INSTRUCTION MANUAL

NEBNext® Enzymatic Methyl-seq v2 Kit

NEB E8015S/L

24/96 reactions

Version 1.3_4/26

Table of Contents

The Library Kit Includes

The volumes provided are sufficient for preparation of up to 24 reactions (NEB #E8015S) or 96 reactions (NEB #E8015L).
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
- (red) NEBNext Carrier DNA
- (white) Elution Buffer
- (yellow) TET2 Reaction Buffer
- (yellow) TET2 Reaction Buffer Supplement
- (yellow) UDP-Glucose
- (yellow) DTT
- (yellow) T4-BGT
- (yellow) T4-BGT Diluent
- (yellow) TET2
- (yellow) Fe(II) Solution
- (yellow) Stop Reagent
- (orange) APOBEC
- (orange) Deamination Reaction Buffer
- (orange) Recombinant Albumin
- (blue) NEBNext Q5U® Master Mix

NEBNext Sample Purification Beads

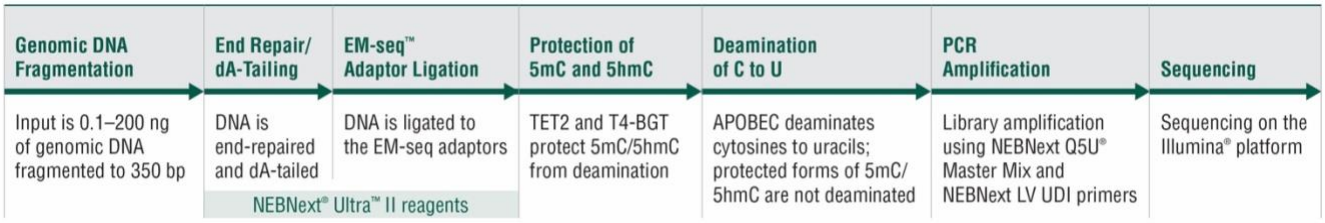
Required Materials Not Included

- 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 0.05 N NaOH. Formamide is preferred. If using NaOH, please see FAQ on NEB #E8015 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 #S1515)
- Metal cooling block, such as Diversified Biotech® (#CHAM-1000)
- PCR machine
- Agilent® TapeStation®, Bioanalyzer® or other fragment analyzer and associated consumables

Overview

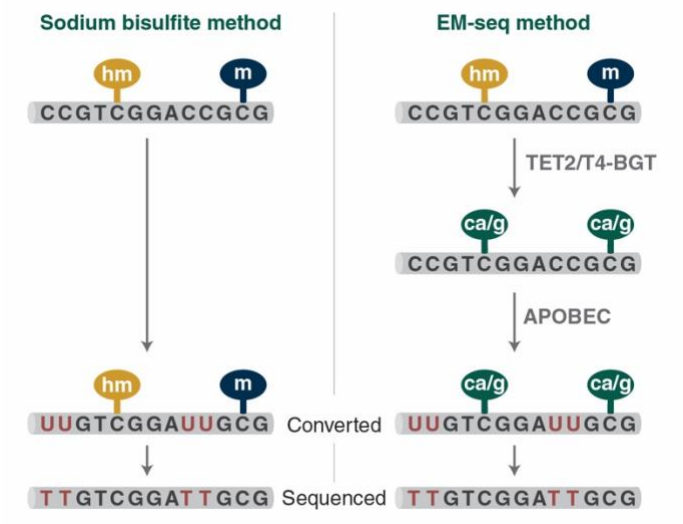
NEBNext Enzymatic Methyl-seq v2 (EM-seq v2, NEB #E8015) is an updated and streamlined version of the original NEBNext Enzymatic Methyl-seq (EM-seq, NEB #E7120) protocol for enzymatic conversion to identify 5mC and 5hmC at single base resolution. The EM-seq v2 kit contains the components needed to make Illumina-compatible libraries that are enzymatically modified to detect 5-methylcytosines (5mC) and 5-hydroxymethylcytosines (5hmC) when used in conjunction with any NEBNext LV Unique Dual Index Primer Sets (NEB #E3390, #E3392, #E3400, #E3402, #E3404, #E3406 and #E3408). The updated user-friendly v2 workflow enables 5mC and 5hmC detection using an expanded input range of 0.1–200 ng and has a faster library preparation time through reaction optimizations. The EM-seq v2 workflow does not differentiate between 5mC and 5hmC.

Figure 1. NEBNext Enzymatic Methyl-seq v2 Kit Workflow.



Overview of the EM-seq v2 workflow. First, a library is made by ligating the EM-seq adaptor to fragmented end repaired/dA-tailed 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. PCR primers are provided separately as NEBNext LV Unique Dual Index Primer Sets (NEB #E3900, #E3902, #E3400, #E3402, #E3404, #E3406 and #E3408).

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



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.

EM-seq is a two-step enzymatic conversion process to detect modified cytosines. The first step uses TET2 and T4-BGT 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 5gmC using T4-BGT. 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 bwa-meth and Bismark.

The workflow for the NEBNext Enzymatic Methyl-seq v2 Kit is user-friendly and enables methylation detection from inputs ranging between 0.1–200 ng. EM-seq-converted DNA is intact compared to bisulfite-converted DNA, resulting in libraries with longer insert sizes, reduced GC bias and more even genome coverage.

The NEBNext libraries for Illumina resemble TruSeq® libraries and the adaptor sequences can be trimmed similar to TruSeq:

Adaptor Read 1 AGATCGGAAGAGCACACGTCTGAACTCCAGTCA

Adaptor Read 2 AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

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 the Illumina sequencing platform.

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

Protocol for EM-seq v2 Library Construction

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 endpoint.

•

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

Starting Material: 0.1–200 ng double-stranded 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 v2 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
0.1 ng	1:1000
1 ng	1:250
10 ng	1:100
200 ng	1:50

The above dilutions are useful to perform a QC of conversion before deep sequencing using approximately 10 million paired-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 (0.1–200 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
Total Volume	**50 µl**

The sequences for pUC19 and lambda can also be found in our GitHub demo pipeline:
https://github.com/nebiolabs/EM-seq/blob/master/assets/methylation_controls.fa

1.1.2. **Fragmenting DNA:** The combined 50 µl sample DNA and control DNAs are fragmented to an average size of ~350 bp (420–620 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: 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
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 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 ≥ 75°C or on, and run the following program:
15 minutes at 20°C
15 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 (from 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: The 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 EM-seq 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°C
Hold at 4°C

Safe Stopping Point: Samples can be stored overnight at -20°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 Sample Purification Beads to resuspend.

1.4.2. Add 93 µl (1X ratio) of resuspended NEBNext Sample Purification Beads to each sample. Mix well by pipetting up and down at least 10 times. Be careful to expel all of the liquid out of the tip during the last mix.

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. Be sure to remove all visible liquid after the second wash using a p10 pipette tip.

1.4.8. Air dry the beads for 1–2 minutes while the tubes are on the magnetic stand with the lid open.

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

1.4.9. Elution Options A or B

Option 1.4.9A: For > 10 ng DNA input

1.4.9A.1 Remove the tubes from the magnetic stand. Elute the DNA targets from the beads by adding 29 µl of ○ (white) Elution Buffer.

1.4.9A.2 Mix well by pipetting up and down 10 times. Incubate for at least 1 minute at room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube before placing back on the magnetic stand.

1.4.9A.3 Place the tube on the magnetic stand. After 3 minutes (or whenever the solution is clear), transfer 28 µl of the supernatant to a new PCR tube.

Option 1.4.9B: For ≤ 10 ng DNA input

Note: Only to be added if Adaptor Ligation has occurred. Do not use with DNA that will not have adaptors ligated before conversion.

1.4.9B.1 Remove the tubes from the magnetic stand. Elute the DNA targets from the beads by adding 28 µl of ○ (white) Elution Buffer.

1.4.9B.2 Mix well by pipetting up and down 10 times. Incubate for at least 1 minute at room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube before placing back on the magnetic stand.

1.4.9B.3 Place the tube on the magnetic stand. After 3 minutes (or whenever the solution is clear), transfer 27 µl of the supernatant to a new PCR tube.

1.4.9B.4 Add 1 µl of the ● (red) NEBNext Carrier DNA to 27 µl of DNA from Step 1.4.9B.3.

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 #E8015S/#E8015G (24 reactions/G size) and Option B for #E8015L (96 reactions).

Note: The 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 (for the 24-reaction/G size kit). 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 (for the 96-reaction kit). Write date on tube.

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

1.5.2. Prepare Diluted ∘ (yellow) T4-BGT.

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

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

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

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

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

Diluted T4-BGT is used for samples ≤ 10 ng

COMPONENT	VOLUME
EM-seq adaptor ligated DNA (from Step 1.4.9A.3. or 1.4.9B.4.)	28 µl
∘ (yellow) TET2 Reaction Buffer (TET2 Reaction Buffer Supplement reconstituted in TET2 Reaction Buffer)	10 µl
∘ (yellow) UDP-Glucose	1 µl
∘ (yellow) DTT	1 µl
∘ (yellow) T4-BGT or Diluted T4-BGT	1 µl
∘ (yellow) TET2	4 µl
Total Volume	**45 µl**

Mix thoroughly by vortexing 1–2 seconds or by pipetting up and down at least 10 times and centrifuge briefly. For multiple reactions, a master mix of the reaction components can be prepared before addition to the sample DNA. 5mC/5hmC oxidation is initiated by the addition of the Fe(II) Solution to the reaction in the next step.

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

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

Combine diluted Fe(II) Solution and reaction mixture (from Step 1.5.3.) as described below:

COMPONENT	VOLUME
Reaction mixture (from Step 1.5.3.)	45 µl
Diluted Fe(II) Solution (from Step 1.5.4.)	5 µl
Total Volume	**50 µl**

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

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

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

COMPONENT	VOLUME
Protected DNA (Step 1.5.5.)	50 µl
∘ (yellow) Stop Reagent	1 µl
Total Volume	**51 µl**

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

1.5.7. Place in a thermal cycler with the heated lid set to ≥ 45°C or on, and run the following program:
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 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 50 µl (1X ratio) of resuspended NEBNext Sample Purification Beads to each sample. Mix well by pipetting up and down at least 10 times. Be careful to expel all of the liquid out of the tip during the last mix.

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 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.

1.6.8. Air dry the beads for 30 seconds–1 minute while the tubes are on the magnetic stand with the lid open.

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

1.6.9. Remove the tubes from the magnetic stand. Elute the DNA targets 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.

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

Note: All sample input ranges (0.1–200 ng) follow the same denaturation and deamination conditions

Denaturation Options A or B

The DNA can be denatured using either Formamide or 0.05 N Sodium Hydroxide. Use Option A for denaturing using Formamide and Option B for denaturing using 0.05 N Sodium hydroxide.

Option 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 protected DNA (from Step 1.6.11.). Mix thoroughly by vortexing 1–2 seconds or by pipetting up and down at least 10 times and centrifuge briefly.

1.7A.3. Incubate at 85°C for 10 minutes in the pre-heated thermal cycler.

1.7A.4. Immediately place in cooling block on ice and allow the sample to fully cool (~ 2 minutes) before proceeding to Section 1.8.

Option 1.7B: Sodium Hydroxide

Optional, See FAQ about preparing NaOH.

1.7B.1. Prepare freshly diluted 0.05 N NaOH.

1.7B.2. Pre-heat thermal cycler to 85°C with the heated lid set to ≥ 105°C or on.

1.7B.3. Add 4 µl 0.05 N NaOH to the 16 µl of protected DNA (from Step 1.6.11.). 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 85°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 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:

COMPONENT	VOLUME
Denatured DNA (from Step 1.7A.4. or 1.7B.5.)	20 µl
Nuclease-free water	14 µl
• (orange) Deamination Reaction Buffer	4 µl
• (orange) Recombinant Albumin	1 µl
• (orange) APOBEC	1 µl
Total volume	**40 µl**

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

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

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

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

Note: The samples move directly into PCR with no bead clean up.

1.9. PCR Amplification

1.9.1. On ice, add the following components to the deaminated DNA from Step 1.8.3.:

COMPONENT	VOLUME
Deaminated DNA (from Step 1.8.3.)	40 µl
UDI Primer Pair*	5 µl
• (blue) NEBNext Q5U Master Mix	45 µl
Total Volume	90 µl

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

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

1.9.3. Place the tube in a thermal cycler with the heated lid set to 105°C and perform PCR amplification using the following cycling conditions:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	4-14
Annealing	62°C	30 seconds	
Extension	65°C	60 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

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

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

1.10. Clean-Up of Amplified Libraries

Note: The ratios recommended for NEBNext Sample Purification Beads in this manual have been experimentally optimized for every step; this is critical since buffer compositions differ between steps and across protocols e.g., post ligation recommendations will not apply to samples post PCR. Please adhere to these guidelines and not those recommended by other sources or for other kits.

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

1.10.1. Vortex Sample Purification Beads to resuspend.

1.10.2. Add 72 µl (0.8X ratio) of resuspended NEBNext Sample Purification Beads to each sample. Mix well by pipetting up and down at least 10 times. Be careful to expel all of the liquid out of the tip during the last mix.

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

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

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

1.10.6. Add 200 µl of freshly prepared 80% ethanol to the tubes while on the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

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

1.10.8. Air dry the beads for 1–2 minutes while the tubes are on the magnetic stand with the lid open.

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

1.10.9. Remove the tubes from the magnetic stand. Elute the DNA targets from the beads by adding 21 µl of ° (white) Elution Buffer. Optional: For long-term storage of libraries, 21 µl of 1X TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) or Low TE (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA) can be used.

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

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

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

1.11. Library Quantification and Sequencing

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

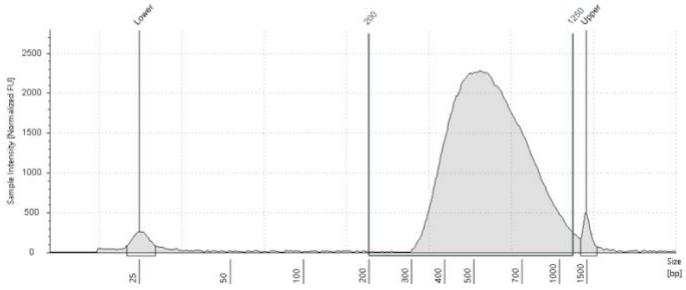


Figure 3. Representative TapeStation trace for an EM-seq v2 library prepared using 200 ng of NA12878 genomic DNA. The library was run on a HS D1000 tape.

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.

Kit Components

NEB #E8015S Table of Components

NEB #	PRODUCT	VOLUME
E7122AVIAL	Control DNA CpG methylated pUC19	0.024 ml
E7123AVIAL	Control DNA Unmethylated Lambda	0.024 ml
E7647AVIAL	NEBNext Ultra II End Prep Reaction Buffer	0.168 ml
E7646AVIAL	NEBNext Ultra II End Prep Enzyme Mix	0.072 ml
E7648AVIAL	NEBNext Ultra II Ligation Master Mix	0.72 ml
E7374AVIAL	NEBNext Ligation Enhancer	0.024 ml
E3351AVIAL	NEBNext Carrier DNA	0.024 ml
E7165AVIAL	NEBNext EM-seq Adaptor	0.06 ml
E3355AVIAL	NEBNext Sample Purification Beads	5.2 ml
E7124AVIAL	Elution Buffer	2.1 ml
E7126AVIAL	TET2 Reaction Buffer	0.3 ml
E8013AVIAL	TET2 Reaction Buffer Supplement (x 3)	Lyophilized
E3353AVIAL	UDP-Glucose	0.024 ml
E7139AVIAL	DTT	0.5 ml
E3354AVIAL	T4-BGT	0.024 ml
E8014AVIAL	T4-BGT Diluent	0.216 ml
E7130AVIAL	TET2	0.096 ml
E7131AVIAL	Fe(II) Solution	0.024 ml
E7132AVIAL	Stop Reagent	0.024 ml
E7133AVIAL	APOBEC	0.024 ml
E3356AVIAL	Deamination Reaction Buffer	0.096 ml
E3357AVIAL	Recombinant Albumin	0.024 ml
E3369AVIAL	NEBNext Q5U Master Mix	1.08 ml

NEB #E8015L Table of Components

NEB #	PRODUCT	VOLUME
E7122AAVIAL	Control DNA CpG methylated pUC19	0.096 ml
E7123AAVIAL	Control DNA Unmethylated Lambda	0.096 ml
E7647AAVIAL	NEBNext Ultra II End Prep Reaction Buffer	0.672 ml
E7646AAVIAL	NEBNext Ultra II End Prep Enzyme Mix	0.288 ml
E7648AAVIAL	NEBNext Ultra II Ligation Master Mix	2.88 ml
E7374AAVIAL	NEBNext Ligation Enhancer	0.096 ml
E3351AAVIAL	NEBNext Carrier DNA	0.096 ml
E7165AAVIAL	NEBNext EM-seq Adaptor	0.24 ml
E3355AAVIAL	NEBNext Sample Purification Beads	20.64 ml
E7124AAVIAL	Elution Buffer	8.6 ml
E7126AAVIAL	TET2 Reaction Buffer	1.2 ml
E8013AAVIAL	TET2 Reaction Buffer Supplement (x 3)	Lyophilized
E3353AAVIAL	UDP-Glucose	0.096 ml
E7139AAVIAL	DTT	0.5 ml
E3354AAVIAL	T4-BGT	0.096 ml
E8014AAVIAL	T4-BGT Diluent	0.864 ml
E7130AAVIAL	TET2	0.384 ml
E7131AAVIAL	Fe(II) Solution	0.096 ml
E7132AAVIAL	Stop Reagent	0.096 ml
E7133AAVIAL	APOBEC	0.096 ml
E3356AAVIAL	Deamination Reaction Buffer	0.384 ml
E3357AAVIAL	Recombinant Albumin	0.096 ml
E3369AAVIAL	NEBNext Q5U Master Mix	4.32 ml

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	11/24
1.1	Updated legal footer.	1/25
1.2	Updated legal footer.	9/25
1.3	Updated link for GitHub Demo Pipeline page 4	4/26