NEBNext® Enzymatic Methyl-seq Conversion Module

NEB #E7125S/L

24/96 reactions

Version 4.0_5/23

Table of Contents

The Conversion Module Includes

The volumes provided are sufficient for preparation of up to 24 reactions (NEB #E7125S) and 96 reactions (NEB #E7125L). 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
- (white) Elution Buffer
- (yellow) TET2 Reaction Buffer
- (yellow) TET2 Reaction Buffer Supplement
- (yellow) Oxidation Supplement
- (yellow) DTT
- (yellow) Oxidation Enhancer
- (yellow) TET2
- (yellow) Fe(II) Solution
- (yellow) Stop Reagent
- (orange) APOBEC
- (orange) APOBEC Reaction Buffer
- (orange) BSA

Required Materials Not Included

- 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
- 0.1X TE, pH 8.0
- Nuclease-free Water
- Clean-up Beads: NEBNext Sample Purification Beads (supplied with Enzymatic Methyl-seq Kit, NEB #E7120), SPRIselect Reagent Kit (Beckman Coulter, Inc. #B23317), AMPure® XP Beads (Beckman Coulter, Inc. #A63881) or preferred bead manufacturer.
- Magnetic rack/stand, such as NEBNext Magnetic Separation Rack (NEB #S1515S)
- PCR machine
- Bioanalyzer®, TapeStation® and associated consumables or other fragment analyzer

Overview

The Enzymatic Methyl-seq Conversion Module (EM-seq) contains the components needed to enzymatically modify and enable detection of 5-methylcytosines (5mC) and 5-hydroxymethylcytosines (5hmC).

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

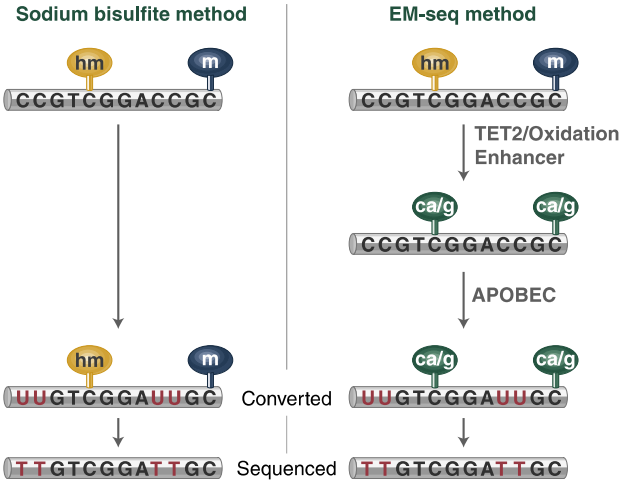

Figure 1 shows a comparison of the sodium bisulfite and EM-seq conversion methods. Sodium bisulfite treatment of DNA results in the deamination of cytosines into uracils, however the modified forms of cytosine (5mC and 5hmC) are not deaminated. Therefore, the preference of bisulfite to chemically deaminate 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.

The Enzymatic Methyl-seq Conversion Module 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 cytosine but does not convert 5caC and 5ghmC.

The workflow described in the NEBNext Enzymatic Methyl-seq Conversion Module 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.

Each module component must pass rigorous quality control standards, and for each new lot the entire set of reagents is functionally validated together with Ultra II DNA Library Prep Kit (NEB #E7645), Multiplex Oligos for Enzymatic Methyl-seq (NEB #E7140), and Q5U™ Master Mix (NEB #M0597) to construct EM-seq indexed libraries and sequence on an 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 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: 10 ng–200 ng fragmented double stranded DNA

1. DNA Preparation

1.1. DNA and Control DNA

DNA should not contain any EDTA moving into the Oxidation Reaction and should be in 28 µl of water or 10 mM Tris pH 8.0. Control DNAs for assessing oxidation and deamination are included with this module and their use is dependent on the users requirements. For sequencing on an Illumina platform, refer to the Enzymatic Methyl-seq Kit Manual (NEB #E7120) for usage recommendations. For other downstream applications and sequencing platforms, please refer to manufacturer's guidelines.

2. Oxidation of 5-Methylcytosines and 5-Hydroxymethylcytosines

2.1. Prepare TET2 Buffer. Use option A if you have E7125S/E7125G (24 reactions/G size) and option B if you have E7125L (96 reactions).

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

2.1A. Add 100 µl of TET2 Reaction Buffer to one tube of TET2 Reaction Buffer Supplement and mix well. Write date on tube.

2.1B. Add 400 µl of TET2 Reaction Buffer to one tube of 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.

2.2. On ice, add the following components directly to the 28 µl DNA (from Step 1.1).

COMPONENT	VOLUME
• (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

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.

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

NOTE: Use the solution immediately, do not store it. Discard after use.

Combine diluted Fe(II) Solution and EM-seq DNA with Oxidation Enzymes (from Step 2.2).

COMPONENT	VOLUME
DNA (from Step 2.2)	45 µl
Diluted Fe(II) Solution (from Step 2.3)	5 µl
Total Volume	**50 µl**

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

2.4. Incubate at 37°C for 1 hour in a thermal cycler with the heated lid set to ≥ 45°C or on.

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

COMPONENT	VOLUME
⚬ (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.

2.6. Incubate at 37°C for 30 minutes then at 4°C in a thermal cycler with the heated lid set to ≥ 45°C or on.

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

3. Clean-Up of TET2 Converted DNA

3.1. Vortex Sample Purification Beads to resuspend. SPRIselect or AMPure XP Beads can be used as well. If using AMPure XP Beads, allow the beads to warm to room temperature for at least 30 minutes before use.

3.2. Add 90 µl of resuspended NEBNext Sample Purification Beads to each sample. Mix well by pipetting up and down at least 10 times. Be careful to expel all of the liquid out of the tip during the last mix.

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

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

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)**.

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

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

3.8. Air dry the beads for up to 2 minutes while the tubes are on the magnetic stand with the lid open.

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

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

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.

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.

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

4. 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.

4A: Formamide (Recommended)

4A.1. Pre-heat thermal cycler to 85°C.

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

4A.3. Incubate at 85°C for 10 minutes in the pre-heated thermal cycler with the heated lid on.

4A.4. Immediately place on ice.

4A.5. Proceed immediately to Section 5.

4B: Sodium Hydroxide (Optional, See FAQ about preparing NaOH)

4B.1. Prepare freshly diluted 0.1 N NaOH.

4B.2. Pre-heat thermal cycler to 50°C with the heated lid set to ≥ 60°C or on.

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

4B.4. Incubate at 50°C for 10 minutes in the pre-heated thermal cycler with the heated lid set to ≥ 60°C or on.

4B.5. Immediately place on ice.

4B.6. Proceed immediately to Section 5.

5. Deamination of Cytosines

5.1. On ice, add the following components to the 20 µl of denatured DNA.

COMPONENT	VOLUME
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.

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

5.3. Incubate at 37°C for 3 hours then at 4°C in a thermal cycler with the heated lid set to ≥ 45°C or on.

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

6. 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.

6.1. Vortex Sample Purification Beads to resuspend. SPRIselect or AMPure XP Beads can be used as well. If using AMPure XP Beads, allow the beads to warm to room temperature for at least 30 minutes before use.

6.2. Add 100 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.

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

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

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)**.

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.

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.

6.8. 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.

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

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.

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.

Note: Please see NEB #E7120 for the PCR amplification protocol for downstream sequencing on an Illumina platform.

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

Kit Components

NEB #E7125S Table of Components

NEB #	PRODUCT	VOLUME
E7122A	Control DNA CpG methylated pUC19	0.024 ml
E7123A	Control DNA Unmethylated Lambda	0.024 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

NEB #E7125L Table of Components

NEB #	PRODUCT	VOLUME
E7122AA	Control DNA CpG methylated pUC19	0.096 ml
E7123AA	Control DNA Unmethylated Lambda	0.096 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

Checklist (EM-seq Conversion)

1. DNA Preparation

[_] DNA and Control DNA

DNA should not contain any EDTA moving into the Oxidation Reaction and should be in a 28 µl final volume.

2. Oxidation of 5-Methylcytosines and 5-Hydroxymethylcytosines

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

24 reaction kit/G size: 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

3. 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

4. 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 5

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 5

5. 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

6. 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 the 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

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	3/19
2.0		5/19
3.0	Add DTT to module and update protocol to include DTT	2/20
3.1	Step 2.2 updated component description. Updated Protocol	3/20
4.0	Updated footnote, legal disclaimer and formatting of tables	5/23

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