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Version 1

Protocol for Use with Standard Insert Libraries (370-420 bp) (NEB#E7120) V.1



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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

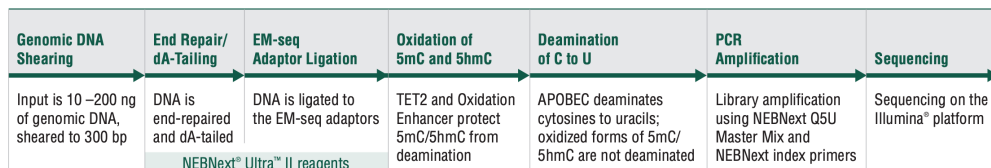


Figure 1. NEBNext Enzymatic Methyl-seq Kit Workflow.

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.

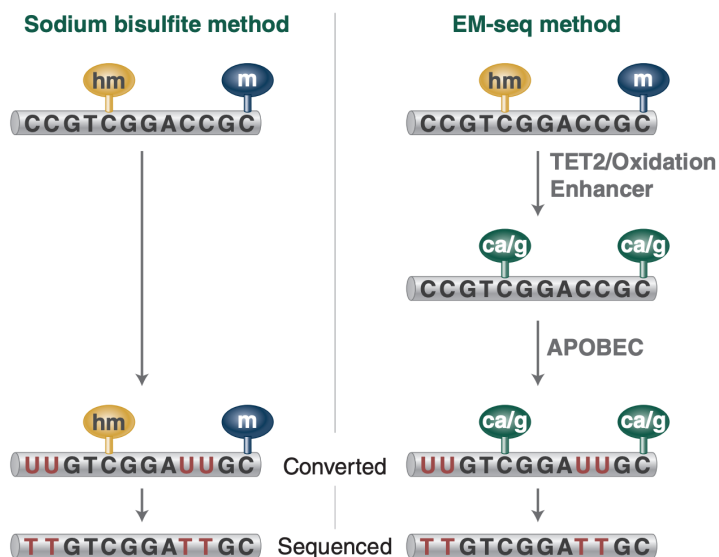


Figure 2. Overview of Sodium Bisulfite Conversion and EM-seq.

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.

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 Custom Solutions department at NEB. Please contact Custom@neb.com for further information.

Materials

MATERIALS

 NEBNext Enzymatic Methyl-seq Kit – 96 rxns **New England Biolabs Catalog #E7120L**

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.

DNA Preparation (Section 1.1)

1 DNA Preparation

Note

Starting materials is 10 ng-200 ng DNA

Combine 10 ng-200 ng of genomic DNA with control DNAs, CpG methylated pUC19 (lilac) and unmethylated lambda DNA (lilac) in  50 μ L made up with 0.1X TE  8 . The amount of control DNA added is dependent on the number of reads required.

Note

If checking library quality on a MiSeq® (2–4 M reads per library) prior to deep sequencing on NovaSeq®, HiSeq® or NextSeq® (100–500 M reads per library) then the amount of controls spiked to the sample DNA is higher than what is required for direct deep sequencing. Having higher ng of control DNA for samples that are sequenced on a MiSeq ensures that there are enough control reads to accurately call cytosine conversion. We recommend this for users who are inexperienced with next generation sequencing library preparation. For libraries sequenced to a depth of 2–4 M paired end reads, approximately 5,000 x 76 base paired end reads of unmethylated lambda and 500 x 76 base paired end reads of CpG methylated pUC19 are needed to give enough reads for accurate conversion estimates. If these same libraries are sequenced to a higher depth of 200–400 M reads per library, then the number of reads associated with the controls would be in vast excess, 500,000 for unmethylated lambda and 50,000 for pUC19.

Recommended Control Inputs:

- Pre-sequencing on MiSeq prior to deep sequencing on NovaSeq, HiSeq or NextSeq: spike in 1 μ L of 0.1 ng/ μ L pUC19 control DNA (lilac) and 1 μ L of 2 ng/ μ L unmethylated lambda DNA (lilac) per 10–200 ng sample DNA.
- Direct Sequencing on NovaSeq, HiSeq or NextSeq: Dilute the pUC19 (lilac) and the unmethylated lambda control (lilac) 1:100 using 0.1X TE, pH 8.0. Spike in 1 μ L diluted pUC19 (0.001 ng) control DNA and 1 μ L diluted unmethylated lambda DNA (0.02 ng) per 10–200 ng sample DNA

2 Shearing DNA

**Note**

The combined  50 μL genomic DNA and control DNAs are fragmented to an average insert size of 240–290 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 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 (Section 1.2)

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

COMPONENT	VOLUME
Fragmented DNA	50 μl
(green) NEBNext Ultra II End Prep Reaction Buffer	7 μl
(green) NEBNext Ultra II End Prep Enzyme Mix	3 μl
<i>Total Volume</i>	60 μl

- 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 thermocycler with the heated lid set to $\geq 75^{\circ}\text{C}$ 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 (Section 1.3)

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



COMPONENT	VOLUME
(red) NEBNext EM-seq Adaptor	2.5 μl
(red) NEBNext Ligation Enhancer	1 μl
(red) NEBNext Ultra II Ligation Master Mix	30 μl
<i>Total Volume</i>	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.



Note

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 Incubate at  20 $^{\circ}\text{C}$ for  00:15:00 in a thermocycler with the heated lid off.



Note

SAFE STOPPING POINT: Samples can be stored overnight at  -20 $^{\circ}\text{C}$.

Clean-Up of Adaptor Ligated DNA (Section 1.4)

- 9 Vortex Sample Purification Beads to resuspend.

- 10 Add  110 μL of resuspended NEBNext Sample Purification Beads to each sample. Mix well by pipetting up and down at least 10 times.



Note

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.

Note

Be careful not to disturb the beads that contain DNA targets.

CAUTION: DO NOT discard the beads.

- 14 Add  200 μ L of 80% freshly prepared ethanol to the tubes while in the magnetic stand.

- 15 Incubate at  Room temperature for  00:00:30 before carefully removing and discarding the supernatant.

**Note**

Be careful not to disturb the beads that contain DNA targets.

- 16 Repeat the ethanol wash once for a total of two washes.

**Note**

Be sure to remove all visible liquid after the second wash using a p10 pipette tip.

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

Note

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.



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

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



Note

If necessary, quickly spin the sample to collect the liquid from the sides of the tube before placing back on the magnetic stand.

20 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 $^{\circ}\text{C}$.

Oxidation of 5-Methylcytosines and 5-Hydroxymethylcytosines (Section 1.5)

21 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.

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.

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.

Note

The reconstituted buffer should be stored at  $-20\text{ }^{\circ}\text{C}$ and discarded after 4 months.

- 22  On ice , add the following components directly to the  28 μL EM-seq adaptor ligated DNA (from Step 20).



COMPONENT	VOLUME
(yellow) TET2 Reaction Buffer (TET2 Reaction Buffer plus reconstituted TET2 Reaction Buffer Supplement)	10 μl
(yellow) Oxidation Supplement	1 μl
(yellow) DTT	1 μl
(yellow) Oxidation Enhancer	1 μl
(yellow) TET2	4 μl

Mix thoroughly by vortexing, centrifuge briefly.

Note

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.

- 23 Dilute the  500 millimolar (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.

Combine Diluted Fe(II) Solution and EM-seq DNA with Oxidation Enzymes (from Step 22).

COMPONENT	VOL UME
EM-seq DNA (from step 22)	45 µl
Diluted Fe(II) Solution (from step 23)	5 µl
<i>Total Volume</i>	50 µl

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

24

Incubate at 37 °C for 01:00:00 in a thermocycler with the heated lid set to ≥ 45 °C or on.



25

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



COMPONENT	VOL UME
(yellow) Stop Reagent	1 µl
<i>Total Volume</i>	51 µl

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

26

Incubate at 37 °C for 00:30:00 then at 4 °C in the thermocycler with the heated lid set to ≥ 45 °C or on.



**Note**

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

Clean-Up of TET2 Converted DNA (Section 1.6)

27 Vortex Sample Purification Beads to resuspend.

28 Add  90 µL of resuspended NEBNext Sample Purification Beads to each sample. Mix well by pipetting up and down at least 10 times.

**Note**

Be careful to expel all of the liquid out of the tip during the last mix.

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

5m



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

31 After  00:05:00 , or when the solution is clear, carefully remove and discard the supernatant.

Note

Be careful not to disturb the beads that contain DNA targets.
CAUTION: DO NOT discard the beads.

32 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.



**Note**

Be careful not to disturb the beads that contain DNA targets.

- 33 Repeat the wash once for a total of two washes.

**Note**

Be sure to remove all visible liquid after the second wash using a p10 pipette tip.

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

Note

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.

- 35 Remove the tubes from the magnetic stand. Elute the DNA target from the beads by adding  17 μ L of Elution Buffer (white) .

- 36 Mix well by pipetting up and down 10 times. Incubate for at least  00:01:00 at  Room temperature .

1m

**Note**

If necessary, quickly spin the sample to collect the liquid from the sides of the tube before placing back on the magnetic stand.

- 37 Place the tube on the magnetic stand. After  00:03:00 , or whenever the solution is clear, transfer  16 μ L of the supernatant to a new PCR tube.



**Note**

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

Denaturation of DNA (Section 1.7)

38

Note

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)

1. Pre-heat thermocycler to 85 °C with the heated lid on.
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.
3. Incubate at 85 °C for 00:10:00 in the pre-heated thermocycler with the heated lid on.
4. Immediately place On ice .
5. Proceed immediately to section 1.8.

Option B: Sodium Hydroxide (Optional, see FAQ about preparing NaOH)

1. Prepare freshly diluted 0.1 N NaOH.
2. Pre-heat thermocycler to 50 °C with the heated lid set to ≥ 60 °C or on.
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.
4. Incubate at 50 °C for 00:10:00 in the pre-heated thermocycler with the heated lid set to ≥ 60 °C or on.
5. Immediately place On ice .
6. Proceed immediately to section 1.8.

Deanimation of Cytosines (Section 1.8)

39  On ice , add the following components to the  20 µL of denatured DNA .

COMPONENT	VOLUME
Nuclease-free water	68 µl
(orange) APOBEC Reaction Buffer	10 µl
(orange) BSA	1 µl
(orange) APOBEC	1 µl
<i>Total Volume</i>	100 µl

Note

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

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

41 Incubate at  37 °C for  03:00:00 , then at  4 °C in a thermocycler with the heated lid set to ≥  45 °C or on.  

Note

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

Clean up of Deanimated DNA (Section 1.9)

42



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.

- 43 Add  100 μ L of resuspended NEBNext Sample Purification Beads to each sample. Mix well by pipetting up and down at least 10 times.



Note

Be careful to expel all of the liquid out of the tip during the last mix.

- 44 Incubate sample on bench top for at least  00:05:00 at  Room temperature .

5m



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

- 46 After  00:05:00 , or when the solution is clear, carefully remove and discard the supernatant.

Note

Be careful not to disturb the beads that contain DNA targets.
CAUTION: DO NOT discard the beads.

- 47 Add  200 μ L 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.



**Note**

Be careful not to disturb the beads that contain DNA targets.

- 48 Repeat the wash once for a total of two washes.

**Note**

Be sure to remove all visible liquid after the second wash using a p10 pipette tip.

- 49 Air dry the beads for up to  00:01:30 while the tubes are on the magnetic stand with the lid open.

1m 30s

Note

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.

- 50 Remove the tubes from the magnetic stand. Elute the DNA target from the beads by adding  21 μ L of Elution Buffer (white) .

- 51 Mix well by pipetting up and down 10 times. Incubate for at least  00:01:00 at  Room temperature .

**Note**

If necessary, quickly spin the sample to collect the liquid from the sides of the tube before placing back on the magnetic stand.

- 52 Place the tube on the magnetic stand. After  00:03:00 , or whenever the solution is clear, transfer  20 μ L of the supernatant to a new PCR tube.



Note

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

PCR Amplification (Section 1.10)

- 53  On ice , add the following components to the  20 µL of deaminated DNA from Step 52:

COMPONENT	VOLUME
EM-seq Index Primer*, **	5 µl
(blue) NEBNext Q5U Master Mix	25 µl
<i>Total Volume</i>	50 µl

* Refer to section 3 in the manual for barcode pooling guidelines

(<https://www.neb.com/-/media/nebus/files/manuals/manuale7120.pdf>)

** EM-seq primers are supplied in tubes in #E7120S or as a 96 Unique Dual Index Primer Pairs Plate in #E7120L

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

- 55 Place the tube in a thermocycler and perform PCR amplification using the following cycling conditions: 

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

Note

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

Clean-Up of Amplified Libraries (Section 1.11)

56 Vortex Sample Purification Beads to resuspend.

57 Add 45 µL resuspended NEBNext Sample Purification Beads to each sample. Mix well by pipetting up and down at least 10 times.



Note

Be careful to expel all of the liquid out of the tip during the last mix.

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

5m



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

60 After 00:05:00 , or when the solution is clear, carefully remove and discard the supernatant.

**Note**

Be careful not to disturb the beads that contain DNA targets.

CAUTION: DO NOT discard the beads.

- 61 Add  200 μ L 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.

**Note**

Be careful not to disturb the beads that contain DNA targets.

- 62 Repeat the wash once for a total of two washes.

**Note**

Be sure to remove all visible liquid after the second wash using a p10 pipette tip.

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

2m

Note

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.

- 64 Remove the tubes from the magnetic stand. Elute the DNA target from the beads by adding  21 μ L of Elution Buffer (white) or



🧪 21 μ L of TE 10 mM Tris, 0.1 mM EDTA, pH 8.0) or low TE (for long term storage) .

- 65 Mix well by pipetting up and down 10 times. Incubate for at least ⌚ 00:01:00 at 🌡 Room temperature .



Note

If necessary, quickly spin the sample to collect the liquid from the sides of the tube before placing back on the magnetic stand.

- 66 Place the tube on the magnetic stand. After ⌚ 00:03:00 , or whenever the solution is clear, transfer 🧪 20 μ L of the supernatant to a new PCR tube.

Note

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

Library Quantification (Section 1.12)

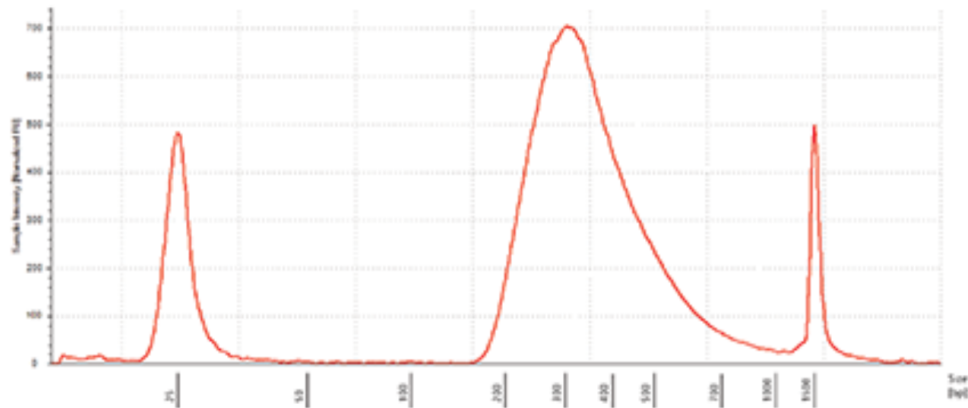
- 67 Use a Bioanalyzer or TapeStation to determine the size distribution and concentration of the libraries.



Note

A typical EM-seq library would have the following TapeStation trace.

50 ng of NA12878 genomic DNA



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