Twist EM-Seq v2 Methylation Detection System Library Preparation Protocol

For use with the Twist NGS Methylation Detection System

Factors beyond the genomic DNA sequence, including the methylation of adenines and cytosines, influence cellular functions and health. These epigenetic modifications regulate gene expression in a cell type-specific manner. Cytosine methylation is found in common genomic sequences called CpG sites, and methylation has been linked to several kinds of cancers and genetic disorders. Being able to accurately detect methylation patterns is not only important for understanding many biological processes in health and disease, but also for diagnostic applications.

Twist Bioscience and New England Biolabs (NEB) have partnered to revolutionize methylome analysis. The NEBNext® EM-seq v2 kit for Twist Methylation is a tool for identifying CpG sites without the use of damaging chemical conversion processes. Instead, EM-seq uses a two-step enzymatic conversion process that is less damaging to the DNA, resulting in high-quality libraries that can be sequenced to identify 5mC and 5hmC sites. Coupled with Twist Methylation Panels and Target Enrichment Kits, the end-to-end system provides unparalleled sequencing efficiency through state-of-the-art enzymology, targeted panel design algorithms, and optimized workflows.

This protocol describes library generation and should only be performed with reagents specified or their equivalents. For the following hybrid capture step, Twist Bioscience offers Methylation Target Enrichment workflows that combine these libraries with custom targeted methylation panels to sequence custom regions of interest. For that protocol, please visit the Twist Bioscience resource library on twistbioscience.com.

Twist NGS Workflow. The complete NGS workflow takes you from sample preparation to NGS sequencing and data analysis.

For Research Use Only. *Not intended for use in diagnostic procedures.*

PROTOCOL COMPONENTS

Please read the product packaging and storage recommendations carefully for each kit and store components as recommended immediately upon arrival.

CATALOG #	NAME	DESCRIPTION	STORAGE
NEBNEXT EM-SEQ V2 LIBRARY PREPARATION (FOR TWIST NGS METHYLATION DETECTION SYSTEM)			
129335	NEBNext(R) EM-seq v2 kit for Twist Methylation	· Control DNA CpG methylated pUC19 · Control DNA unmethylated Lambda · Ultra II End Prep Reaction Buffer · Ultra II End Prep Enzyme Mix · Ultra II Ligation Master Mix · Ligation Enhancer · TET2 Reaction Buffer · TET2 Reaction Buffer Supplement · DTT · TET2 · Fe (II) Solution · Stop Reagent · APOBEC · NEBNext® Q5U® Master Mix · Elution Buffer · UDP-Glucose · T4-BGT · T4-BGT Diluent · Deamination Reaction Buffer · Recombinant Albumin · NEBNext® Carrier DNA · NEBNext® EM-seq Adaptor	–25 to –15°C
	Twist Total Purification Beads, EM-seq v2	Twist Total Purification Beads	2 to 8°C
TWIST ADAPTERS (ORDERED SEPARATELY)			
127377, 127378, 127379, 127380: 96 rxn	NEBNext® LV Unique Dual Index Primers (Plate A, B, C, and D)	UDI Primers, provide unique dual-indexed combinations with 1 reaction per index pair	–25 to –15°C

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This product is for research use only. This product is not intended for the diagnosis, prevention, or treatment of a disease or condition. Twist Bioscience assumes no liability regarding use of the product for applications in which it is not intended.

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MATERIALS SUPPLIED BY USER

The following materials or their equivalent are required to generate libraries using the NEBNext® EM-seq v2 kit for Twist Methylation.

PRODUCT	SUGGESTED SUPPLIER
REAGENTS AND CONSUMABLES	
1 N NaOH	—
Ethanol (200 proof)	—
Molecular biology grade water	—
10 mM Tris-HCl pH 8 (optional)	—
Buffer EB (optional)	Qiagen
1.5-ml microcentrifuge tubes	VWR
5-ml Eppendorf tubes	Eppendorf
Thin-walled PCR 0.2-ml strip-tubes	Eppendorf
96-well thermal cycling plates	VWR
Qubit dsDNA Broad Range Quantification Assay	Thermo Fisher Scientific
Agilent D5000 ScreenTape Assay	Agilent Technologies
EQUIPMENT	
Pipettes and tips	—
Vortex mixer	—
96-well compatible magnetic plate	Alpaqua, Permagen Labware
1.5-ml compatible magnetic stand	Beckman Coulter
Benchtop mini centrifuge for 0.2-ml tubes	—
Thermomixer (preferably) or heat block	Eppendorf
Thermal cycler (96-well) with heated lid	—
Fluorometer (Qubit 4.0)	Thermo Fisher Scientific
4200 TapeStation	Agilent Technologies
Focused-ultrasonicator platform	Covaris or similar

GENERAL NOTES AND PRECAUTIONS

Wear appropriate protective equipment (lab coat, gloves, and protective glasses or goggles) at all times when performing this protocol.

For best results, read this document before performing the protocol and follow the instructions provided. Twist cannot guarantee the performance of the Twist EM-seq v2 Methylation Detection System Library Preparation if modifications are made to the protocol.

This library preparation method may yield more material than needed for target enrichment. Excess product can be stored at –20°C for later use.

Do NOT mix or combine the same reagents from different lots.

Test the compatibility of your thermal cycler and PCR tubes by incubating them at 95°C for up to 5 minutes to ensure the PCR tubes do not crack under heat and pressure. Adjust the tightness of the thermal cycler lid and/or use a spacer specific to the thermal cycler model.

This protocol details different methods for mixing reagents (gentle pipetting, flicking or tapping, vortexing), depending on the volume, vessel, and reagents involved.

FOR TECHNICAL SUPPORT, CONTACT CUSTOMERSUPPORT@TWISTBIOSCIENCE.COM.

PROTOCOL OVERVIEW

This protocol converts methylated genomic DNA into double-stranded, adapter-ligated DNA libraries that are ready for hybrid capture. It uses a two-step enzymatic conversion process to distinguish between unmethylated and methylated cytosines: TET2 oxidizes 5-methylcytosine (5mC) and 5-hydromethylcytosine (5hmC) sites in the first step, protecting them from enzymatic deamination by APOBEC in the second step. Because unmethylated cytosines are converted to thymines, sequenced cytosines in the resulting library represent 5mC or 5hmC sites. This workflow uses mechanical fragmentation and the NEBNext EM-seq adapter and barcoded primer system. This protocol allows you to generate libraries ready for methylation detection (Steps 1–7) in approximately 10 hours. If planning to perform target enrichment after library preparation, please see Appendix A.

STEP	NEB NEXT ENZYMATIC METHYL-SEQ LIBRARY PREP (AMPLIFIED, INDEXED LIBRARIES)	TIME
1	**Prepare and shear genomic DNA** Diluted and sheared experimental samples	**0.5 hour**
2	**Perform end prep on sheared DNA** dA-Tailed DNA fragments	**1 hour**
3	**Ligate EM-seq adapters** DNA libraries with Universal Adapters	**1 hour**
4	**Oxidize 5mC and 5hmC sites** Oxidized 5mC and 5hmC sites within the DNA libraries	**2 hours**
5	**Denature DNA** Denatured DNA libraries	**0.5 hour**
6	**Deaminate cytosines** APOBEC deaminates cytosines but not oxidized 5mC and 5hmC	**3.5 hours**
7	**PCR amplify libraries** Amplified and purified final library product	**1.5 hours**

STEP 1 PREPARE AND SHEAR gDNA

Reagents Required

- gDNA
- Control DNA Unmethylated Lambda (lilac)
- Control DNA CpG methylated pUC19 (lilac)

Before You Begin

- Thaw all required reagents on ice, then pulse-vortex for 2 seconds to mix and pulse-spin.
- Turn on and program the focused-ultrasonicator platform to the preferred size distribution

NOTE: Optional DNA Controls (CpG methylated pUC19 and unmethylated lambda DNA) are included in this kit. These included controls can be spiked-in to the target DNA of interest and included in the workflow to control for efficient conversion.

PREPARE SAMPLE gDNA

1.1

Use the below table as a starting point for the recommended amount of controls (Control DNA Unmethylated Lambda and Control DNA CpG methylated pUC19) to add to the sample gDNA for evaluation of conversion efficiencies.

NOTE: Control DNA spike-in levels for different size panels and hybridization systems should be empirically determined to obtain an optimal ratio of target to spike-in reads for target enrichment sequencing.

SAMPLE DNA MASS INPUT	CONTROL DNA DILUTION RECOMMENDATIONS (WGS)	CONTROL DNA DILUTION RECOMMENDATIONS (TARGET ENRICHMENT)*
0.1 ng	1:1,000	–
1 ng	1:250	–
10 ng	1:100	1:1,000
200 ng	1:50	1:500

**Twist does not recommend using DNA inputs below 10 ng for libraries destined for target enrichment.*

1.2

Suspend the sample gDNA in 48 µl with 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).

1.3

Add the diluted DNA controls to the sample gDNA at the dilution levels listed above.

COMPONENT	VOLUME
Sample gDNA	48 µl
Control DNA Unmethylated Lambda (lilac)	1 µl
Control DNA CpG methylated pUC19 (lilac)	1 µl
Total	**50 µl**

SHEAR COMBINED DNA

1.4

Sample gDNA and control DNA are mechanically fragmented to an average insert size of ~350 bp (420–620 bp final Illumina library). It is recommended that fragmentation is performed using a focused-ultrasonicator platform, such as the R230 Focused-ultrasonicator from Covaris. Enzymatic fragmentation is not recommended as it may result in the removal of methyl groups from DNA.

1.5

Transfer the 50 µl of sheared DNA to a clean thin-walled PCR 0.2-ml strip-tube or a well of a 96-well thermal cycling plate for End Prep.

NOTE: Combined sample gDNA and control DNA do not need to be cleaned up or size-selected before End Prep.

PROCEED TO STEP 2: PERFORM END PREP ON SHEARED DNA

STEP 2 PERFORM END PREP ON SHEARED DNA

Reagents Required

- Sheared combined sample DNA (from Step 1.5)
- From the NEBNext Enzymatic Methyl-seq v2 Kit:
  - Ultra II End Prep Reaction Buffer (green)
  - Ultra II End Prep Enzyme Mix (green)

Before You Begin

- Thaw on ice:
  - Ultra II End Prep Reaction Buffer (green)
  - Ultra II End Prep Enzyme Mix (green)
  - Equilibrate Twist Total Purification Beads for 30 minutes at room temperature (for use in Steps 3, 4, and 7 of the protocol)

RESUSPEND THE PRE-HYBRIDIZATION SOLUTION

2.1

On ice, add the following components to the tube of sheared DNA:

REAGENT	VOLUME
Sheared combined sample DNA	50 µl
Ultra II End Prep Reaction Buffer (green)	7 µl
Ultra II End Prep Enzyme Mix (green)	3 µl
Total	**60 µl**

2.2

Set a 100-µl or 200-µl pipette to 50 µl and then gently pipette the entire volume up and down at least 10 times to mix thoroughly. Pulse-spin for 2 seconds to collect all liquid at the bottom of the tube.

NOTE: It is important to mix well. The presence of a small amount of bubbles will not interfere with the performance.

2.3

Place in a thermal cycler with the heated lid set to ≥75°C or on, and run the following program:

STEP	TEMPERATURE	TIME
1	20°C	15 minutes
2	65°C	15 minutes
3	4°C	HOLD

PROCEED TO STEP 3: LIGATE EM-SEQ ADAPTERS

STEP 3 LIGATE EM-SEQ ADAPTERS

Reagents Required

- End Prep reaction mix (from Step 2.3)
- From the NEBNext Enzymatic Methyl-seq v2 Kit:
  - NEBNext EM-seq Adaptor (red)
  - Ligation Enhancer (red)
  - Ultra II Ligation Master Mix (red)
  - NEBNext Carrier DNA (red)
  - Elution Buffer (white)
- 80% Ethanol
- Equilibrated Twist Total Purification Beads

Before You Begin

- Thaw on ice:
  - NEBNext EM-seq Adaptor (red)
  - Ligation Enhancer (red)
  - Ultra II Ligation Master Mix (red)
  - Elution Buffer (white)
  - NEBNext Carrier DNA (red)

PREPARE THE LIGATION REAGENTS

3.1

On ice, add the following components directly to the 60 µl End Prep reaction mixture and mix well:

REAGENT	VOLUME
NEBNext EM-seq Adaptor (red)	2.5 µl
Ligation Enhancer (red)	1 µl
Ultra II Ligation Master Mix (red)	30 µl
Total (including End Prep reaction mixture)	93.5 µl

NOTE: Ligation Enhancer and Ligation Master Mix can be mixed ahead of time and will remain stable for at least 8 hours at 4°C. Adding adapters to a premix in the adapter ligation step is not recommended. Premix adapters and sample and then add the other ligation reagents.

3.2

Set a 100-µl or 200-µl pipette to 80 µl and then pipette the entire volume up and down 10 times or vortex for 3 seconds to homogenize solution. Pulse-spin for 2 seconds to collect all liquid from the sides of the tube.

⚠IMPORTANT: 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.

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

STEP	TEMPERATURE	TIME
1	20°C	15 minutes
2	4°C	HOLD

STOPPING POINT: If not immediately proceeding to the next step, store the ligated libraries at –20°C for up to 24 hours.

PURIFY

3.4 Vortex the pre-equilibrated Twist Total Purification Beads until mixed well.

3.5 Add 93 µl (1X ratio) of resuspended Twist Total Purification Beads to each sample. Mix well by vortexing for 2-3 seconds.

3.6 Incubate the samples for at least 5 minutes at room temperature.

3.7 Place the samples on a magnetic plate for 5 minutes or until the supernatant is clear.

3.8 The Twist Total Purification Beads form a pellet, leaving a clear supernatant. Without removing the plate or tube(s) from the magnetic plate, remove and discard the supernatant.

3.9 Wash the bead pellet by gently adding 200 µl freshly prepared 80% ethanol (do not disturb the pellet). Incubate for 1 minute, then remove and discard the ethanol.

3.10 Repeat the wash twice (Step 3.9) while keeping the samples on the magnetic plate.

3.11 Carefully remove all remaining ethanol with a 10-µl pipette, making sure not to disturb the bead pellet.

3.12 Air-dry the bead pellet on the magnetic plate for 1–3 minutes or until the bead pellet is dry. Do not overdry the bead pellet.

IMPORTANT: Do not overdry the beads. This may result in lower recovery of DNA. 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.

ELUTION OPTION A OR B

Option A: >10ng DNA Input

3.13.A Remove the plate or tubes from the magnetic plate and add 29 µl of Elution Buffer (white) to each sample. Mix by pipetting up and down at least 10 times.

3.14.A Incubate at room temperature for at least 1 minute.

3.15.A Place the plate or tubes on a magnetic plate and let stand for 3 minutes, or until the beads form a pellet.

3.16.A Transfer 28 µl of the clear supernatant containing the ligated and indexed libraries to a clean thin-walled PCR 0.2-ml strip-tube or a well of a 96-well thermal cycling plate, making sure not to disturb the bead pellet.

Option B: ≤10 ng DNA input

3.13.B Remove the plate or tubes from the magnetic plate and add 28 µl of Elution Buffer (white) to each sample. Mix by pipetting up and down at least 10 times.

3.14.B Incubate at room temperature for at least 1 minute.

3.15.B Place the plate or tubes on a magnetic plate and let stand for 3 minutes, or until the beads form a pellet.

3.16.B Transfer 27 µl of the clear supernatant containing the adapter-ligated libraries to a clean thin-walled PCR 0.2-ml strip-tube or a well of a 96-well thermal cycling plate, making sure not to disturb the bead pellet.

3.17.B Add 1 µl of NEBNext Carrier DNA (red) to the 27 µl clear supernatant from step 3.16.B.

PROCEED TO STEP 4: OXIDIZE 5MC AND 5HMC SITES

STEP 4 OXIDIZE 5mC AND 5hmC SITES

Reagents Required

- EM-seq adapter ligated DNA (from Step 3.16.A or 3.17.B)
- From the NEBNext Enzymatic Methyl-seq v2 Kit:
  - TET2 Reaction Buffer (yellow)
  - TET2 Reaction Buffer Supplement (yellow)
  - UDP-Glucose (yellow)
  - DTT (yellow)
  - T4-BGT (yellow)
  - Diluted T4-BGT (yellow) using ≤ 10 ng DNA input
  - TET2 (yellow)
  - Fe (II) Solution (yellow)
  - Stop Reagent (yellow)
  - Elution Buffer (white)
- 80% Ethanol
- Equilibrated Twist Total Purification Beads

Before You Begin

- Thaw on ice:
  - UDP-Glucose (yellow)
  - DTT (yellow)
  - T4-BGT (yellow)
  - Diluted T4-BGT (yellow) using ≤ 10 ng DNA input
  - TET2 (yellow)
  - Fe (II) Solution (yellow)
  - Stop Reagent (yellow)
  - Elution Buffer (white)
- Prepare the reconstituted TET2 Reaction Buffer using the following information:
  - The TET2 Reaction Buffer Supplement (yellow) is a powder. Centrifuge before use to ensure it is at the bottom of the tube.
  - Add 400 µl of TET2 Reaction Buffer (yellow) to one tube of TET2 Reaction Buffer Supplement (yellow) and mix well to generate the reconstituted TET2 Reaction Buffer (yellow). Write today's date on the tube.
  - NOTE: The reconstituted buffer should be stored at –20°C and discarded after 4 months.
- If using ≤ 10 ng DNA input, prepare diluted T4-BGT (yellow) using the following information:
  - Dilute the T4-BGT (yellow) 1:10 using T4-BGT Diluent (yellow).
  - Example: Add 9 µl T4-BGT Diluent (yellow) to 1 µl T4-BGT (yellow) and mix well by vortexing for 2-3 seconds. Pulse-spin for 2 seconds before use.
  - NOTE: The diluted T4-BGT should be used immediately and discarded after use.

PROTECTION OF 5-METHYLCYTOSINES AND 5-HYDROXYMETHYLCYTOSINES

4.1 On ice, prepare the oxidation master mix in a 1.5-ml microcentrifuge tube as indicated below:

REAGENT	VOLUME PER REACTION*
Reconstituted TET2 Reaction Buffer	10 µl
UDP-Glucose (yellow)	1 µl
DTT (yellow)	1 µl
T4-BGT or Diluted T4-BGT (yellow)	1 µl
TET2 (yellow)	4 µl
Total Volume	**17 µl**

**Prepare a master mix for multiple reactions*

NOTES:
- Use T4-BGT for > 10 ng DNA input
- Use Diluted T4-BGT for ≤ 10 ng DNA input

4.2 On ice, add 17 µl of the oxidation master mix to each well or tube containing the EM-seq adapter ligated DNA from Step 3.16.A or 3.17.B.

4.3 Mix thoroughly by vortexing or by pipetting the entire volume up and down at least 10 times. Then, pulse-spin for 2 seconds.

4.4 Dilute the Fe (II) Solution (yellow) by adding 1 µl to 1249 µl of water.

NOTES:
- Fe (II) Solution (yellow) color can vary between colorless and yellow, this is normal.
- Use the solution immediately, do not store it. Discard after use.

4.5 Add the diluted Fe(II) Solution to the reaction mixture (from Step 4.3).

REAGENT	VOLUME PER REACTION*
Reaction mixture (from Step 4.3)	45 µl
Diluted Fe(II) Solution (from Step 4.4)	5 µl
Total Volume	**50 µl**

4.6 Mix thoroughly by vortexing or by pipetting the entire volume up and down at least 10 times. Then, pulse-spin for 2 seconds.

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

STEP	TEMPERATURE	TIME
1	37°C	30 minutes
2	4°C	HOLD

4.8 When the thermal cycler program is complete, place samples on ice and add 1 µl of Stop Reagent (yellow) to each sample.

4.9 Mix thoroughly by vortexing or by pipetting the entire volume up and down at least 10 times. Then, pulse-spin for 2 seconds.

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

STEP	TEMPERATURE	TIME
1	37°C	30 minutes
2	4°C	HOLD

STOPPING POINT: If not immediately proceeding to the next step, store the ligated libraries at –20°C.

PURIFICATION

4.11 Vortex the pre-equilibrated Twist Total Purification Beads until well mixed.

4.12 Add 50 µl (1X ratio) of resuspended Twist Total Purification Beads to each sample from Step 4.10. Mix well by vortexing for 2-3 seconds.

4.13 Incubate the samples for 5 minutes at room temperature.

4.14 Place the samples on a magnetic plate for 5 minutes or until the supernatant is clear.

4.15 The Twist Total Purification Beads form a pellet, leaving a clear supernatant. Without removing the plate or tubes from the magnetic plate, remove and discard the supernatant.

4.16 Gently wash the bead pellet by adding 200 µl of freshly prepared 80% ethanol (do not disturb the pellet). Incubate for 1 minute, then remove and discard the ethanol.

4.17 Repeat the wash once for a total of two washes (Step 4.16) while keeping the samples on the magnetic plate or stand.

4.18 Carefully remove all remaining ethanol with a 10-µl pipette, making sure not to disturb the bead pellet.
NOTE: Before pipetting, the bead pellet may be briefly spun to collect ethanol at the bottom of the plate or tube and returned to the magnetic plate.

4.19 Air-dry the bead pellet on the magnetic plate for 30 seconds to 2 minutes or until the bead pellet is dry. Do not overdry the bead pellet.

⚠ IMPORTANT: Do not overdry 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.

4.20 Remove the plate or tubes from the magnetic plate and add 18 µl of Elution Buffer (white) to each sample. Mix by pipetting up and down 10 times until homogenized.

4.21 Incubate at room temperature for at least 2 minutes. If necessary, pulse-spin for 2 seconds to collect the liquid from the sides of the tube before placing back on the magnetic stand.

4.22 Place the plate or tubes on a magnetic plate and let stand for at least 3 minutes, or until the beads form a pellet.

4.23 Transfer 16 µl of the clear supernatant containing the oxidized libraries to a clean thin-walled PCR 0.2-ml strip-tube or a well of a 96-well thermal cycling plate, making sure not to disturb the bead pellet.
CAUTION: Carrying even a small amount of beads forward can lead to inefficient deamination.

STOPPING POINT: If not immediately proceeding to the next step, store the purified, oxidized libraries at –20°C.

PROCEED TO STEP 5: DENATURE DNA

STEP 5 DENATURE DNA

Reagents Required

- Purified, oxidized libraries (from Step 4.23)
- Formamide or 1 N Sodium Hydroxide

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.

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

Option A: Sodium Hydroxide (Recommended)

5.1.A Prepare freshly diluted 0.05 N NaOH.

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

5.3.A Add 4 µl 0.05 N NaOH to the 16 µl of protected DNA (from Step 4.23). Mix thoroughly by vortexing 2–3 seconds or by pipetting up and down at least 10 times and pulse-spin for 2 seconds.

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

5.5.A Immediately place on ice and allow the sample to fully cool (~ 2 minutes) before proceeding to Step 6.

Option B: Formamide

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

5.2.B Add 4 µl Formamide to the 16 µl of protected DNA (from Step 4.23). Mix thoroughly by vortexing 2–3 seconds or by pipetting up and down at least 10 times and pulse-spin for 2 seconds.

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

5.4.B Immediately place in a cooling block on ice and allow the sample to fully cool (~2 minutes) before proceeding to Step 6.

STEP 6 DEAMINATE CYTOSINES

Reagents Required

- Denatured, oxidized libraries (from Step 5.5.A or 5.4.B)
- From the NEBNext Enzymatic Methyl-seq v2 Kit:
  - Deamination Reaction Buffer (orange)
  - Recombinant Albumin (orange)
  - APOBEC (orange)
- Nuclease-free water

Before You Begin

- Thaw on ice:
  - Deamination Reaction Buffer (orange)
  - Recombinant Albumin (orange)
  - APOBEC (orange)

PREPARE THE APOBEC MIX

6.1 On ice, prepare the APOBEC master mix in a 1.5-ml microcentrifuge tube as indicated below.

REAGENT	VOLUME PER REACTION*
Nuclease-free water	14 µl
Deamination Reaction Buffer (orange)	4 µl
Recombinant Albumin (orange)	1 µl
APOBEC (orange)	1 µl
Total Volume	**20 µl**

**Prepare a master mix for multiple reactions*

6.2 On ice, add 20 µl of the APOBEC master mix to each well or tube containing the denatured, oxidized libraries from Step 5.5.A or 5.4.B.

6.3 Mix thoroughly by vortexing 2–3 seconds or by pipetting up and down at least 10 times and pulse-spin for 2 seconds.

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

STEP	TEMPERATURE	TIME
1	37°C	3 hours
2	4°C	HOLD

STOPPING POINT: If not immediately proceeding to the next step, store the purified, oxidized libraries overnight in the thermal cycler at 4°C.

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

STEP 7 PCR AMPLIFY LIBRARIES

Reagents Required

- Deaminated libraries (from Step 6.4)
- From the NEBNext Enzymatic Methyl-seq v2 Kit:
  - NEBNext LV Unique Dual Index Primer plate
  - NEBNext Q5U Master Mix (blue)
  - Elution Buffer (white)
- 80% Ethanol
- Equilibrated Twist Total Purification Beads

Before You Begin

- Thaw on ice:
  - NEBNext LV Unique Dual Index Primer plate
  - NEBNext Q5U Master Mix (blue)
  - Elution Buffer (white)

PREPARE THE PCR MIX AND THERMAL CYCLER

7.1

On ice, add the following components to the deaminated DNA from Step 6.4:

REAGENT	VOLUME PER REACTION*
Deaminated DNA	40 µl
UDI Primer Pair*	5 µl
NEBNext Q5U Master Mix (blue)	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.*

NOTE: Refer to the NEBNext EM-seq manual and Appendix B for barcode pooling guidelines.

7.2

Mix thoroughly by vortexing or by pipetting up and down at least 10 times, pulse-spin for 2 seconds.

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

	STEP	TEMPERATURE	TIME	NUMBER OF CYCLES
1	Initialization	98°C	30 seconds	1
2	Denaturation	98°C	10 seconds	*See below table for specific initial cycle recommendations.*
	Annealing	62°C	30 seconds	
	Extension	65°C	60 seconds	
3	Final Extension	65°C	5 minutes	1
4	Final Hold	4°C	HOLD	–

DNA INPUT	PCR CYCLES (WGS)	PCR CYCLES (TARGET ENRICHMENT)*
200 ng	5	8
50 ng	6	9
10 ng	8	11
1 ng	11	–
0.1 ng	14	–

**Twist does not recommend using DNA inputs below 10 ng for libraries destined for target enrichment.*

STOPPING POINT: If not immediately proceeding to the next step, store the purified, oxidized libraries at –20°C.

PREPARE THE PCR MIX AND THERMAL CYCLER

7.4 Vortex the pre-equilibrated Twist Total Purification Beads until well mixed.

7.5 Add 72 µl (0.8X ratio) of resuspended Twist Total Purification Beads to each sample. Mix well by vortexing for 2-3 seconds and pulse-spin for 2 seconds.

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

7.7 Place the samples on a magnetic plate for 5 minutes.

7.8 The Twist Total Purification Beads form a pellet, leaving a clear supernatant. Without removing the plate or tubes from the magnetic plate, remove and discard the supernatant.

7.9 Gently wash the bead pellet by adding 200 µl of freshly prepared 80% ethanol (do not disturb the pellet). Incubate for 1 minute, then remove and discard the ethanol.

7.10 Repeat the wash once for a total of two washes (Step 7.9).

7.11 Carefully remove all remaining ethanol with a 10 µl pipette, making sure not to disturb the bead pellet.

7.12 Air-dry the bead pellet on the magnetic plate for 1–2 minutes or until the bead pellet is dry. Do not overdry the bead pellet.

⚠ IMPORTANT: Do not overdry the beads. This may result in lower recovery of DNA. 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.

7.13 Remove the tubes from the magnetic stand and add 22 µl of Elution Buffer (white) to each sample. Mix well by vortexing or pipette mixing,* then pulse-spin for 2 seconds. If the beads have pelleted, re-vortex before pulse spinning again.
*NOTE: The beads may be difficult to resuspend via pipette mixing during this step. If pipette mixing to resuspend the beads, the beads may stick to the pipette tips and lead to bead loss and variation in the final library yield.

Optional: For long-term storage of libraries, 22 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.

7.14 Incubate at room temperature for 2 minutes.

7.15 Re-vortex beads and pulse-spin for 2 seconds before incubating for another 2 minutes. This should help maximize recovery of DNA from the beads and minimize variation in the final library.

7.16 Place the plate or tubes on a magnetic plate or stand for at least 3 minutes, or until the beads form a pellet.

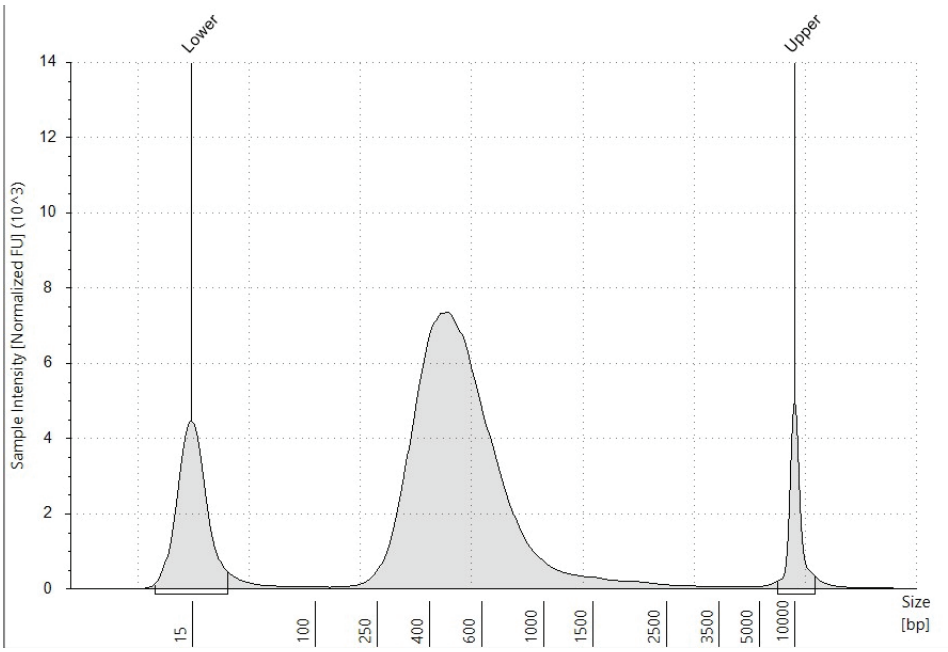
7.17 Transfer 20 µl of the clear supernatant containing the final library solution to a clean thin-walled PCR 0.2-ml strip-tube or a well of a 96-well thermal cycling plate, making sure not to disturb the bead pellet

STOPPING POINT: If not immediately proceeding to the next step, store the final product at –20°C. For long-term storage of libraries, 22 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.

PERFORM QC

7.18

Quantify and validate the size range of each library using the Thermo Fisher Scientific Qubit dsDNA Broad Range Quantitation Assay and Agilent 4200 TapeStation D5000 ScreenTape Assay. The average fragment length should be approximately 450–550 bp when using a range setting of 150–1,000 bp.



STOPPING POINT: If not immediately proceeding to either of the Twist Methylation Target Enrichment protocols, store the final product at –20°C. Please refer to Appendix A for details and reagents required for target enrichment compatible with methylation libraries.

END OF WORKFLOW

APPENDIX A: TWIST TARGETED METHYLATION SEQUENCING WORKFLOW USING THE TWIST TARGETED METHYLATION SEQUENCING PROTOCOL

The Twist Targeted Methylation Sequencing workflow is compatible with this library generation protocol and can be used to detect methylated sequences. The workflow uses hybridization times between 30 minutes and 4 hours, but Twist Bioscience recommends starting with a 2-hour hybridization time. The protocol uses a similar experimental set up as the Twist Fast Hybridization Target Enrichment protocol, but has been edited to include suggestions specific to methylation detection. This protocol can be found on Twist Bioscience's resource library at twistbioscience.com.

CATALOG #	NAME	DESCRIPTION	STORAGE
More information at twistbioscience.com/products/ngs	Custom Methylation Panel	E.g. Specific CpG Regions, Genes of Interest, etc	–25 to –15°C
TWIST METHYLATION TARGET ENRICHMENT WITH FAST HYBRIDIZATION			
129336: 96x12 rxn	Twist Fast Hybridization Reagents	· Fast Hybridization Mix · Hybridization Enhancer · Amplification Primers, ILMN	–25 to –15°C
	Twist Fast Wash Buffers	· Fast Binding Buffer · Fast Wash Buffer 1 · Wash Buffer 2	2 to 8°C
	Twist TrueAmp Polymerase Mix	Twist TrueAmp Polymerase Mix	–25 to –15°C
	Twist Universal Blocker Kit	For the prevention of nonspecific capture: · Universal Blockers · Blocker Solution	–25 to –15°C
	Twist Binding and Purification Beads	For target enrichment and purification: · Streptavidin Binding Beads · DNA Purification Beads	2 to 8°C
	Twist Methylation Enhancer	For the prevention of methylation-related nonspecific capture: · Methylation Enhancer	–25 to –15°C
TWIST METHYLATION TARGET ENRICHMENT WITH STANDARD HYBRIDIZATION V2			
129337: 96x12 rxn	Twist Hybridization Reagents	· Hybridization Mix · Hybridization Enhancer · Amplification Primer, ILMN	–25 to –15°C
	Twist Standard Wash Buffers v2	· Binding Buffer · Standard Wash Buffer 1 · Wash Buffer 2	2 to 8°C
	Twist TrueAmp Polymerase Mix	Twist TrueAmp Polymerase Mix	–25 to –15°C
	Twist Universal Blocker Kit	· For the prevention of nonspecific capture: · Universal Blockers · Blocker Solution	–25 to –15°C
	Twist Binding and Purification Beads	For target enrichment and purification: · Streptavidin Binding Beads · DNA Purification Beads	2 to 8°C
	Twist Methylation Enhancer	For the prevention of methylation-related nonspecific capture: · Methylation Enhancer	–25 to –15°C

APPENDIX B: BARCODE SEQUENCES AND SAMPLE SHEETS

For a complete guide to the barcodes associated with the NEBNext Multiplex Oligos for EM-Seq v2 library generation, please see the following sample sheets below:

LV Uniqe Dual Index Primers Set 1:
E3400 Forward Strand Workflow Sample Sheet: https://www.neb.com/en-us/-/media/nebus/worksheet-samples/e3400_-forward-strand-workflow-sample-sheet.csv?rev=3310c60d19eb4ec5ba7db02523ee4f2d&sc_lang=en-us

E3400 Reverse Complement Workflow Sample Sheet: https://www.neb.com/en-us/-/media/nebus/worksheet-samples/e3400_-reverse-complement-workflow-sample-sheet.csv?rev=9d2438682e154f3a9a78506ea68f2c77&sc_lang=en-us

LV Uniqe Dual Index Primers Set 2:
E3402 Forward Strand Workflow Sample Sheet: https://www.neb.com/en-us/-/media/nebus/worksheet-samples/e3402_-forward-strand-workflow-sample-sheet.csv?rev=3d513f325ec34fe5aa8ec7e39b550ba0&sc_lang=en-us

E3402 Reverse Complement Workflow Sample Sheet: https://www.neb.com/en-us/-/media/nebus/worksheet-samples/e3402_-reverse-complement-workflow-sample-sheet.csv?rev=c53ef139e7e847079a16625e13313d70&sc_lang=en-us

LV Uniqe Dual Index Primers Set 3:
E3404 Forward Strand Workflow Sample Sheet: Does not have direct web link. Parent page here where xlsx file can be downloaded under "Usage Guidelines" section: https://www.neb.com/en-us/products/e3404-nebnext-lv-unique-dual-index-primers-set-3

E3404 Reverse Complement Workflow Sample Sheet: Same as above

LV Uniqe Dual Index Primers Set 4:
E3406 Forward Strand Workflow Sample Sheet: https://www.neb.com/en-us/-/media/nebus/worksheet-samples/e3406_-forward-strand-workflow-sample-sheet.csv?rev=1216b3036bd6429ab3e2c507738fcf80&sc_lang=en-us

E3406 Reverse Complement Workflow Sample Sheet: https://www.neb.com/en-us/-/media/nebus/worksheet-samples/e3406_-reverse-complement-workflow-sample-sheet.csv?rev=a7b686d808524ee996dab55eb1278c68&sc_lang=en-us

APPENDIX C: KIT COMPONENTS

The following table details part numbers for each component provided in the kits required for this protocol.

BOX	COMPONENT	COMPONENT PART NUMBER
NEBNext Enzymatic Methyl-seq v2 Kit	Control DNA CpG methylated pUC19	E7122AAVIAL
	Control DNA unmethylated Lambda	E7123AAVIAL
	Ultra II End Prep Reaction Buffer	E7647AAVIAL
	Ultra II End Prep Enzyme Mix	E7646AAVIAL
	Ultra II Ligation Master Mix	E7648AAVIAL
	Ligation Enhancer	E7374AAVIAL
	TET2 Reaction Buffer	E7126AAVIAL
	TET2 Reaction Buffer Supplement	E8013AAVIAL
	DTT	E7139AAVIAL
	TET2	E7130AAVIAL
	Fe (II) Solution	E7131AAVIAL
	Stop Reagent	E7132AAVIAL
	APOBEC	E7133AAVIAL
	NEBNext® Q5U® Master Mix	E3369AAVIAL
	Elution Buffer	E7124AAVIAL
	UDP-Glucose	E3353AAVIAL
	T4-BGT	E3354AAVIAL
	T4-BGT Diluent	E8014AAVIAL
	Deamination Reaction Buffer	E3356AAVIAL
	Recombinant Albumin	E3357AAVIAL
	NEBNext® Carrier DNA	E3351AAVIAL
	NEBNext® EM-seq Adaptor	E7165AAVIAL
Twist Total Purification Beads, EM-seq v2	Total Purification Beads, EM-seq v2	128387
NEBNext® LV Unique Dual Index Primers		E3400AVIAL (Plate A) E3402AVIAL (Plate B) E3404AVIAL (Plate C) E3406AVIAL (Plate D)

END OF APPENDIX