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Protocol for Use with Standard Insert Libraries (370-420 bp) (NEB#E7120) V.2

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We use this protocol and it's working

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Abstract

This protocol details how to construct DNA libraries from start to finish using NEBNext reagents.

The corresponding NEB manual is here: https://www.neb.com/-/media/nebus/files/manuals/manuale7120.pdf and this protocol relates to section 1.

Overview

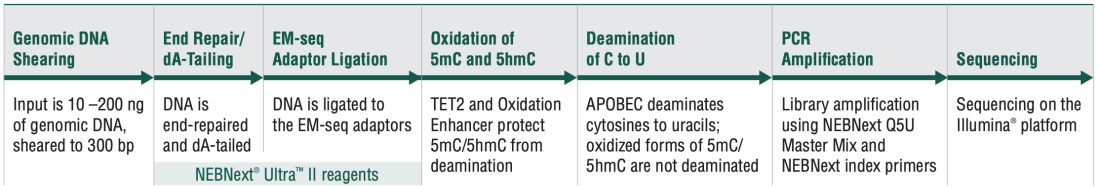


Figure 1. NEBNext Enzymatic Methyl-seq Kit Workflow.

The Enzymatic Methyl-seq kit (EM-seq) for Illumina contains all the components needed to make libraries that are enzymatically modified to detect 5-methylcytosines (5mC) and 5-hydroxymethylcytosines (5hmC).

Figure 1 is an overview of the EM-seq workflow. Firstly, a library is made by ligating EM-seq adaptor to sheared end repaired/dA-tailed genomic DNA. This is followed by two sets of enzymatic conversion steps to differentiate cytosines from 5mC and 5hmC. Finally, libraries are PCR amplified before sequencing.

Figure 2. Overview of Sodium Bisulfite Conversion and EM-seq.

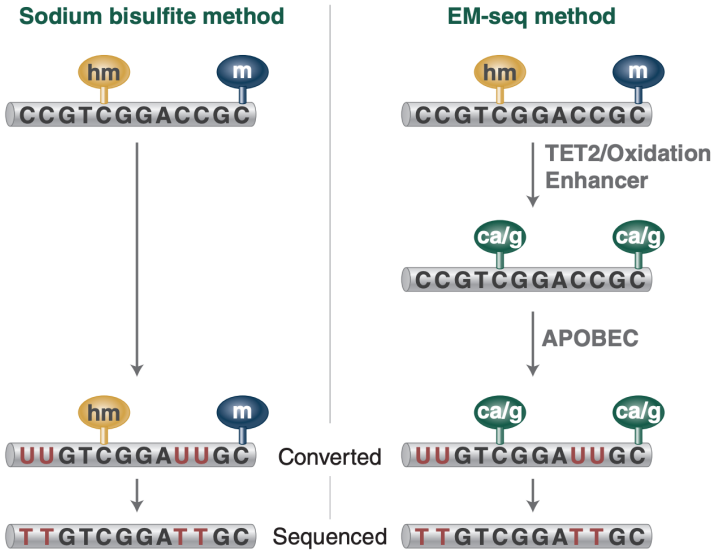


Figure 2 shows a comparison of the sodium bisulfite and EM-seq methods. Sodium bisulfite treatment of DNA results in the deamination of cytosines into uracils, however the modified forms of cytosine (5mC and 5hmC) are not deaminated. Therefore, the preference of bisulfite to chemically deaminate cytosines enables the methylation status of cytosines to be

determined. When bisulfite treated DNA is PCR amplified, uracils are replaced by thymines and the 5mC/5hmC are replaced by cytosines. Once sequenced, unmethylated cytosines are represented by thymines and 5mC and 5hmC are represented by cytosines. By comparing sequences to non-converted genomes the appropriate methylation status can be assessed.

Enzymatic Methyl-seq 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 C but does not convert 5caC and 5ghmC. The resulting converted sequence can be analyzed like bisulfite-treated DNA. Typical aligners used to analyze data include but are not limited to Bismark and BWAMeth.

The workflow described in the NEBNext Enzymatic Methyl-seq Kit 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.

Please note that the bead volumes provided are sufficient for building standard size libraries described in Section 1, Protocol for use with Standard Size Libraries (370–420 bp). If following the Section 2 Protocol, for use with Large Size Libraries (470–520 bp), users need to supply additional beads due to the increased volumes needed for cleanups. We recommend using SPRIselect® Reagent Kit (Beckman Coulter, Inc. #B23317) or AMPure® XP Beads (Beckman Coulter, Inc. #A63881). If you are combining leftover beads from E7120 with the above products, we recommend combing with SPRIselect® Reagent Kit (Beckman Coulter, Inc. #B23317).

Each kit component must pass rigorous quality control standards, and for each new lot the entire set of reagents is functionally validated together by construction of indexed libraries and sequenced on an Illumina sequencing platform.

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

Materials

The Library Kit Includes

The volumes provided are sufficient for preparation of up to 24 reactions (NEB #E7120S) and 96 reactions (NEB #E7120L).

The NEBNext Sample Purification Beads should be stored at room temperature and all other 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
(green) NEBNext Ultra™ II End Prep Reaction Buffer
(green) NEBNext Ultra II End Prep Enzyme Mix
(red) NEBNext Ultra II Ligation Master Mix
(red) NEBNext Ligation Enhancer
(red) NEBNext EM-seq™ Adaptor
(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 (Please Note: This buffer may be clear or yellow in color.)
(yellow) Stop Reagent
(orange) APOBEC
(orange) APOBEC Reaction Buffer
(orange) BSA
(blue) NEBNext Q5U™ Master Mix
NEBNext Multiplex Oligos for Enzymatic Methyl-seq (24 Unique Dual Index Primer Pairs) or (96 Unique Dual Index Primer Pairs)
NEBNext Sample Purification Beads

Required Materials Not Included:

- Covaris® S2 instrument or other fragmentation equipment
- 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
- Magnetic rack/stand, such as NEBNext Magnetic Separation Rack (NEB #S1515S)
- PCR machine
- Bioanalyzer®, TapeStation® and associated consumables or other fragment analyzer

Safety warnings

Please see SDS (Safety Data Sheet) for hazards and safety warnings.

Before start

Safe Stopping Point: 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.

Caution: This signifies a step in the protocol that has two paths leading to the same end point.

(Color): Colors in parenthesis indicate the cap color of the reagent to be added.

Starting Material: 10 ng–200 ng DNA

DNA Preparation

1 DNA and Control DNA
Combine genomic DNA (10–200 ng) with control DNAs specified below. Genomic DNA can be in any of the following buffers:
10 mM Tris pH 8.0, 1X TE (10mM Tris pH 8.0, 1mM EDTA), or low TE (10 mM Tris pH 8.0, 0.1 mM EDTA).

	A	B
	COMPONENT	VOLUME
	gDNA	48 µl
	(lilac) Control DNA Unmethylated Lambda (see Table 1.1)	1 µl
	(lilac) Control DNA CpG methylated pUC19 (see Table 1.1)	1 µl
	Total Volume	50 µl

The following table is a guide for the amount of (lilac) Control DNA CpG methylated pUC19 and (lilac) Control DNA Unmethylated Lambda DNA to be added to samples prior to EM-seq library construction. Expected read numbers along with read length should be considered to ensure that enough controls are included for the users individual sequencing goals.

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

	A	B	C
	DILUTION OF (LILAC) LAMBDA CONTROL AND (LILAC) pUC19 CONTROL		
	DNA Input Amount	Pre-sequencing 2-4 Milliio n Paired Reads	Deep Sequencing 100-150 Million Paired Reads
	10 ng	1:20	1:100
	200 ng	No Dilution	1:50

Regardless of sequencing depth, a minimum of 5,000 paired end reads with a read length of 76 bases, for unmethylated Lambda DNA, and 500 paired end reads with a read length of 76 bases, for CpG methylated pUC19, are needed to give enough coverage for accurate conversion estimates.

Different applications may require different sequencing depths 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 other may require 50 million paired end reads or even 300 million 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 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.

Shearing DNA

2 The combined 50 µl genomic DNA and control DNAs are fragmented to an average fragment size of ~300 bp (370–420 bp final Illumina library). Fragmentation can be done using a preferred fragmentation device such as a Covaris instrument. Enzymatic fragmentation is not recommended as this may result in the removal of methylation marks.

Transfer the 50 µl of sheared DNA to a new PCR tube for End Prep.

Note: DNA does not need to be cleaned up or size selected before End Prep

End Prep of Sheared DNA

3 On ice, mix the following components in a sterile nuclease-free PCR tube:

	A	B
	COMPONENT	VOLUME
	Sheared DNA (Step 2)	50 µl
	(green) NEBNext Ultra II End Prep Reaction Buffer	7 µl
	(green) NEBNext Ultra II End Prep Enzyme Mix	3 µl
	Total Volume	60 µl

Note: NEBNext Ultra II End Prep Reaction Buffer and Enzyme Mix can be pre-mixed ahead of time as a master mix.

4 Set a 100 µL or 200 µL pipette to 50 µL and then 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.

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

00:30:00 at 20 °C
00:30:00 at 65 °C
Hold at 4 °C

Ligation of EM-seq Adaptor

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

	A	B
	COMPONENT	VOLUME
	End Prep reaction mixture (Step 5)	60 µl
	(red) NEBNext EM-seq Adaptor	2.5 µl
	(red) NEBNext Ligation Enhancer	1 µl
	(red) NEBNext Ligation Master Mix	30 µl
	Total Volume	93.5 µl

Note: Ligation Enhancer and Ligation Master Mix can be mixed ahead of time and is stable for at least 8 hours at 4 °C. We do not recommend adding adaptor to a premix in the adaptor ligation step. Premix adaptor and sample and then add the other ligation reagents.

7 Set a 100 µL or 200 µL pipette to 80 µL and then 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.

CAUTION: 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.

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

15 minutes at 20°C
Hold at 4°C

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

Clean-Up of Adaptor Ligated DNA

9 Vortex Sample Purification Beads to resuspend.

10 Add 110 µl (~1.1X) 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.

11 Incubate samples on bench top for at least 00:05:00 at Room temperature .

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

13 After 00:05:00 (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.

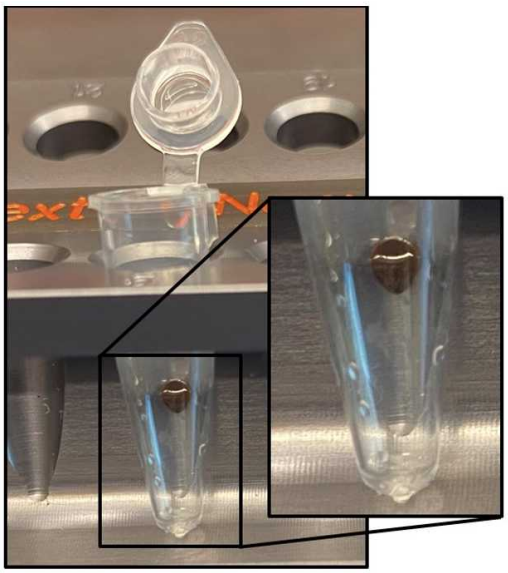
14 Add 200 µL of 80% freshly prepared ethanol to the tubes while in the magnetic stand. Incubate at Room temperature for 00:00:30 and then carefully removing and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

30s

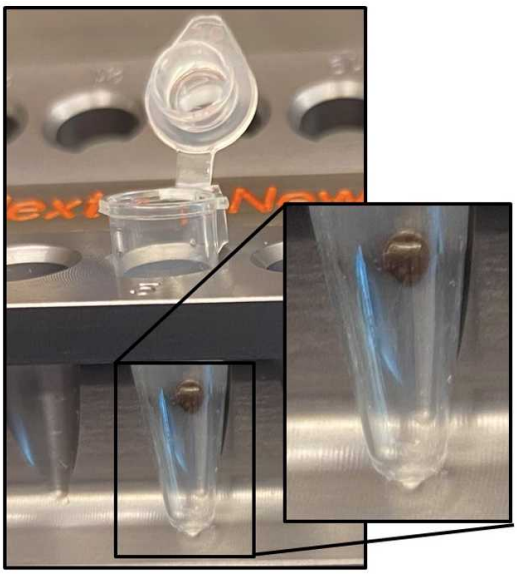
15 Repeat the ethanol wash once for a total of two washes. Be sure to remove all visible liquid after the second wash using a p10 pipette tip.

16 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.



After ethanol is removed the beads will be shiny and droplets of ethanol will be on the inside of the tube



When the beads are ready to elute visible droplets are gone and the beads are still dark brown and look a little matte

17 Remove the tubes from the magnetic stand. Elute the DNA target from the beads by adding 29 µL of Elution Buffer (white).

18 Mix well by pipetting up and down 10 times. Incubate for at least 00:01:00 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.

19 Place the tube on the magnetic stand. After 00:03:00, or whenever the solution is clear, transfer 28 µL of the supernatant to a new PCR tube.

Note

SAFE STOPPING POINT: Samples can be stored overnight at -20 °C.

Oxidation of 5-Methylcytosines and 5-Hydroxymethylcytosines

20 Prepare **TET2 Buffer**. Use option A if you have E7120S/ E7120G (24 Reactions/G size) and option B if you have E7120L (96 reactions).

Note

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

21 **Option A: E7120S/E7120G**

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

22 **Option B: E7120L**

Add 400 µL of TET2 Reaction Buffer to one tube of TET2 Reaction Buffer Supplement and mix well. Write date on tube.

The reconstituted buffer should be stored at -20 °C and discarded after 4 months.

23 On ice, add the following components directly to the EM-seq adaptor ligated DNA.

	A	B
	COMPONENT	VOLUME
	EM-seq adaptor ligated DNA (Step 19)	28 µl
	(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
	Total Volume	45 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.

24 Dilute the 500 mM (yellow) Fe(II) Solution by adding 1 µL to 1249 µL of water .

Note

Note: The 500 mM (yellow) Fe(II) solution color can vary between colorless to yellow, this is normal. Use the diluted solution immediately, do not store it. Discard after use.

Combine Diluted (yellow) Fe(II) Solution and Reaction DNA Oxidation Enzymes as described below.

	A	B
	COMPONENT	VOLUME
	Reaction Mixture (Step 23)	45 µl
	Diluted Fe(II) Solution (from step 24)	5 µl
	Total Volume	50 µl

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

25 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

26 Transfer the samples to ice and add 1 µL of Stop Reagent (yellow).

	A	B
	COMPONENT	VOLUME
	Oxidized DNA (Step 25)	50 µl
	(yellow) Stop Reagent	1 µl
	Total Volume	

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

27 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

Note

SAFE STOPPING POINT: Samples can be stored overnight at either 4 °C in the thermal cycler or at -20 °C in the freezer.

Clean-Up of TET2 Converted DNA

28 Vortex Sample Purification Beads to resuspend.

29 Add 90 µl (1.8X) 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.

30 Incubate samples on bench top for at least 00:05:00 at Room temperature . 5m

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

32 After 00:05:00 (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.

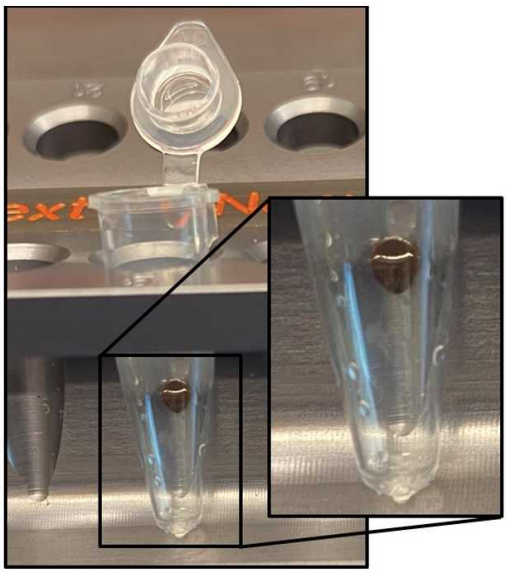
33 Add 200 µL of 80% freshly prepared ethanol to the tubes while in the magnetic stand. Incubate at Room temperature for 00:00:30 , then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

34 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.

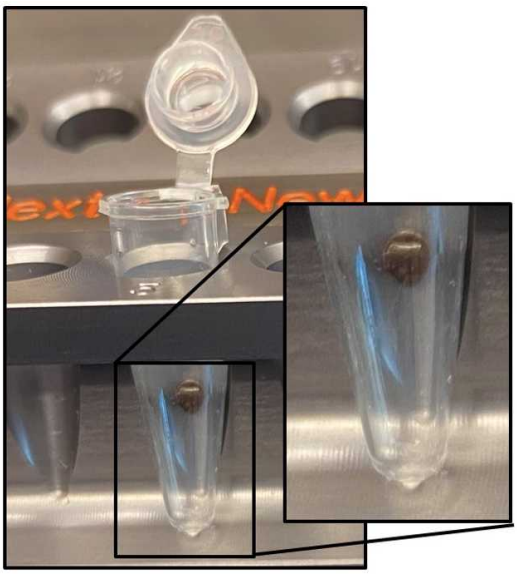
35 Air dry the beads for up to 2 minutes while the tubes are on the magnetic stand with the lid open.

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.



After ethanol is removed the beads will be shiny and droplets of ethanol will be on the inside of the tube



When the beads are ready to elute visible droplets are gone and the beads are still dark brown and look a little matte

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

37 Mix well by pipetting up and down 10 times. Incubate for at least 00:01:00 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.

1m

38 Place the tube on the magnetic stand. After 00:03:00 (or whenever the solution is clear), transfer 16 µL of the supernatant to a new PCR tube.

Note

SAFE STOPPING POINT: Samples can be stored Overnight **at** -20 °C **.**

Denaturation of DNA

39

Note

Caution: 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.

Option A: Formamide (Recommended)

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

41 Add 4 µL Formamide to the 16 µL of oxidized DNA (Step 39). Vortex to mix or by pipetting up and down at least 10 times, centrifuge briefly.

42 Incubate at 85 °C for 00:10:00 in the pre-heated thermal cycler. 10m

43 Immediately place On ice and allow the sample to fully cool (~2 minutes) before proceeding to Deamination of Cytosines.

Option B: Sodium Hydroxide (Optional, see FAQ at NEB.com about preparing NaOH)

44 Prepare freshly diluted 0.1 N NaOH.

45 Pre-heat thermal cycler to 50 °C with the heated lid set to ≥ 105 °C or on.

46 Add 4 µL 0.1 N NaOH to the 16 µL of oxidized DNA (Step 39). Vortex to mix or by pipetting up and down at least 10 times, centrifuge briefly.

47 Incubate at 50 °C for 00:10:00 in the pre-heated thermal cycler. 10m

48 Immediately place On ice and allow the sample to fully cool (~2 minutes) before proceeding to Deamination of Cytosines.

Deamination of Cytosines

49 On ice, add the following components to the denatured DNA.

	A	B
	COMPONENT	VOLUME
	Denatured DNA (Step 43 or 48)	20 µl
	Nuclease-free water	68 µl
	(orange) APOBEC Reaction Buffer	10 µl
	(orange) BSA	1 µl
	(orange) APOBEC	1 µl
	Total Volume	100 µl

Note

For multiple reactions, a master mix of the reaction components can be prepared before addition to the denatured DNA.

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

51 Place in a thermal cycler, and run the following program wiht the heated lid set to ≥ 45 °C or on.

03:00:00 at 37 °C

Hold at 4 °C

Note

SAFE STOPPING POINT: Samples can be stored overnight at either 4 °C in the thermal cycler or at -20 °C in the freezer.

Clean up of Deaminated DNA

52

Note

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.

Vortex Sample Purification Beads to resuspend.

53 Add 100 µl (1.0X) 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.

54 Incubate sample on bench top for at least 00:05:00 at Room temperature . 5m

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

56 After 00:05:00 (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

Note

CAUTION: DO NOT discard the beads.

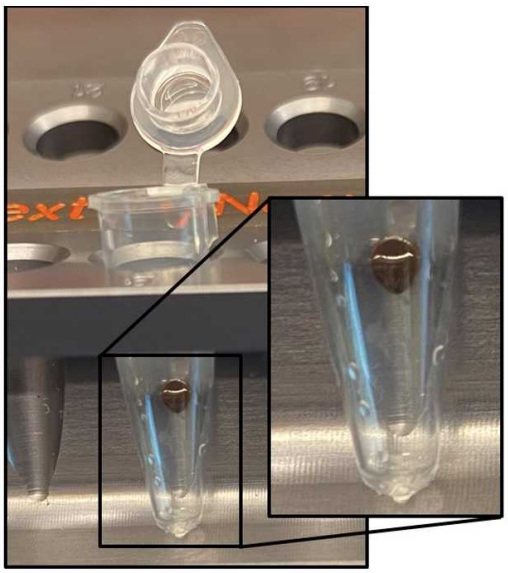
57 Add 200 µL of 80% freshly prepared ethanol to the tubes while in the magnetic stand. Incubate at Room temperature for 00:00:30 , then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

58 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.

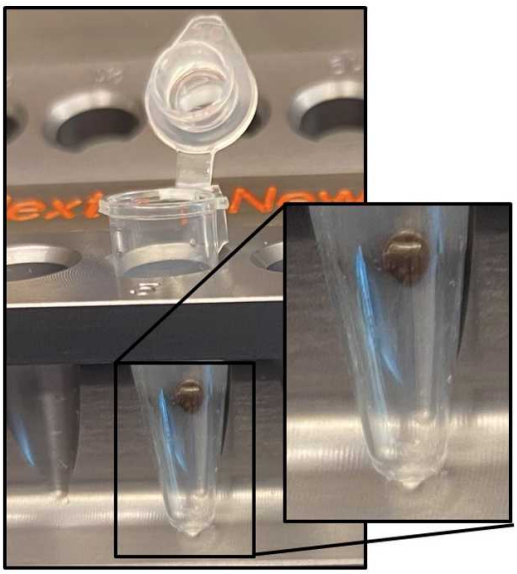
59 Air dry the beads for up to 00:01:30 while the tubes are on the magnetic stand with the lid open. 1m 30s

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.



After ethanol is removed the beads will be shiny and droplets of ethanol will be on the inside of the tube



When the beads are ready to elute visible droplets are gone and the beads are still dark brown and look a little matte

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

61 Mix well by pipetting up and down 10 times. Incubate for at least 00:01:00 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.

62 Place the tube on the magnetic stand. After 00:03:00 (or whenever the solution is clear), transfer 20 µL of the supernatant to a new PCR tube.

Note

SAFE STOPPING POINT: Samples can be stored Overnight **at** -20 °C **.**

PCR Amplification

63 On ice, add the following components to the deaminated DNA:

	A	B
	COMPONENT	VOLUME
	Deaminated DNA (Step 62)	20 µl
	EM-seq Index Primer*, **	5 µl
	(blue) NEBNext Q5U Master Mix	25 µl
	Total Volume	50 µl

*Refer to Section 3 in the manual at NEB.com for barcode pooling guidelines.
**EM-seq primers are supplied in tubes in NEB #E7120S or as a 96 Unique Dual Index Primers Pairs Plate in NEB #E7120L.

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

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

	A	B	C	D
	CYCLE STEP	TEMP	TIME	CYCLES
	Initial Denaturation	98°C	30 seconds	1
	Denaturation	98°C	10 seconds	4-8*
	Annealing	62°C	30 seconds	
	Extension	65°C	60 seconds	
	Final Extension	65°C	5 minutes	1
	Hold	4°C	∞	-

*Cycle Recommendations:

- 10 ng DNA input: 8 cycles
- 50 ng DNA input: 5-6 cycles
- 200 ng DNA input: 4 cycles

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

Clean-Up of Amplified Libraries

66 Vortex Sample Purification Beads to resuspend.

67 Add 45 µl (0.9X) 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.

68 Incubate samples on bench top for at least 00:05:00 at Room temperature . 5m

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

70 After 00:05:00 (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

Note

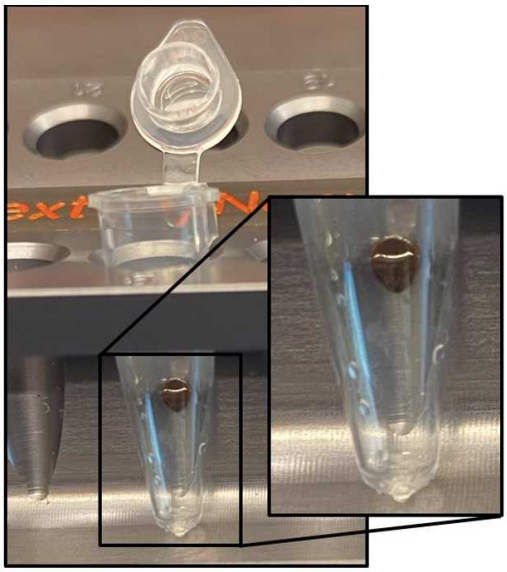
CAUTION: DO NOT discard the beads.

71 Add 200 µL of 80% freshly prepared ethanol to the tubes while in the magnetic stand. Incubate at Room temperature for 00:00:30 , then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

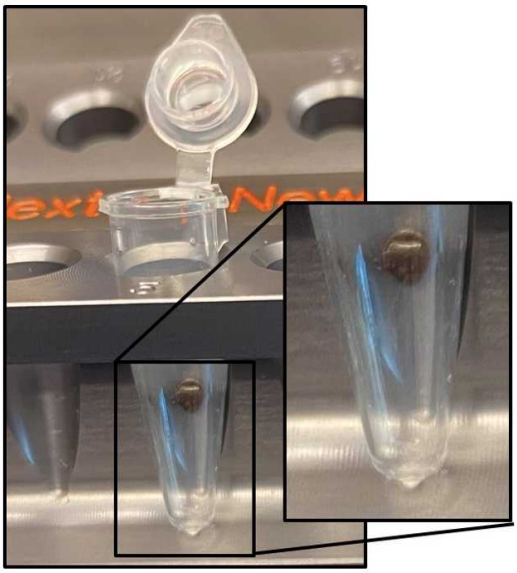
72 Repeat the wash once for a total of two washes. Be sure to remove all visible liquid after the second wash using a p10 pipette tip.

73 Air dry the beads for up to 00:02:00 while the tubes are on the magnetic stand with the lid open. 2m

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.



After ethanol is removed the beads will be shiny and droplets of ethanol will be on the inside of the tube



When the beads are ready to elute visible droplets are gone and the beads are still dark brown and look a little matte

74 Remove the tubes from the magnetic stand. Elute the DNA target from the beads by adding 21 µL of Elution Buffer (white) For long terms storage use, 21 µl of 1XTE (10 mM Tris, 1 mM EDTA, pH 8.0), Low TE (10 mM Tris, 0.2 mM EDTA, pH 8.0) or 0.1XTE (1 mM Tris, 0.1 mM EDTA, pH 8.0).

75 Mix well by pipetting up and down 10 times. Incubate for at least 00:01:00 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.

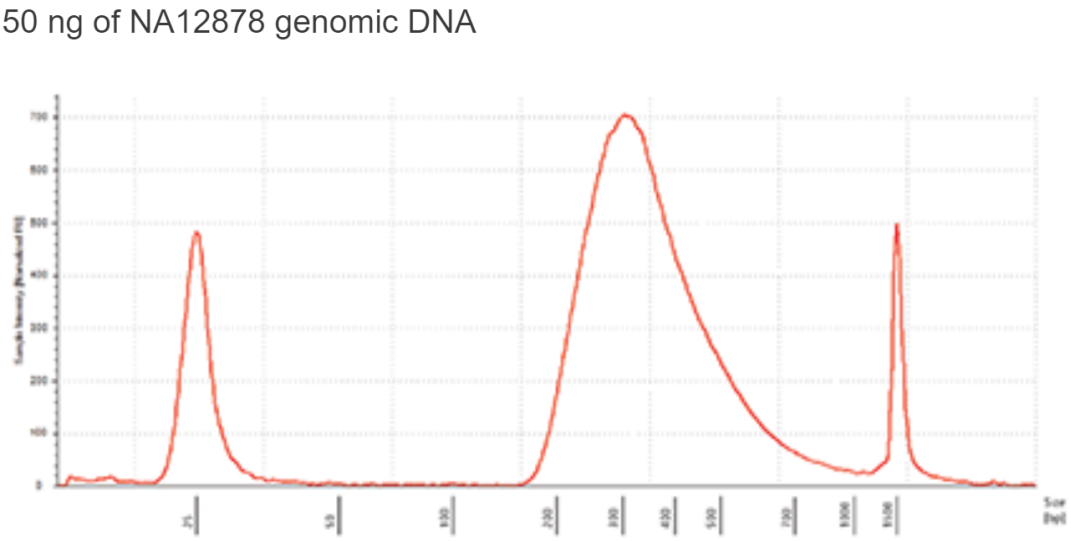
76 Place the tube on the magnetic stand. After 00:03:00 (or whenever the solution is clear), transfer 20 µL of the supernatant to a new PCR tube.

Note

SAFE STOPPING POINT: Samples can be stored overnight at -20 °C .

Library Quantification

77 Use a Bioanalyzer or TapeStation to determine the size distribution and concentration of the libraries. A typical EM-seq library would have the following TapeStation trace.



Sequence using the preferred Illumina platform. 2 x 76 base reads or 2 x 100 base reads for standard sized libraries.