INSTRUCTION MANUAL

NEBNext® Enzymatic 5hmC-seq Conversion Module

NEB E3365S/L

24/96 reactions

Version 1.1_2/24

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The Conversion Module Includes

The volumes provided are sufficient for preparation of up to 24 reactions (NEB #E3365S) or 96 reactions (NEB #E3365L). 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 Unmethylated Lambda
- (lilac) Control DNA 5hmC T4
- (red) NEBNext Carrier DNA
- (yellow) NEBNext Glucosylation Reaction Buffer
- (yellow) UDP-Glucose
- (yellow) T4-BGT
- (yellow) Stop Reagent
- (white) Elution Buffer
- (orange) APOBEC
- (orange) Deamination Reaction Buffer
- (orange) Recombinant Albumin

Required Materials Not Included

- If DNA requires fragmentation: NEBNext UltraShear™ (NEB #M7634) or Covaris® instrument and the required tubes or other fragmentation equipment
- PCR strip tubes or 96-well plates
- Clean-up beads: SPRIselect™ Reagent Kit (Beckman Coulter®, Inc. #B23317), AMPure® XP beads (Beckman Coulter, Inc. #A63881) or preferred bead manufacturer.
- Formamide (Sigma #F9037-100 ml), Hi-Di™ Formamide (Thermo Fisher Scientific® #4401457) or 0.1 N NaOH. Formamide is preferred. If using NaOH, please see FAQ on NEB #E3365 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® Bioanalyzer®, TapeStation® or other fragment analyzer and associated consumables

Overview

The NEBNext Enzymatic 5hmC-seq Conversion Module contains the reagents required to enzymatically modify and enable specific detection of 5-hydroxymethylcytosine (5hmC). The user-friendly workflow enables 5hmC identification from 0.1 – 200 ng of input DNA.

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 that are sequenced on an Illumina® sequencing platform.

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

Figure 1. Overview of the E5hmC-seq conversion method

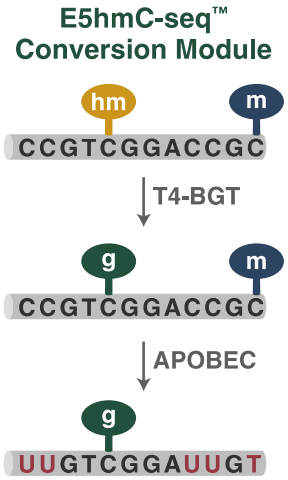

Figure 1 shows the two-step enzymatic conversion process used to detect 5hmC. The first step uses T4-BGT to glucosylate 5hmC. This protects 5hmC but not 5mC or cytosines from deamination by APOBEC. Note that the final DNA obtained from the NEBNext Enzymatic 5hmC-seq Conversion Module will contain U and glucosylated 5hmC.

Protocol for E5hmC-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: 0.1–200 ng fragmented double stranded DNA

1.1. DNA Preparation

1.1.1. DNA and Control DNA

DNA for the Glucosylation Reaction needs to be fragmented as applicable for the downstream application.

For sequencing on an Illumina platform, refer to the Enzymatic 5hmC-seq Kit Manual (NEB #E3350) for usage recommendations. For other downstream applications and sequencing platforms, please refer to manufacturer's guidelines.

The following table is a guide for the amount of • (lilac) Control DNA Unmethylated Lambda and • (lilac) Control DNA 5hmC T4 to be added to samples going into E5hmC-seq conversion. Expected read numbers along with read length should be considered to ensure that enough controls are included for the user's individual sequencing goals or other downstream applications.

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) for example on a MiSeq®, prior to deep sequencing (approx. 100–150 million paired reads) on NovaSeq®, HiSeq® or NextSeq®.

DILUTION OF • (LILAC) UNMETHYLATED LAMBDA CONTROL AND • (LILAC) 5hmC T4 CONTROL		
DNA Input Amount	**Pre-sequencing** 2–4 Million Paired Reads	**Deep Sequencing** 100–150 Million Paired Reads
0.1 ng	1:500	1:1000
1 ng	1:80	1:250
10 ng	1:20	1:100
200 ng	No Dilution	1:50

For example, samples going into Illumina library preparation can be mechanically or enzymatically fragmented using NEBNext UltraShear (NEB #M7634) 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. Samples can be fragmented in 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. If the volume needs to be adjusted going into the Glucosylation Reaction, fragmented samples can be cleaned up using beads (~2X bead ratio) or column and eluted as specified below.

We do not recommend fragmenting 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

COMPONENT	VOLUME
Sample DNA	48 µl
• (lilac) Control DNA Unmethylated Lambda (see Table 1.1)	1 µl
• (lilac) Control DNA 5hmC T4 (see Table 1.1)	1 µl
Total Volume	**50 µl**

Note: While DNA and controls must be fragmented in a buffer containing 10 mM Tris-HCl (see above), the Glucosylation Reaction itself in Step 1.2. is compatible with samples in water, 1X TE, 0.1X TE, low TE, or 10 mM Tris pH 7.5 or 8.0.

Note: The volume going into conversion is 28 µl.

Regardless of sequencing depth, a minimum of 5,000 paired end reads with a read length of 76 bases for • (lilac) Control DNA Unmethylated Lambda and 15,000 paired end reads with a read length of 76 bases for • (lilac) Control DNA 5hmC T4 are needed to give enough coverage 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 libraries may only need 2 million paired end reads whereas others may require 50 million paired end reads or even 1 billion 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 for 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.

Post fragmentation:

- **Inputs > 10 ng:** DNA must be in 28 µl of water, 1X TE, 0.1X TE, low TE, or 10 mM Tris pH 7.5 or 8.0 to go into the Glucosylation Reaction.
- **Inputs ≤ 10 ng:** For adaptor ligated DNA add 1 µl of • (red) NEBNext Carrier DNA to sample in 27 µl of water, 1X TE, 0.1X TE, low TE, or 10 mM Tris pH 7.5 or 8.0 to go into the Glucosylation Reaction.
- **Inputs ≤ 10 ng:** For DNA with no adaptors ligated, DNA must be in 28 µl of water, 1X TE, 0.1X TE, low TE, or 10 mM Tris pH 7.5 or 8.0 to go into the Glucosylation Reaction.

Note: For companion EM-seq product (NEB #E7120/E7125), there should be no EDTA in the sample going into conversion.

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. Glucosylation of 5-Hydroxymethylcytosines (5hmC)

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

COMPONENT	VOLUME
Fragmented DNA (from Step 1.1.1.)	28 µl
Nuclease-free water	15 µl
○ (yellow) NEBNext Glucosylation Reaction Buffer	5 µl
○ (yellow) UDP-Glucose	1 µl
○ (yellow) T4-BGT	1 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. For multiple reactions, a master mix of the reaction components can be prepared before addition to the sample DNA.

1.2.2. 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

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

COMPONENT	VOLUME
Glucosylated DNA (from Step 1.2.1.)	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.

1.2.4 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.

1.3. Clean-up of Glucosylated DNA

The ratios recommended for SPRIselect 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 SPRIselect beads to resuspend.

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

Denaturation Options A or B

The DNA can be denatured using either Formamide or 0.1 N Sodium Hydroxide. Use Option A for denaturing with Formamide and Option B for denaturing with 0.1 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 glucosylated 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 in cooling block 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 0.1 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 0.1 N NaOH to the 16 µl of glucosylated 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 in cooling block on ice and allow the sample to fully cool (~2 minutes) before proceeding to Section 1.5.

1.5. Deamination of 5mC and Cytosines

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

COMPONENT	VOLUME
Denatured DNA (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, 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.

1.6. Clean-up of Deaminated DNA

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

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

1.6.1. Vortex SPRIselect beads to resuspend.

1.6.2. Add 40 µl (1X ratio) of resuspended SPRIselect 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 an 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.

Checklist

1.1 DNA Preparation

1.1.1. Combine DNA and control DNA

See Table 1.1 for recommended control dilutions

[_] Fragment 0.1 ng – 200 ng sample DNA with ● (lilac) Control DNA Unmethylated Lambda and ● (lilac) Control DNA 5hmC T4

[_] 1 µl of ● (red) NEBNext Carrier DNA for inputs ≤ 10 ng (**application dependent; do not include if adaptors have not been ligated**)

1.2. Glucosylation of 5-Hydroxymethylcytosines

Add Glucosylation Reagents to 28 µl DNA

[_] 15 µl Nuclease-free water

[_] 5 µl ○ (yellow) NEBNext Glucosylation Reaction Buffer

[_] 1 µl ○ (yellow) UDP-Glucose

[_] 1 µl ○ (yellow) T4-BGT

[_] Vortex 1–2 seconds or pipette mix 10 times, centrifuge briefly

[_] Incubate in a thermal cycler (heated lid ≥ 45°C or on)

60 minutes at 37°C

Hold at 4°C

[_] Add 1 µl ○ (yellow) Stop Reagent

[_] Vortex 1–2 seconds or pipette mix 10 times, centrifuge briefly

[_] Incubate in a thermal cycler (heated lid ≥ 45°C or on)

30 minutes at 37°C

Hold at 4°C

1.3. Clean-up of Glucosylated DNA

[_] Vortex SPRIselect beads

[_] Add 50 µl of resuspended beads to each sample and mix by pipetting 10 times

[_] Incubate 5 minutes

[_] Place tubes on magnet for 5 minutes

[_] 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 ethanol wash

[_] Air-dry the beads for 30 seconds – 1 minute while on magnet

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

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

1.4. Denaturation of DNA

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

A. Formamide Denaturation (Recommended)

[_] Pre-heat thermal cycler to 85°C (heated lid ≥ 105°C or on)

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

[_] Vortex 1 – 2 seconds or pipette mix 10 times, centrifuge briefly

[_] Incubate in pre-heated thermal cycler

85°C for 10 minutes

Immediately, place in cooling block on ice until fully cooled (~2 minutes)

[_] Proceed immediately into Section 1.5.

B. Sodium Hydroxide Denaturation (Formamide is preferred)

[_] Prepare freshly diluted 0.1 N NaOH

[_] Pre-heat thermal cycler to 85°C (heated lid ≥ 105°C or on)

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

[_] Vortex 1–2 seconds or pipette mix 10 times, centrifuge briefly

[_] Incubate in pre-heated thermal cycler

85°C for 10 minutes

Immediately, place in cooling block on ice until fully cooled (~2 minutes)

[_] Proceed immediately into Section 1.5.

1.5. Deamination of 5mC and Cytosines

Add Deamination Reagents to 20 µl denatured DNA on ice

[_] 14 µl Nuclease-free water

[_] 4 µl ● (orange) Deamination Reaction Buffer

[_] 1 µl ● (orange) Recombinant Albumin

[_] 1 µl ● (orange) APOBEC

[_] Vortex 1–2 seconds or pipette mix 10 times, centrifuge briefly

[_] Incubate in a thermal cycler (heated lid ≥ 45°C or on)

3 hours at 37°C

Hold at 4°C

1.6. Clean-up of Deaminated DNA

[_] Vortex SPRIselect beads

[_] Add 40 µl of resuspended beads to each sample and mix by pipetting 10 times

[_] Incubate 5 min

[_] Place tubes on magnet for 5 minutes

[_] 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 ethanol wash

[_] Air-dry the beads for 30 seconds – 1 minute while on magnet

[_] Remove the samples from the magnet and resuspend in 21 µl of ○ (white) Elution Buffer or appropriate buffer for downstream application.

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

Kit Components

NEB #E3365S Table of Components

NEB #	PRODUCT	VOLUME
E7123A	Control DNA Unmethylated Lambda	0.024 ml
E3349A	Control DNA 5hmC T4	0.024 ml
E3351A	NEBNext Carrier DNA	0.024 ml
E3352A	NEBNext Glucosylation Reaction Buffer	0.12 ml
E3353A	UDP-Glucose	0.024 ml
E3354A	T4-BGT	0.024 ml
E7132A	Stop Reagent	0.024 ml
E7124A	Elution Buffer	2.1 ml
E7133A	APOBEC	0.024 ml
E3356A	Deamination Reaction Buffer	0.096 ml
E3357A	Recombinant Albumin	0.024 ml

NEB #E3365L Table of Components

NEB #	PRODUCT	VOLUME
E7123AA	Control DNA Unmethylated Lambda	0.096 ml
E3349AA	Control DNA 5hmC T4	0.096 ml
E3351AA	NEBNext Carrier DNA	0.096 ml
E3352AA	NEBNext Glucosylation Reaction Buffer	0.480 ml
E3353AA	UDP-Glucose	0.096 ml
E3354AA	T4-BGT	0.096 ml
E7132AA	Stop Reagent	0.096 ml
E7124AA	Elution Buffer	8.6 ml
E7133AA	APOBEC	0.096 ml
E3356AA	Deamination Reaction buffer	0.384 ml
E3357AA	Recombinant Albumin	0.096 ml

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	12/23
1.1	Clarified post fragmentation verbiage in Section 1.1. Updated header and footer.	2/24