INSTRUCTION MANUAL

NEBNext® Enzymatic Methyl-seq v2 Conversion Module

NEB E8020S/L

24/96 reactions

Version 2.0_9/25

Table of Contents

The Conversion Module Includes

The volumes provided are sufficient for preparation of up to 24 reactions (NEB #E8020S) or 96 reactions (NEB #E8020L). All 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
- (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

Required Materials Not Included

- NEBNext UltraShear® (M7634) or Covaris® instrument and the required tubes or other fragmentation equipment
- PCR strip tubes or 96-well plates
- Cleanup beads: SPRIselect™ Reagent Kit (Beckman Coulter®, Inc. #B23317), AMPure® XP beads (Beckman Coulter, Inc. #A63881) or preferred bead manufacturer
- 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 associated with NEB #E8015.
- 80% Ethanol
- 10 mM Tris-HCl pH 7.5 or 8.0 or low TE (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA)
- 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

Overview

The Enzymatic Methyl-seq v2 Conversion Module (EM-seq v2 Conversion Module) contains all the components needed to enzymatically modify and enable detection of 5-methylcytosines (5mC) and 5-hydroxymethylcytosines (5hmC). The updated user-friendly workflow enables 5mC and 5hmC identification from 0.1–200 ng of input DNA. The EM-seq v2 workflow does not differentiate between 5mC and 5hmC.

Figure 1. Overview of Sodium Bisulfite Conversion and EM-seq Conversion.

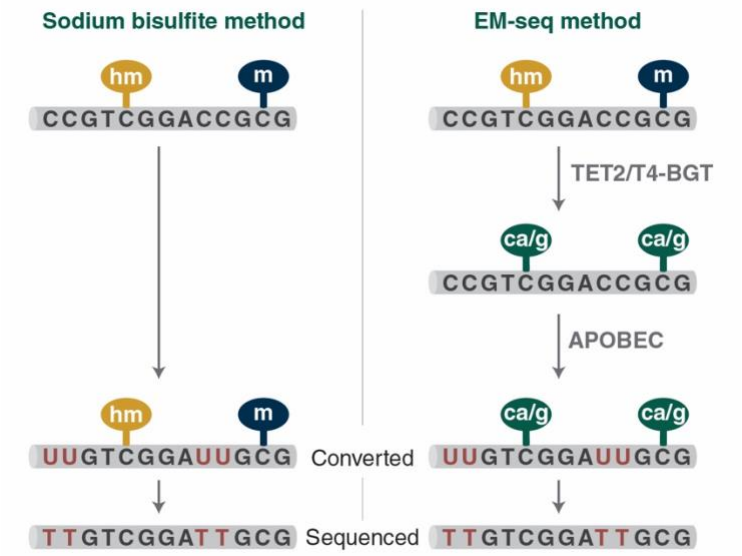

Figure 1 shows a comparison of the sodium bisulfite and EM-seq methods.

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.

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 the 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 described in the NEBNext Enzymatic Methyl-seq v2 Conversion Module 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 size, reduced GC bias and more even genome coverage.

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 Conversion Module

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: 0.1–200 ng double stranded DNA

1.1. DNA Preparation

1.1.1. Sample DNA and Control DNA

DNA going into the first enzymatic reaction (protection of 5mC and 5hmC) reaction needs to be fragmented as applicable for the downstream application.

For sequencing on an Illumina platform, refer to the Enzymatic Methyl-seq Kit v2 Manual (NEB #E8015) for usage recommendations. For other downstream applications and sequencing platforms, please refer to manufacturer's guidelines. If downstream application would not typically require fragmentation, fragment DNA to 2 kb or less, so that DNA can denature. This is necessary for the efficiency of the deamination step.

Addition of control DNA is recommended for next generation sequencing applications to assess protection and conversion efficiencies. The following table is a guide for the amount of • (lilac) Control DNA Unmethylated Lambda and • (lilac) Control DNA CpG methylated pUC19 to be added to samples prior to EM-seq library construction to evaluate conversion efficiencies.

Table 1.1 Dilutions of control DNAs for a range of genomic DNA inputs.

Sample DNA Input Amount	Control DNA Dilution Recommendations
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. The actual dilution factor at time of use will be lower.

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.

For example, samples going into Illumina library preparation can be enzymatically fragmented using NEBNext UltraShear (NEB #M7634) or mechanically sheared to an average size of ~ 350 bp. Control DNAs should be added prior to fragmentation to provide a means to qualify the final sequencing data as specified below. When using Covaris, samples must be fragmented in a buffer containing 10 mM Tris-HCl pH 7.5 or 8.0. If EDTA is present in the buffer or to adjust for volume going into the Protection Reaction, sheared samples must be cleaned up using beads (~2X bead ratio) or column to remove EDTA and eluted as specified below.

NOTE: When using Covaris, do not fragment input DNA in 0.1X TE (1 mM Tris-HCl pH 8.0, 0.1 mM EDTA) or water.

Table 1.2 An example setup for combining sample DNA and control DNAs for fragmentation using Covaris .

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**

Post fragmentation:

If fragmenting DNA on Covaris the sample will contain EDTA and a cleanup must be performed to bring the sample into 28 µl or less of nuclease free water. Perform a 2 x bead cleanup or column cleanup and elute in ≤ 28 µl depending on whether addition of the carrier DNA is required.

- Inputs > 10 ng:
  - DNA must be in 28 µl of water or 10 mM Tris-HCl pH 7.5 or 8.0 to go into the Protection Reaction.
- Inputs ≤ 10 ng:
  - DNA ligated to sequencing adaptor: Add 1 µl of • (red) NEBNext Carrier DNA to sample in 27 µl of water or 10 mM Tris-HCl pH 7.5 or 8.0 before going into the Protection Reaction**.**
  - DNA without sequencing adaptors ligated: DNA must be in 28 µl of water or 10 mM Tris-HCl pH 7.5 or 8.0 to go into Oxidation Reaction**.**

Note: Addition of the NEBNext Carrier DNA may not be appropriate for every application. For example, if sequencing, do not use with DNA that does not have adaptors ligated before conversion.

1.2. Protection of 5-Methylcytosines and 5-Hydroxymethylcytosines

1.2.1. Prepare TET2 Buffer. Use Option A if you have #E8020S/E8020G (24 reactions/G size) and Option B if you have #E8020L (96 reactions).

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

1.2.1A. 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.

1.2.1.B. 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.

1.2.2. ⚠ Prepare Diluted • (yellow) T4-BGT <u>for samples using ≤ 10 ng DNA input.</u>

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 any leftovers should be discarded after use.

1.2.3. On ice, add the following components directly to fragmented DNA:

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

Diluted T4-BGT is used for samples ≤ 10 ng

COMPONENT	VOLUME
Fragmented DNA (from Step 1.1.1.)	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.

1.2.4. 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 1.2.3.) as described below:

COMPONENT	VOLUME
Reaction mixture (from Step 1.2.3.)	45 µl
Diluted Fe(II) Solution (from Step 1.2.4.)	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.

1.2.5. 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

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

COMPONENT	VOLUME
Protected DNA (Step 1.2.5.)	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.

1.2.7. 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.

1.3. Clean-up of Protected DNA

Note: The ratios recommended for SPRIselect/AMPure XP 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.

1.3.1. Vortex Sample Purification Beads to resuspend.

1.3.2. Add 50 µl (1X ratio) of resuspended SPRIselect/AMPure XP 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.

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

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

1.3.5. 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)**.

1.3.6. 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.

1.3.7. 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.

1.3.8. 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.

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

1.3.10. 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.

1.3.11. 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.

1.4. 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 1.4A: Formamide (Recommended)

1.4A.1. Pre-heat thermal cycler to 85°C with the heated lid set to ≥ 105°C or on.

1.4A.2. Add 4 µl Formamide to the 16 µl of protected DNA (from Step 1.3.11.). Mix thoroughly by vortexing 1–2 seconds or by pipetting up and down at least 10 times and centrifuge briefly.

1.4A.3. Incubate at 85°C for 10 minutes in the pre-heated thermal cycler.

1.4A.4. Immediately place on ice and allow the sample to fully cool (~2 minutes) before proceeding to Section 1.5.

Option 1.4B: Sodium Hydroxide

Optional, See FAQ about preparing NaOH.

1.4B.1. Prepare freshly diluted 0.05 N NaOH.

1.4B.2. Pre-heat thermal cycler to 85°C with the heated lid set to ≥ 105°C or on.

1.4B.3. Add 4 µl 0.05 N NaOH to the 16 µl of protected DNA (from Step 1.3.11.). Mix thoroughly by vortexing 1–2 seconds or by pipetting up and down at least 10 times and centrifuge briefly.

1.4B.4. Incubate at 85°C for 10 minutes in the pre-heated thermal cycler.

1.4B.5. Immediately place on ice and allow the sample to fully cool (~2 minutes) before proceeding to Section 1.5.

1.5. Deamination of Cytosines

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

COMPONENT	VOLUME
Denatured DNA (from Step 1.4A.4. or 1.4B.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.

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

1.5.3. 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

1.6.Clean-up of Deaminated DNA

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

Caution: The Sample Purification Beads behave differently during the APOBEC cleanup. After the bead washes, do not over dry the beads as they become very difficult to resuspend.

Note: Depending on the downstream application, you can skip the cleanup.

1.6.1. Vortex Sample Purification Beads to resuspend.

1.6.2. Add 40 µl (1X ratio) of resuspended SPRIselect/AMPure XP 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 21 µl of ∘ (white) Elution Buffer or appropriate buffer for downstream application.

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 20 µl of the supernatant to a new PCR tube.

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
E3351AVIAL	NEBNext Carrier DNA	0.024 ml
E7124AVIAL	Elution Buffer	2.1 ml
E7126AVIAL	TET2 Reaction Buffer	0.3 ml
E8013AVIAL	TET2 Reaction Buffer Supplement (x3)	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

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
E3351AAVIAL	NEBNext Carrier DNA	0.096 ml
E7124AAVIAL	Elution Buffer	8.6 ml
E7126AAVIAL	TET2 Reaction Buffer	1.2 ml
E8013AAVIAL	TET2 Reaction Buffer Supplement (x3)	Lyophilized
E3353AAVIAL	UDP-Glucose	0.096 ml
E7139AAVIAL	DTT	0.500 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

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	11/24
1.1	Updated legal footer	1/25
2.0	Updated fragmentation instructions for non NEBNext Applications	9/25