NEBNext Enzymatic Methyl-seq 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 diagnosing cancer.

Twist Bioscience and New England Biolabs (NEB) have partnered to revolutionize methylome analysis. The NEB Enzymatic Methyl-seq (EM-seq) Library Preparation Kit is a new 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 Custom Methylation Panels and 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. For the following hybrid capture step, Twist Bioscience offers a Targeted Methylation Sequencing workflow that combines 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.

WORKFLOW 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
101977: 96 rxn	NEBNext Enzymatic Methyl-Seq	· Reagents for library construction: · Control DNA CpG methylated pUC19 · Control DNA Unmethylated Lambda · NEBNext Ultra™ II End Prep Reaction Buffer · NEBNext Ultra II End Prep Enzyme Mix · NEBNext Ultra II Ligation Master Mix · NEBNext Ligation Enhancer · NEBNext EM-seq™ Adaptor · Elution Buffer · TET2 Reaction Buffer · TET2 Reaction Buffer Supplement · Oxidation Supplement · DTT · Oxidation Enhancer · TET2 · Fe(II) Solution · Stop Reagent · APOBEC · APOBEC Reaction Buffer · BSA · NEBNext Q5U™ Master Mix	–25 to –15°C
101978, 107518, 107523, 107524: 96 rxn	NEBNext Multiplex Oligos for Enzymatic Methyl-Seq	UDI Primers, provides unique dual-indexed combinations with 1 reaction per index pair	–25 to –15°C
101979: 96 rxn (33.1 mL)	Twist Total Purification Beads	DNA Purification Beads	2 to 8°C
OPTIONAL			
110830: 96 rxn	Twist Methylated UMI Adapters - TruSeq Compatible, 96 Samples	Methylated UMI Adapters	–25 to –15°C

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INTENDED USE

This product is for research use only. It 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 TO BE SUPPLIED BY USER

The following materials or their equivalents are required to generate libraries using the NEBNext EM-seq Library Preparation Protocol.

PRODUCT	SUGGESTED SUPPLIER
REAGENTS AND CONSUMABLES	
Ethanol (200 proof)	—
Molecular biology grade water	—
10 mM Tris-HCl pH 8	—
1 mM EDTA pH 8	—
Formamide or 0.1 N Sodium Hydroxide	Sigma (Formamide), Teknova (Sodium Hydroxide)
Buffer EB	Qiagen
1.5-ml microcentrifuge tubes	VWR
Thin-walled PCR 0.2-ml strip-tubes	Eppendorf
96-well thermal cycling plates	VWR
1.5-ml compatible magnetic stand	Beckman Coulter
96-well compatible magnetic plate	Alpaqua, Permagen Labware
Qubit dsDNA Broad Range Quantitation Assay	Thermo Fisher Scientific
Qubit dsDNA High Sensitivity Quantitation Assay	Thermo Fisher Scientific
Agilent DNA 7500 Kit	Agilent Technologies
Focused-ultrasonicator platform consumable tubes	Covaris or similar
EQUIPMENT	
Pipettes and tips	—
Vortex mixer	—
Benchtop mini centrifuge for 0.2-ml tubes	—
Thermal cycler (96-well) with heated lid	—
Fluorometer (Qubit 3.0)	Thermo Fisher Scientific
2100 Bioanalyzer Instrument	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 provided instructions. Twist Bioscience cannot guarantee performance 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.

Test the compatibility of your PCR tubes with your thermal cycler 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.

IMPORTANT NOTES

Store all DNA samples at –20°C when not in use. Keep on ice at all times when in use.

Certain applications like those using cell-free DNA as sample input may benefit from improved duplicate resolution during analysis. For these applications, Twist Methylated UMI Adapters can be used as a drop-in replacement for NEBNext EM-seq Adapters.

Measuring DNA concentration by absorbance at 260 nm is not recommended. Use the Thermo Fisher Scientific Qubit dsDNA Broad Range Quantitation Assay to quantify DNA accurately.

For technical support and troubleshooting, contact Twist Bioscience at 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 C.

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

STEP 1 SHEAR AND PREPARE GENOMIC DNA

Reagents and Equipment Required

- Genomic DNA
- Focused-ultrasonicator platform

Before You Begin

- Thaw on ice:
  - Genomic DNA
- 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 combined together and included in the workflow to control for efficient conversion. Please see Appendix A for a detailed protocol on how to utilize these controls.

SHEAR GENOMIC DNA

1.1 Fragment genomic DNA to average insert size of 240–290 bp (370–420 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.

PREPARE SHEARED GENOMIC DNA

1.2 Quantify sheared DNA using a Qubit dsDNA High Sensitivity Quantitation Assay

1.3 Transfer the 200 ng of sheared DNA to a clean thin-walled PCR 0.2-ml strip-tube or a well of a 96-well thermal cycling plate. Bring the volume in the well to 50 µl with 0.1X TE pH 8.0.

NOTE: Combined sample DNA does not need to be cleaned up or size-selected before End Repair.

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 1.3 above)
- From the NEBNext Enzymatic Methyl-seq kit:
  - NEBNext Ultra II End Prep Reaction Buffer (green)
  - NEBNext Ultra II End Prep Enzyme Mix (green)

Before You Begin

- Thaw on ice:
  - NEBNext Ultra II End Prep Reaction Buffer (green)
  - NEBNext Ultra II End Prep Enzyme Mix (green)

PREPARE END REPAIR REAGENTS

2.1

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

REAGENT	VOLUME
Sheared combined sample DNA	50 µl
NEBNext Ultra II End Prep Reaction Buffer (green)	7 µl
NEBNext 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. Perform a quick spin to collect all liquid from the sides 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 turned “on” (if the lid cannot have a set temperature), and run the following program:

STEP	TEMPERATURE	TIME
1	20°C	30 min
2	65°C	30 min
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 above)
- From the NEBNext Enzymatic Methyl-seq kit:
  - NEBNext EM-seq Adapter (red)
  - NEBNext Ligation Enhancer (red)
  - NEBNext Ultra II Ligation Master Mix (red)
  - Elution Buffer (white)
- 80% Ethanol
- Equilibrated Twist Total Purification Beads
- Twist Methylated UMI Adapter (Optional)

Before You Begin

- Thaw on ice:
  - NEBNext EM-seq Adapter (red)
  - NEBNext Ligation Enhancer (red)
  - NEBNext Ultra II Ligation Master Mix (red)
  - Elution Buffer (white)
  - Twist Methylated UMI Adapter (Optional)

PREPARE THE LIGATION REAGENTS

3.1

On ice, add 2.5 µl of NEBNext EM-seq Adapter (red) to each well or tube containing the End Prep reaction mixture from Step 2.3. Mix gently by pipetting and keep on ice.

NOTE: Twist Methylated UMI Adapters can be used in place of the NEBNext EM-seq Adapter (red). Add 2.5 µl of Twist Methylated UMI Adapter to each well containing the reaction mixture. Continue the protocol as written.

3.2

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

REAGENT	VOLUME PER REACTION*
NEBNext Ligation Enhancer (red)	1 µl
NEBNext Ultra II Ligation Master Mix (red)	30 µl
Total	**31 µl**

**Prepare a master mix for multiple reactions.*

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.

3.3

On ice, add 31 µl of the ligation master mix to each reaction from Step 3.1.

3.4 Set a 100 µl or 200 µl pipette to 80 µl and then pipette the entire volume up and down 10 times to mix thoroughly. Perform a quick spin 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.

3.5 Incubate at 20°C for 15 minutes in a thermal cycler with the heated lid turned "off."

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

PURIFY

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

3.7 Add 110 µl (1.18x) of homogenized Twist Total Purification Beads to each ligation sample from Step 3.5. Mix well by vortexing.

3.8 Incubate the samples for 5 minutes at room temperature.

3.9 Place the samples on a magnetic plate for 1 minute.

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

3.11 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.

3.12 Repeat the wash twice while keeping the samples on the magnetic plate.

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

3.14 Air-dry the bead pellet on the magnetic plate for 2–5 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.

3.15 Remove the plate or tubes from the magnetic plate and add 30 µl of Elution Buffer (white) to each sample. Mix by pipetting until homogenized.

3.16 Incubate at room temperature for 2 minutes.

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

3.18 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.

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

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 3.18)
- From the NEBNext Enzymatic Methyl-seq kit:
  - TET2 Reaction Buffer (yellow)
  - TET2 Reaction Buffer Supplement (yellow)
  - Oxidation Supplement (yellow)
  - DTT (yellow)
  - Oxidation Enhancer (yellow)
  - TET2 (yellow)
  - 500 mM Fe(II) Solution (yellow)
  - Stop Reagent (yellow)
  - Elution Buffer (white)
- 80% Ethanol
- Equilibrated Twist Total Purification Beads

Before You Begin

- Thaw on ice:
  - Oxidation Supplement (yellow)
  - DTT (yellow)
  - Oxidation Enhancer (yellow)
  - TET2 (yellow)
  - 500 mM 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 is a powder. Centrifuge before use to ensure it is at the bottom of the tube.
  - Add 400 µl of TET2 Reaction Buffer to one tube of TET2 Reaction Buffer Supplement and mix well to generate the reconstituted TET2 Reaction Buffer. Write today's date on the tube.
    NOTE: The reconstituted buffer should be stored at –20°C and discarded after 4 months.

PREPARE THE THERMAL CYCLER AND PCR MIX

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 (TET2 Reaction Buffer plus reconstituted TET2 Reaction Buffer Supplement) (yellow)	10 µl
Oxidation Supplement (yellow)	1 µl
DTT (yellow)	1 µl
Oxidation Enhancer (yellow)	1 µl
TET2 (yellow)	4 µl
Total	**17 µl**

**Prepare a master mix for multiple reactions.*

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 3.18.

4.3 Mix thoroughly by vortexing and centrifuge briefly.

4.4 Dilute the 500 mM Fe(II) Solution (yellow) by adding 1 µl to 1249 µl of water.
NOTE: Use the solution immediately, do not store it. Discard after use.

4.5 Add the diluted Fe(II) Solution to the EM-seq DNA with Oxidation Enzymes (from Step 4.2):

REAGENT	VOLUME PER REACTION*
EM-seq DNA with Oxidation Enzymes (from Step 4.2)	45 µl
Diluted Fe(II) Solution (from Step 4.4)	5 µl
Total	**50 µl**

4.6 Mix thoroughly by vortexing or by pipetting up and down at least 10 times. Centrifuge briefly.

4.7 Incubate at 37°C for 1 hour in a thermal cycler with the heated lid set to ≥45°C or turned “on” (if the lid cannot have a set temperature).

4.8 Transfer the samples to ice and add 1 µl of Stop Reagent (yellow).
NOTE: grains of particulates may be present in the tube for Stop Reagent (yellow), but this will not affect performance. This reagent does not require any special pre-treatment, mixing, or heat applied.

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

4.10 Incubate at 37°C for 30 minutes then at 4°C in a thermal cycler with the heated lid set to ≥45°C or turned “on” (if the lid cannot have a set temperature).

STOPPING POINT: If not immediately proceeding to the next step, store the oxidized libraries at 4°C or –20°C for up to 24 hours.

PURIFY

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

4.12 Add 90 µl (1.8x) of homogenized Twist Total Purification Beads to each oxidized sample from Step 4.10. Mix well by vortexing.

4.13 Incubate the samples for 5 minutes at room temperature.

4.14 Place the samples on a magnetic plate for 1 minute.

4.15 The Twist Total Purification Beads form a pellet, leaving a clear supernatant. Without removing 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 twice while keeping the samples on the magnetic plate.

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

4.19 Air-dry the bead pellet on the magnetic plate for 2–5 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 until homogenized.

4.21 Incubate at room temperature for 2 minutes.

4.22 Place the plate or tubes on a magnetic plate and let stand for 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.

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

PROCEED TO STEP 5: DENATURE DNA

STEP 5 DENATURE DNA

Reagents Required

- Purified, oxidized libraries (from Step 4.23)
- Formamide or 0.1 N Sodium Hydroxide for denaturing step

PREPARE THE DENATURING MIX

5.1 The DNA can be denatured using either Formamide or 0.1 N Sodium Hydroxide.
NOTE: Use option 5.2.A for denaturing using Formamide and option 5.2.B for denaturing using 0.1 N Sodium Hydroxide.

5.2.A Formamide (Recommended):

5.2.A.1 Pre-heat thermal cycler to 85°C with the heated lid set to 105°C or turned “on” (if the lid cannot have a set temperature).

5.2.A.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.

5.2.A.3 Incubate at 85°C for 10 minutes in the pre-heated thermal cycler with the heated lid set to 105°C or turned “on” (if the lid cannot have a set temperature).

5.2.A.4 Immediately place on ice.

5.2.B Sodium Hydroxide (Optional, see NEB FAQ about preparing NaOH):

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

5.2.B.2 Pre-heat thermal cycler to 50°C with the heated lid set to ≥60°C or turned “on” (if the lid cannot have a set temperature).

5.2.B.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.

5.2.B.4 Incubate at 50°C for 10 minutes in the pre-heated thermal cycler with the heated lid set to ≥60°C or turned “on” (if the lid cannot have a set temperature).

5.2.B.5 Immediately place on ice.

PROCEED TO STEP 6: DEAMINATE CYTOSINES

STEP 6 DEAMINATE CYTOSINES

Reagents Required

- Denatured, oxidized libraries (from Step 5.2)
- From the NEBNext Enzymatic Methyl-seq kit:
  - APOBEC Reaction Buffer (orange)
  - BSA (orange)
  - APOBEC (orange)
  - Elution Buffer (white)
- 80% Ethanol
- Equilibrated Twist Total Purification Beads

Before You Begin

- Thaw on ice:
  - APOBEC Reaction Buffer (orange)
  - BSA (orange)
  - APOBEC (orange)
  - Elution Buffer (white)

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	68 µl
APOBEC Reaction Buffer (orange)	10 µl
BSA (orange)	1 µl
APOBEC (orange)	1 µl
Total	**80 µl**

**Prepare a master mix for multiple reactions.*

6.2 On ice, add 80 µl of the APOBEC master mix to each well or tube containing the denatured DNA from Step 5.2.

6.3 Mix thoroughly by vortexing or by pipetting up and down at least 10 times. Centrifuge briefly.

6.4 Incubate at 37°C for 3 hours, then at 4°C, in a thermal cycler with the heated lid set to ≥45°C or turned "on" (if the lid cannot have a set temperature).

STOPPING POINT: If not immediately proceeding to the next step, store the oxidized libraries 4°C or –20°C for up to 24 hours.

⚠ IMPORTANT: The Twist Total 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.

PURIFY

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

6.6 Add 100 µl (1.0x) of homogenized Twist Total Purification Beads to each deaminated sample from Step 6.4. Mix well by vortexing.

6.7 Incubate the samples for 5 minutes at room temperature

6.8 Place the samples on a magnetic plate for 1 minute.

6.9 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.

6.10 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.

6.11 Repeat the wash twice while keeping the samples on the magnetic plate.

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

6.13 Air-dry the bead pellet on the magnetic plate for 2–5 minutes or until the bead pellet is dry. Do not overdry the bead pellet, as overdrying will cause the beads to become difficult to resuspend.

⚠ 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.

6.14 Remove the plate or tubes from the magnetic plate and add 22 µl of Elution Buffer (white) to each sample. Mix by pipetting until homogenized.

6.15 Incubate at room temperature for 2 minutes.

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

6.17 Transfer 20 µl of the clear supernatant containing the deaminated 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.

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

PROCEED TO STEP 7: PCR AMPLIFY LIBRARIES

STEP 7 PCR AMPLIFY LIBRARIES

Reagents Required

- Deaminated libraries (from Step 6.17)
- From the NEBNext Enzymatic Methyl-seq kit:
  - EM-seq Index Primer plate
  - NEBNext Q5U Master Mix (blue)
  - Elution Buffer (white)
- 80% Ethanol
- Equilibrated Twist Total Purification Beads

Before You Begin

- Thaw on ice:
  - EM-seq Index Primer plate
  - NEBNext Q5U Master Mix (blue)
  - Elution Buffer (white)

PREPARE THE PCR MIX AND THERMAL CYCLER

7.1 On ice, add the EM-seq Index Primer plate and NEBNext Q5U Master Mix (blue) to the deaminated DNA libraries from Step 6.17:

REAGENT	VOLUME
EM-seq Index Primer plate	5 µl
NEBNext Q5U Master Mix (blue)	25 µl
Deaminated DNA libraries (from Step 6.17)	20 µl
Total	**50 µl**

NOTE: Refer to the NEBNext EM-seq manual and Appendix B for barcode pooling guidelines. EM-seq primers are supplied as a 96 Unique Dual Index Primer Pairs Plate.

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

7.3 Place the tube in a thermal cycler and perform PCR amplification using the following cycling conditions:

	STEP	TEMPERATURE	TIME	NUMBER CYCLES
1	Initialization	98°C	30 seconds	1
2	Denaturation	98°C	10 seconds	9
	Annealing	62°C	30 seconds	
	Extension	65°C	60 seconds	
3	Final Extension	65°C	5 minutes	1
4	Final Hold	4°C	HOLD	–

STOPPING POINT: If not immediately proceeding to the next step, store the amplified libraries at 4°C or –20°C for up to 24 hours.

PURIFY

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

7.5 Add 45 µl (0.9x) of homogenized Twist Total Purification Beads to each library from Step 7.3. Mix well by vortexing.

7.6 Incubate the samples for 5 minutes at room temperature.

7.7 Place the samples on a magnetic plate for 1 minute.

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 twice while keeping the samples on the magnetic plate.

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

7.12 Air-dry the bead pellet on the magnetic plate for 2–5 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 plate or tubes from the magnetic plate and add 22 µl of Elution Buffer (white) to each sample. Mix by pipetting until homogenized.

7.14 Incubate at room temperature for 2 minutes.

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

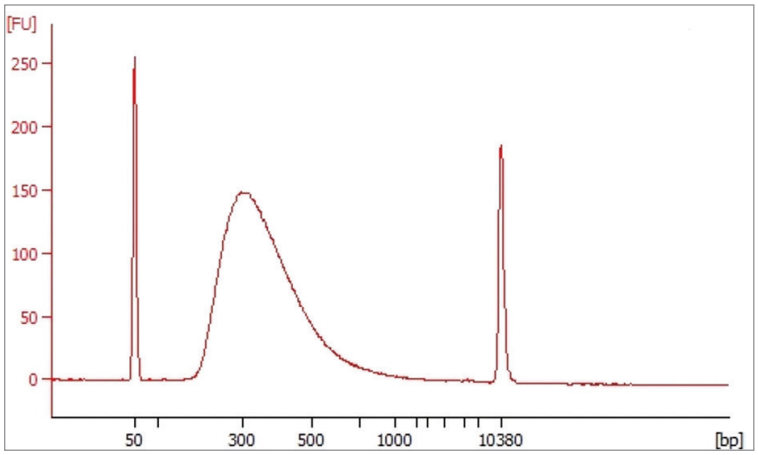
7.16 Transfer 20 µl of the clear supernatant containing the final EM-seq library to a clean thin-walled PCR 0.2 ml strip-tube or a well of a 96-well thermal cycling plate, making sure not to disturb the bead pellet.

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

PERFORM QC

7.17

Quantify and validate the size range of each EM-seq library using the Thermo Fisher Scientific Qubit dsDNA Broad Range Quantitation Assay and Agilent DNA 7500 Assay. The average fragment length should be approximately 350bp to 450bp when using a range setting of 150–1,000 bp.



Electropherogram generated by an Agilent 7500 DNA analysis of EM-seq libraries that were prepared as described. Note the single prominent peak.

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

END OF WORKFLOW

APPENDIX A: UTILIZING AND PREPARING THE DNA CONTROLS

Two DNA controls are included in this kit: CpG methylated pUC19 and unmethylated lambda DNA. These optional controls are methylated at known amounts so that an accurate conversion rate can be determined after sequencing. These controls can be spiked directly into test samples for processing through library preparation and target enrichment. Additional target enrichment probes against the control genomes are required for this method, please contact Twist Bioscience at **customersupport@twistbioscience.com** for details. To use these samples as stand-alone controls for library preparation, please follow the procedure detailed below.

A.1 Prepare CpG methylated pUC19 and unmethylated lambda DNA for use as a stand-alone control by mixing each sample together in a clean 1.5-ml microcentrifuge tube using the volumes in the table below.

REAGENT	VOLUME	MASS	EXPECTED METHYLATION LEVEL	EXPECTED CONVERSION EFFICIENCY
CpG Methylated pUC19	48 µl	4.8 ng	95-98%	>=99.5%
Unmethylated Lambda DNA	48 µl	96 ng	Up to 0.5%	>=99.5%
Total	**96 µl**	**100.8 ng**	—	—

A.2 Concentrate the DNA control mixture using SPRI bead clean-up, a column concentrator, or a speed vacuum concentrator.

A.3 Resuspend DNA controls in 50 µl 0.1X TE pH 8.0 and mechanically fragment to an average insert size of 200–300 bp (350–450 bp final Illumina library).
NOTE: Fragmentation should be done using a focused-ultrasonicator platform, such as a Covaris instrument, and following a similar process as with the sample genomic DNA.

A.4 Generate the control DNA library following Step 2 of the protocol and onwards.
NOTE: The control library does not go through the capture process. Save until the final pooling process.

A.5 Sequence the final targeted libraries on a platform of your choice.

NOTES:

- Target sequencing depth can vary between 50–250X depending on application. For specific recommendations on sequencing depth, please contact customersupport@twistbioscience.com.
- After sequencing, further analysis will be required. Bed files for the pUC19 and Lambda DNA genomes can be found at the following links:
  - https://www.twistbioscience.com/resources/data-files/puc19-dna-control-bed-files
  - https://www.twistbioscience.com/resources/data-files/lambda-dna-control-bed-files

END OF APPENDIX A

APPENDIX B: BARCODE SEQUENCES AND SAMPLE SHEETS

BARCODE SEQUENCES

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

NEBNext Multiplex Oligo Sample Sheets: https://www.twistbioscience.com/resources/data-files/nebnext-multiplex-oligos-sequences-sample-sheet-templates

END OF APPENDIX B

APPENDIX C: 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
100856: 2 rxn 100578:12 rxn 100767: 96 rxn	Twist Universal Blockers	For the prevention of nonspecific capture: · Universal Blockers · Blocking Solution	–25 to –15°C
103556: 2 rxn 103557:12 rxn 103557: 96 rxn	Twist Methylation Enhancer	For the prevention of methylation-related nonspecific capture: · Methylation Enhancer	–25 to –15°C
101262: 2 rxn 100983: 12 rxn 100984: 96 rxn	Twist Binding and Purification Beads	For target enrichment and purification: Streptavidin Binding Beads · DNA Purification Beads	2 to 8°C
101278: 2 rxn 101174: 12 rxn 101175: 96 rxn	Twist Fast Hybridization and Wash Kit (2 Boxes) Twist Fast Hybridization Reagents (Box 1 of 2) Twist Fast Wash Buffers (Box 2 of 2)	For target enrichment with fast hybridization: Fast Hybridization Mix · Hybridization Enhancer · Amplification Primers Fast Binding Buffer · Fast Wash Buffer 1 · Wash Buffer 2	–25 to –15°C 2 to 8°C

END OF APPENDIX C

LAST REVISED: January 22, 2025

REVISION	DATE	DESCRIPTION
7.0	Jan 22, 2025	• Added Twist Methylated UMI Adapters to workflow components table • Added paragraph regarding use of Twist Methylated UMI Adapters to Important Notes section • Added note regarding use of Twist Methylated UMI Adapters to Step 3.1 • Changed drying time in Steps 3.14, 4.19, 6.13, and 7.12
6.0	Sep 3, 2024	• Added stopping points to table in Protocol Overview section • Clarified statements regarding mixing DNA controls before library preparation in Step 1 • Added note regarding potential particulates in Step 4.8 • Clarified steps in Appendix A • Added additional details to Stopping Point on page 21 regarding target enrichment • Updated workflow components table and Appendix B to include information for plates B, C, and D • Minor stylistic and editorial edits throughout
5.0	Apr 18, 2023	• Added missing components to NEBNext Enzymatic Methyl-Seq description in Workflow Components table • Slightly revised initial steps in Steps 3, 4, and 6 and updated language throughout to improve clarity
4.0	Nov 1, 2022	• Materials to be supplied by user updated. • Appendix B updated. • Minor text additions and typos fixed throughout document.
3.0	Dec 2, 2021	• Materials to be supplied by user updated.
2.0	Apr 16, 2021	• Materials to be supplied by user updated.