA, pH 8.0 (for long term storage)

[_] Place back on the magnet, wait until the supernatant clears and transfer 20 µl of sample to fresh PCR tubes

1.12 Library Quantification and Sequencing

[_] Use a Bioanalyzer or TapeStation to determine the size distribution and concentration of the libraries

[_] Sequence using the preferred Illumina platform. 2 x 76 base or 2 x 100 base reads for standard sized libraries.

CheckList (Section 2)

2.1. DNA Preparation

2.1.1.Combine DNA and control DNA

Sequencing on MiSeq before NovaSeq, HiSeq or NextSeq (Check 2-4 M paired end reads per EM-seq Library)

[_] 10–200 ng sample DNA

[_] 1 µl of 0.1 ng/µl pUC19 control DNA • (lilac)

[_] 1 µl of 2 ng/µl unmethylated lambda DNA • (lilac)

[_] Add 0.1X TE pH 8.0 to 50 µl

For direct sequencing on NovaSeq, HiSeq or NextSeq

[_] 10–200 ng sample DNA

[_] 1 µl of pUC19 DNA • (lilac) diluted 100X to 0.001 ng/µl pUC19

[_] 1 µl of unmethylated lambda DNA • (lilac) diluted 100X to 0.02 ng/µl

[_] Add 0.1X TE pH 8.0 to 50 µl

2.1.2.Shear DNA

[_] Shear to 350–400 bp, use preferred instrument.

[_] Transfer the 50 µl of sheared material directly to a PCR strip tube to begin library construction.

2.2. End Prep of Sheared DNA

Add End Prep Reagents to sample (50 µl):

[_] 7 µl NEBNext Ultra II End Prep Reaction Buffer • (green)

[_] 3 µl NEBNext Ultra II End Prep Enzyme Mix • (green)

[_] Vortex or pipette mix 10 times with pipette, quick spin

[_] Incubate in thermal cycler

30 minutes 20°C

30 minutes 65°C

Hold at 4°C

2.3. Ligation of EM-seq Adaptor

Add Ligation Reagents to End Repaired DNA

[_] 2.5 µl NEBNext EM-seq adaptor • (red)

[_] 1 µl NEBNext Ligation Enhancer • (red)

[_] 30 µl NEBNext Ultra II Ligation Master Mix • (red)

[_] Vortex or pipette mix 10 times with pipette, quick spin

[_] Incubate in thermal cycler

15 minutes 20°C (heated lid off)

Hold at 4°C

2.4. Clean-Up of Adaptor Ligated DNA

[_] Vortex beads

[_] Add 110 µl of resuspended NEBNext Sample Purification Beads to each sample and mix by pipetting 10 times

[_] Incubate 5 min

[_] Place tubes on magnet for 5 min

[_] Remove and discard the supernatant, while keeping the sample on the magnet

[_] On magnet add 200 µl 80% ethanol, wait 30 seconds and then remove and discard the ethanol wash

[_] Repeat the 80% ethanol wash

[_] Airdry the beads for up to 2 min while on magnet

[_] Remove the samples from the magnet and resuspend in 29 µl of Elution Buffer ◦ (white)

[_] Place back on the magnet, wait until the supernatant clears and transfer 28 µl of sample to fresh PCR tubes

2.5. Oxidation of 5-Methylcytosines and 5-Hydroxymethylcytosines

[_] Reconstitute the TET2 Reaction Buffer Supplement • (yellow) using TET2 Reaction Buffer • (yellow)

24 Reaction kit: 1 tube TET2 Reaction Buffer Supplement • (yellow) & 100 µl TET2 Reaction Buffer • (yellow)

96 Reaction kit: 1 tube TET2 Reaction Buffer Supplement • (yellow) & 400 µl TET2 Reaction Buffer • (yellow)

Once reconstituted the Tet2 Reaction Buffer should be used within 4 months

[_] Add Oxidation Reagents to 28 µl Adaptor Ligated DNA

[_] 10 µl Reconstituted TET2 Reaction Buffer • (yellow)

[_] 1 µl Oxidation Supplement • (yellow)

[_] 1 µl DTT • (yellow)

[_] 1 µl Oxidation Enhancer • (yellow)

[_] 4 µl TET2 • (yellow)

[_] Mix by vortexing or pipette mix 10 times, centrifuge briefly

[_] Make diluted Fe(II) Solution.

Add 1 µl 500 mM Fe(II) Solution • (yellow) to 1,249 µl of water. Use immediately, do not store.

[_] Add 5 µl Diluted Fe(II) Solution to sample.

[_] Mix by vortexing or pipette mix 10 times, centrifuge briefly, then incubate in a thermal cycler

60 minutes 37°C

Hold at 4°C

[_] Add 1 µl Stop Reagent • (yellow)

[_] Mix by vortexing or pipette mix 10 times, centrifuge briefly

[_] Incubate in a thermal cycler

30 minutes 37°C

Hold at 4°C

2.6. Clean-Up of TET2 converted DNA

[_] Vortex beads

[_] Add 90 µl of resuspended NEBNext Sample Purification Beads to each sample and mix by pipetting 10 times

[_] Incubate 5 min

[_] Place tubes on magnet for 5 min

[_] Remove and discard the supernatant, while keeping the sample on the magnet

[_] On magnet add 200 µl 80% ethanol, wait 30 seconds and then remove and discard the ethanol wash

[_] Repeat the 80% ethanol wash

[_] Airdry the beads for up to 2 min while on magnet

[_] Remove the samples from the magnet and resuspend in 17 µl of Elution Buffer ∘ (white)

[_] Place back on the magnet, wait until the supernatant clears and transfer 16 µl of sample to fresh PCR tubes

2.7. Denaturation of DNA

Use either Formamide (A) or Sodium Hydroxide (B)

A. Formamide Denaturation (Recommended)

[_] Pre-heat thermal cycler to 85°C

[_] Add 4 µl Formamide to the 16 µl oxidized DNA

[_] Mix by vortexing or pipette mix 10 times, centrifuge briefly

[_] Incubate in a thermal cycler

85°C for 10 min

Immediately, place on ice

[_] Proceed immediately into Section 2.8

B. Sodium Hydroxide Denaturation (Optional: Formamide is preferred)

[_] Prepare freshly diluted 0.1 N NaOH

[_] Pre-heat thermal cycler to 50°C

[_] Add 4 µl 0.1 N NaOH to the 16 µl oxidized DNA

[_] Mix by vortexing or pipette mix 10 times, centrifuge briefly

[_] Incubate in a thermal cycler

50°C for 10 min

Immediately, place on ice

[_] Proceed immediately into Section 2.8

2.8. Deamination of Cytosines

Add Deamination Reagents to 20 µl denatured DNA on ice

[_] 68 µl Nuclease-free water

[_] 10 µl APOBEC Reaction Buffer • (orange)

[_] 1 µl BSA • (orange)

[_] 1 µl APOBEC • (orange)

[_] Mix by vortexing or pipette mix 10 times, centrifuge briefly

[_] Incubate in a thermal cycler

37°C for 3 hours

Hold at 4°C

2.9. Clean-Up of Deaminated DNA

[_] Vortex beads

[_] Add 100 µl of resuspended NEBNext Sample Purification Beads to each sample and mix by pipetting 10 times

[_] Incubate 5 min

[_] Place tubes on magnet for 5 min

[_] Remove and discard the supernatant, while keeping the sample on the magnet

[_] On magnet add 200 µl 80% ethanol, wait 30 seconds and then remove and discard the ethanol wash

[_] Repeat the 80% ethanol wash

[_] Airdry the beads for up to 90 sec while on magnet. Do not over-dry as beads become difficult to resuspend

[_] Remove the samples from the magnet and resuspend in 21 µl of Elution Buffer ◦ (white)

[_] Place back on the magnet, wait until the supernatant clears and transfer 20 µl of sample to fresh PCR tubes

2.10. PCR Amplification

Add Amplification Reagents to 20 µl deaminated DNA

[_] 5 µl NEBNext Unique Dual Index Primer Pairs

[_] 25 µl NEBNext Q5U Master Mix • (blue)

[_] Mix by vortexing or pipette mix 10 times, centrifuge briefly

[_] Amplify in thermal cycler

CYCLE STEP	TEMPERATURE	TIME	CYCLES
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	10 s	4–8*
Annealing	62°C	30 s	
Extension	65°C	60 s	
Final Extension	65°C	5 min	1
Hold	4	∞	

Cycle recommendation:

10 ng DNA: 8 cycles

50 ng DNA: 5–6 cycles

200 ng DNA: 4 cycles

2.11. Clean-Up of Amplified Libraries

Vortex beads

[_] Add 90 µl of water to each sample

[_] Add 91 µl of resuspended NEBNext Sample Purification Beads to each sample and mix by pipetting 10 times

[_] Incubate 5 min

[_] Place tubes on magnet for 5 min

[_] Remove and discard the supernatant, while keeping the sample on the magnet

[_] On magnet add 200 µl 80% ethanol, wait 30 seconds and then remove and discard the ethanol wash

[_] Repeat the 80% ethanol wash

[_] Airdry the beads for up to 2 min while on magnet

[_] Remove the samples from the magnet and resuspend in 21 µl of Elution Buffer ∘ (white) or 10 mM Tris, 0.1 mM EDTA, pH 8.0 (for long term storage)

[_] Place back on the magnet, wait until the supernatant clears and transfer 20 µl of sample to fresh PCR tubes

2.12. Library Quantification and Sequencing

[_] Use a Bioanalyzer or TapeStation to determine the size distribution and concentration of the libraries

[_] Sequence using the preferred Illumina platform. 2 x 100 base or 2 x 150 base reads for large sized libraries.

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

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