EpiNext™ 5-mC RNA Bisulfite-Seq Easy Kit (Illumina)

Base Catalog # P-9007

PLEASE READ THIS ENTIRE USER GUIDE BEFORE USE

Uses: The EpiNext™ 5-mC RNA Bisulfite-Seq Easy Kit (Illumina) is designed for easily carrying out RNA bisulfite conversion, followed by a "post-bisulfite" library preparation process for Illumina platform-based bisulfite sequencing, all in one kit. Intended applications include whole transcriptome RNA bisulfite sequencing and various other RNA bisulfite-based next generation sequencing techniques for RNA methylation analysis. The optimized protocol and components of the kit allow the RNA to be bisulfite converted and fragmented simultaneously, followed by quick non-barcoded (singleplexed) and barcoded (multiplexed) library construction using low-nanogram quantities of bisulfite converted RNA.

Input RNA: Starting materials can be total RNA isolated from various tissue/cell samples such as fresh and frozen tissues, cultured cells from a flask or microplate, microdissection samples, and body fluid samples, etc. The amount of RNA for each bisulfite reaction can be 5 ng to 500 ng. For an optimal reaction, the input RNA amount should be 100 ng to 200 ng. The yield of RNA purified after bisulfite conversion depends on the amount of input RNA, nature of RNA, and source of the starting material.

Precautions: To avoid cross-contamination, the following precautions are necessary for handling EpigenTek columns or tubes: Carefully pipette the sample or solution into the columns or tubes. Use aerosol-barrier pipette tips and always change pipette tips between liquid transfers. Always cap the F-Spin Columns before placing them in a microcentrifuge. Wear gloves throughout the entire procedure. In case of contact between gloves and sample, change gloves immediately.

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KIT CONTENTS

Component	12 reactions Cat. #P-9007-12	24 reactions Cat. #P-9007-24	Storage Upon Receipt
Conversion Buffer	3 ml	6 ml	RT
Conversion Powder	2 vials	4 vials	RT
NA Binding Solution	6 ml	12 ml	RT
F-Spin Column**	15	30	RT
F-Collection Tube	15	30	RT
Desulphonation Solution	100 µl	200 µl	RT
5X Reaction Buffer*	100 µl	200 µl	-20ºC
Reaction Enzyme Mix*	50 µl	100 µl	-20°C
Adaptor-A (10 µM)*	28 µl	56 µl	-20°C
Adaptor-B (10 µM)*	28 µl	56 µl	-20°C
RNA Digestion Buffer	30 µl	60 µl	-20°C
RNA Digestion Enzyme*	15 µl	30 µl	-20°C
MQ Binding Beads*	1.8 ml	2X (1.8 ml)	4°C
2X HiFi PCR Master Mix*	160 µl	320 µl	-20°C
Primer U (10 µM)*	15 µl	30 µl	-20°C
Primer I (10 µM)*	15 µl	30 µl	-20°C
Elution Buffer*	1000 µl	2000 µl	-20°C

**Spin the solution down to the bottom before use.*
***Always cap spin columns before placing them in the microcentrifuge.*

SHIPPING & STORAGE

The kit is shipped in two parts: the first part at ambient room temperature and the second part on frozen ice packs at 4°C

Upon receipt: (1) Store the following components at -20°C immediately: **5X Reaction Buffer**, **Reaction Enzyme Mix**, **Adaptor-A**, **Adaptor-B**, **RNA Digestion Buffer**, **RNA Digestion Enzyme**, **2X HiFi PCR Master Mix**, **Primer U**, **Primer I**, and **Elution Buffer**. (2) Store the following components at 4°C: **MQ Binding Beads**. (3) Store all other components at room temperature away from light.

The kit is stable for up to 6 months from the shipment date, when stored properly.

MATERIALS REQUIRED BUT NOT SUPPLIED

- ☐ Vortex mixer
- ☐ Agilent® Bioanalyzer® or comparable method to assess the quality of DNA library
- ☐ Thermocycler

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- ☐ Centrifuge including desktop centrifuge (up to 14,000 rpm)
- ☐ Magnetic stand (96-well format)
- ☐ Pipettes and pipette tips
- ☐ PCR tubes or plates
- ☐ 1.5 ml microcentrifuge tubes
- ☐ 100% ethanol
- ☐ Distilled water
- ☐ RNA sample

GENERAL PRODUCT INFORMATION

Quality Control: Each lot of EpiNext™ 5-mC RNA Bisulfite-Seq Easy Kit (Illumina) is tested against predetermined specifications to ensure consistent product quality. EpigenTek guarantees the performance of all products in the manner described in our product instructions.

Product Warranty: If this product does not meet your expectations, simply contact our technical support unit or your regional distributor. We also encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

Safety: Suitable lab coat, disposable gloves, and proper eye protection are required when working with this product.

Product Updates: EpigenTek reserves the right to change or modify any product to enhance its performance and design. The information in this User Guide is subject to change at any time without notice. Be sure to use the latest User Guide for this kit which can be accessed online at www.epigentek.com/datasheet.

Usage Limitation: The EpiNext™ 5-mC RNA Bisulfite-Seq Easy Kit (Illumina) is for research use only and is not intended for diagnostic or therapeutic application.

Intellectual Property: The EpiNext™ 5-mC RNA Bisulfite-Seq Easy Kit (Illumina) and methods of use contain proprietary technologies by EpigenTek.

A BRIEF OVERVIEW

5-methylcytosine (5-mC) in DNA occurs by the covalent addition of a methyl group at the 5-carbon of the cytosine ring by DNA methyltransferases. This process has been well studied and is generally associated with repression of gene expression. It was also observed that in humans, 5-mC occurs in various RNA molecules including tRNAs, rRNAs, mRNAs and non-coding RNAs (ncRNAs). At least 10,275 5-mC candidate sites were discovered in mRNAs and ncRNAs, which cover 10.6% of the total cytosine residues in the transcriptome. 5-mC seems to be enriched in some classes of ncRNA, but relatively depleted in mRNAs. However, the majority (83%) of their candidate sites were found in mRNAs. Within these transcripts 5-mC appears to be depleted within protein coding sequences, but enriched in 5' and 3' UTRs. Two different methyltransferases, NSUN2 and Dnmt2 are known to

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catalyze 5-mC modification in eukaryotic RNA. Recent data strongly suggest that RNA cytosine methylation affects the regulation of various biological processes such as RNA stability and mRNA translation. Furthermore, loss of 5-mC in vault RNAs causes aberrant processing into Argonaute-associated small RNA fragments that can function as microRNAs. Thus, impaired processing of vault ncRNA may contribute to the etiology of human disorders related to NSun2-deficiency.

Bisulfite conversion of RNA followed by next generation sequencing yields reliable information about RNA cytosine methylation states on a transcriptome-wide scale. To effectively and efficiently prepare a bisulfite-converted RNA library for use in next generation sequencing, EpigenTek first developed the EpiNext™ RNA Bisulfite-Seq Kit (Illumina) (Cat. #P-9006) by utilizing its "Post-Bisulfite" technology and further refines its methylated RNA assay expertise with the development of the EpiNext™ 5-mC RNA Bisulfite-Seq Easy Kit (Illumina). This kit has the following advantages and features:

- **High sensitivity and efficiency:** By using an innovative method that enables adaptor tagging of bisulfite-converted RNA to be intact through random probing with blocking formation of un-tagged or half-tagged RNA fragments, both non-barcoded (singleplexed) and barcoded (multiplexed) library preparation can be reliably made with high sensitivity and efficiency, thereby providing a robust and reliable means to build 5mC RNA-seq library. The optimized RNA bisulfite method and enhanced adaptor tagging eliminates loss of fragments and selection bias, which enables input RNA to be as low as 5 ng with increased efficiency of completely tagged cDNA library.

- **Easy and streamlined procedure:** The entire procedure can be finished in 6 hours. Gel-free size selection/purification saves time and prevents handling errors, as well as loss of valuable samples.

- **Complete conversion:** The innovative reagent composition converts unmethylated cytosine into uracil at a level greater than 99.9%, with no or negligible inappropriate/error conversion of methylcytosine to thymine (<0.1%) when the indicated range of sample RNA is used.

- **Extremely convenient:** The kit contains all the required components for each step of the RNA library preparation process, which is sufficient for bisulfite conversion, ligation, clean-up, size selection, and library amplification, thereby allowing the bisulfite RNA library preparation to be streamlined for the most reliable and consistent results.

- **Minimal bias:** Ultra HiFi amplification enables achievement of reproducibly high yields of bisulfite converted RNA libraries with minimal sequence bias and low error rates.
- **Broad sample suitability:** Starting materials can be total RNA isolated from various tissue/cell samples such as fresh and frozen tissue, cultured cells from a flask or microplate.

PRINCIPLE & PROCEDURE

This kit includes all reagents required for a successful RNA bisulfite conversion and bisulfite RNA library preparation using bisulfite-converted RNA generated from a wide range of input RNA amounts (5 ng to 500 ng). In this preparation, RNA is simultaneously bisulfite converted and fragmented to the appropriate length during the bisulfite process. The bisulfite-treated RNA, which is in single stranded form, is then simultaneously converted to double stranded cDNA and adaptor tagged. The tagged fragments are size selected and purified using **MQ Binding Beads**, followed by amplification with a high-fidelity PCR Mix, which ensures maximum yields from minimum amounts of starting material and provides highly accurate amplification of library cDNA with low error rates and minimum bias.

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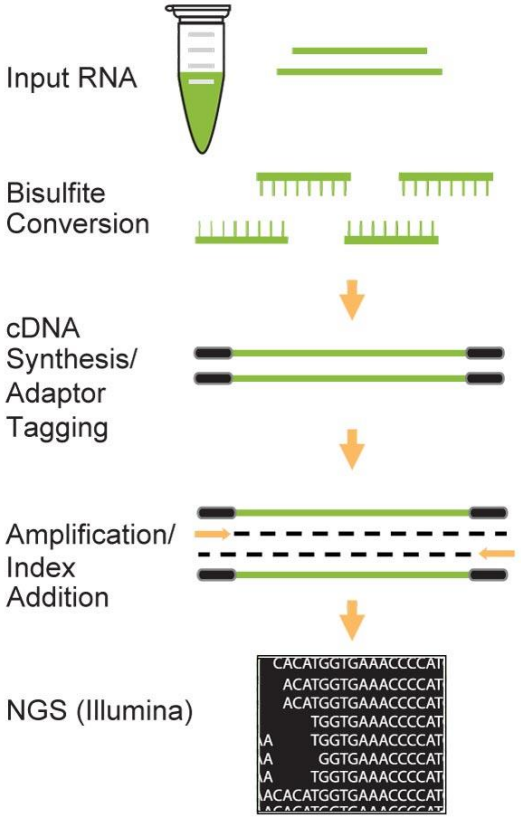

Fig 1: Workflow of the EpiNext™ 5-mC RNA Bisulfite-Seq Easy Kit (Illumina).

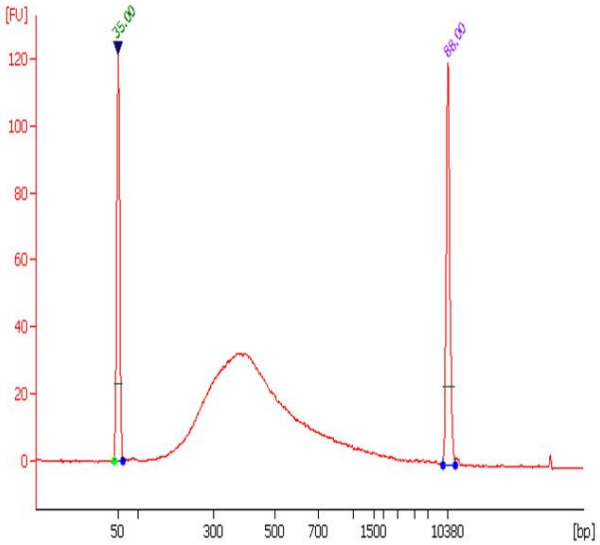

Fig 2: Size distribution of library fragments: Post-bisulfite cDNA library was prepared from 50 ng of input mouse RNA using the EpiNext™ 5-mC RNA Bisulfite-Seq Easy Kit (Illumina).

ASSAY PROTOCOL

For the best results, please read the protocol in its entirety prior to starting your experiment.

Starting Materials

Input RNA Amount: RNA amount can range from 5 ng to 500 ng per reaction. An optimal amount is 100 ng to 200 ng per reaction. Starting RNA may be in water or in a buffer such as TE. RNA should be high quality and relatively free of DNA. DNase I can be used to remove DNA. RNA should be eluted in RNase-free water.

RNA Storage: RNA should be stored at -20°C or -80°C until use.

1. Working Buffer and Solution Preparation

a. Prepare Conversion Solution:

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Add 1.4 ml of **Conversion Buffer** and 40 µl of **Desulphonation Solution** to 1 vial of **Conversion Powder** to generate conversion solution. Mix by inverting and shaking the vial repeatedly for 3-4 minutes (trace amount of undissolved **Conversion Powder** may remain, which is normal as **Conversion Powder** is saturated in solution).

b. Prepare *70% ethanol* by adding 3 ml of distilled water to 7 ml of *100% ethanol*.

c. Prepare *90% ethanol* by adding 1ml of distilled water to 9 ml of *100% ethanol*.

d. Prepare working desulphonation buffer:

First dilute **Desulphonation Solution** at 1:12 ratio by adding 5 µl of **Desulphonation Solution** to 55 µl of distilled water. Next add 2 µl of diluted **Desulphonation Solution** to every 1 ml of *90% ethanol* and mix.

2. RNA Bisulfite Conversion

a. Add 100 µl of the conversion solution to a PCR tube followed by adding 2-10 µl of RNA sample.

Prepared conversion solution can be stored at -20°C for up to 2 weeks without significant loss of efficiency. For the best results, the mixed solution should be used immediately.

b. Tightly close the PCR tubes and place them in a thermal cycler with heated lid. Program and run the thermal cycler:

65°C 5 min
60°C 90 min
Hold 4°C up to 16 h

Meanwhile, insert the number of **F-Spin Columns** into **F-Collection Tubes** as needed by your experiment.

3. Converted RNA Clean-Up

a. Add 250 µl of **NA Binding Solution** to each column. Then transfer the samples from each PCR tube (from Step 2b) to each column containing the **NA Binding Solution**. Centrifuge at 12,000 rpm for 1 minute. Remove columns from collection tubes and discard the flowthrough. Place columns back into collection tubes.

b. Add 200 µl of *70% ethanol* solution to each column. Centrifuge at 12,000 rpm for 1 minute.

c. Add 200 µl of the working desulphonation buffer (diluted **Desulphonation Solution** and *90% ethanol* mixture from Step 1d) to each column. Allow columns to sit for 30 minutes at room temperature, then centrifuge at 12,000 rpm for 1 minute. Remove columns from collection tubes and discard the flowthrough. Place columns back into collection tubes.

d. Add 200 µl of *90% ethanol* to each column. Centrifuge at 12,000 rpm for 1 minute. Remove columns from collection tubes and discard the flowthrough. Place columns back into collection tubes. Add 200 µl of *90% ethanol* to each column again and centrifuge at 12,000 rpm for 1 minute.

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e. Insert each column into a new 1.5 ml tube. Add 12 µl of **Elution Buffer** directly to each column's filter membrane. Centrifuge at 12,000 rpm for 1 minute to elute converted RNA.

Converted RNA is now ready for use, or storage at or below –20°C for up to 2 months. To validate the conversion efficiency, we recommend performing RT-PCR after cDNA synthesis with use of our positive control primers (#P-9003-50-F and #P-9003-50-R).

4. cDNA Synthesis

a. Prepare cDNA synthesis reaction in 0.2 ml PCR tube according to Table 1:

Table 1. cDNA Conversion

Component	Volume
Converted RNA*	10 µl (50-100 ng input RNA)
5X Reaction Buffer	3.5 µl
Adaptor-A (10 µM)	2 µl
Conversion Enzyme Mix	2 µl
Total Volume	**17.5 µl**

***** *If converted RNA volume is less than 10 µl, add distilled water to make the total volume 10 µl.*

Mix and incubate for 60 minutes at 37°C in a thermocycler without heated lid (make sure to set lid temperature to 25°C).

b. Add 1.8 µl of **RNA Digestion Buffer** and 1 µl of **RNA Digestion Enzyme** to each reaction, mix by pipetting and incubate at 37˚C for 10 minutes.

5. cDNA Purification

a. Resuspend **MQ Binding Beads** by vortex.
b. Add 24 µl of resuspended beads to the PCR tube of cDNA synthesis reaction. Mix thoroughly on a vortex mixer or by pipetting up and down at least 10 times.
c. Incubate for 6 minutes at room temperature to allow cDNA to bind to beads.
d. Put the PCR tube on an appropriate magnetic stand until the solution is clear (about 4 minutes). Carefully remove and discard the supernatant. *(Caution: Be careful not to disturb or discard the beads that contain cDNA.)*
e. Keep the PCR tube in the magnetic stand and add 160 µl of freshly prepared *90% ethanol* to the tube. Incubate at room temperature for 1 minute, and then carefully remove and discard the ethanol.
f. Repeat Step 5e once for total of two washes.
g. Open the PCR tube cap and air dry beads for 1-2 minutes while the tube is on the magnetic stand.
h. Resuspend the beads in 11 µl **Elution Buffer** and incubate at room temperature for 6 minutes to release the cDNA from the beads.
i. Capture the beads by placing the tube in the magnetic stand for 2 minutes or until the solution is completely clear.
j. Transfer 11 µl to a new 0.2 ml PCR tube.

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6. Library Synthesis

a. Prepare library synthesis reaction in a 0.2 ml PCR tube according to Table 2:

Table 2. Library Synthesis

Component	Volume
cDNA (from Step 5)	10 µl
5X Reaction Buffer	3.5 µl
Adaptor-B (10 µM)	2 µl
Total Volume	**15.5 µl**

b. Mix and incubate for 2 minutes at 98°C in a thermocycler without heated lid (make sure to set lid temperature to 25°C) followed by incubation on ice for 2 minutes. Add 2 µl of **Conversion Enzyme Mix** and then incubate at 37°C for 60 minutes in a thermocycler without heated lid.

7. Clean-Up of Synthesized Library

a. Resuspend **MQ Binding Beads** by vortex.
b. Add exactly 21 µl of resuspended beads to the tube of library synthesis reaction. Mix thoroughly on a vortex mixer or by pipetting up and down at least 10 times.
c. Incubate for 6 minutes at room temperature to allow cDNA to bind to beads.
d. Put the PCR tube on an appropriate magnetic stand until the solution is clear (about 2 minutes). Carefully remove and discard the supernatant. Be careful not to disturb or discard the beads that contain cDNA.
e. Keep the PCR tube in the magnetic stand and add 160 µl of freshly prepared *90% ethanol* to the tube. Incubate at room temperature for 1 minute, and then carefully remove and discard the ethanol.
f. Repeat Step 7e one more time for a total of two washes.
g. Open the cap of the PCR tube and air dry beads for 1-2 minutes while the tube is on the magnetic stand.
h. Resuspend the beads in 11 µl **Elution Buffer**, and incubate at room temperature for 6 minutes to release the cDNA from the beads.
i. Capture the beads by placing the tube in the magnetic stand for 2 minutes or until the solution is completely clear.
j. Transfer 10.5 µl of clear solution to a new 0.2 ml PCR tube for library amplification and indexing.

***Note**: The synthesis library can be quantified with use of fluorescence method such as Picogreen or real time qPCR so that the concentration of library can be obtained for determining the cycles for library amplification.*

8. Library Amplification

a. Prepare the PCR Reactions

Thaw all reaction components including master mix, DNA/RNase free water, primer solution, and library template. Mix well by vortexing briefly. Keep components on ice while in use, and return to -20˚C immediately following use. Add components into each PCR tube/well according to the following table:

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Component	Size (µl)
HiFi Master Mix (2X)	12.5 µl
Primer U	1 µl
Primer I	1 µl
cDNA Library	10.5 µl
Total Volume	**25 µl**

Important Note: *Use of Primer I included in the kit will generate a singleplexed library. For multiplexed library preparation, replace the Primer I with one of 12 different barcodes (index) from the EpiNext™ NGS Barcode (Index) Set-12 (Cat. No. P-1060). You can also add user-defined barcode (Illumina compatible) instead of Primer I.*

b. Program the PCR Reactions

Place the reaction plate in the instrument and set the PCR conditions as follows:

Cycle Step	Temp	Time	Cycle
Activation	98°C	30 sec	1
Cycling	98°C 55°C 72°C	10 sec 20 sec 20 sec	Variable*
Final Extension	72°C	2 min	1

** PCR cycles may vary depending on the amount of input RNA or concentration of the synthesized cDNA library. In general, 12 PCR cycles for 200 ng, 13 PCR cycles for 100 ng, 14 cycles for 50 ng, and 19 cycles for 5 ng RNA input. Further optimization of PCR cycle number may be required.*

9. Clean-up of Amplified Library cDNA

a. Resuspend **MQ Binding Beads** by vortex.
b. Add 22.5 µl of resuspended beads to the PCR tube of amplification reaction. Mix thoroughly on a vortex mixer or by pipetting up and down at least 10 times.
c. Incubate for 6 minutes at room temperature to allow cDNA to bind to beads.
d. Put the PCR tube on an appropriate magnetic stand until the solution is clear (about 4 minutes). Carefully remove and discard the supernatant. *(Caution: Be careful not to disturb or discard the beads that contain cDNA.)*
e. Keep the PCR tube in the magnetic stand and add 160 µl of freshly prepared *90% ethanol* to the tube. Incubate at room temperature for 1-2 minutes, and then carefully remove and discard the ethanol.
f. Repeat Step 9e once for a total of two washes.
g. Open the PCR tube cap and air dry beads for 1-2 minutes while the tube is on the magnetic stand.
h. Resuspend the beads in 12.5 µl **Elution Buffer**, and incubate at room temperature for 6 minutes to release the DNA from the beads.
i. Capture the beads by placing the tube in the magnetic stand for 2 minutes or until the solution is completely clear.
j. Transfer 12 µl to a new 0.2 ml PCR tube.

Note: *(1) Quality of the prepared library can be assessed using an Agilent® Bioanalyzer® or comparable method. Library fragments should have the correct size distribution (ex: 250 bps at peak size) without adaptors or adaptor-dimers (about 127 bps). (2) To check the size distribution, dilute*

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*library with water (if necessary) and apply it to an Agilent high sensitivity chip. If there is the presence of <150 bp adaptor dimers, it is recommend to use 0.8X **MQ Binding Beads** to remove fragments below 150 bps. (3) The amount of indexed library can be quantified using qPCR, Qubit or Picogreen assays. (4) Each indexed library can be combined in equal amounts to form multiplexed libraries for sequencing.*

The prepared cDNA library can be stored at -20˚C until ready to use for sequencing.

TROUBLESHOOTING

Problem	**Possible Causes**	**Suggestions**
RNA is poorly modified	Poor RNA quality (RNA is severely degraded).	Check if the sample RNA 260/280 ratio is between 1.9 - 2.0.
	Too little RNA or too much RNA (i.e., <1 ng or >1 µg).	Increase or decrease input RNA to within the correct range, or to the optimal range of 100 ng to 200 ng.
	Temperature or thermal cycling condition is incorrect.	Check for appropriate temperature or thermal cycling conditions.
	Insufficient RNA clean-up.	Ensure that 2 µl of diluted **Desulphonation Solution** is added into every 1 ml of *90% ethanol* in Step 1d.
	Kit is not stored or handled properly.	Store all components of the kit according to the storage conditions specified in this user guide.
Eluate contains little or no RNA	Poor input RNA quality (degraded).	Check if RNA is degraded.
	NA Binding Solution is not added into the sample.	Ensure that **NA Binding Solution** is added in Step 3a.
	Concentration of ethanol solution used for RNA clean-up is not correct.	Use *90% ethanol* for RNA clean-up.
Low yield of library	Insufficient amount of starting RNA.	To obtain the best results, the amount of input RNA should be >5 ng.
	Improper reaction conditions at each reaction step.	Check if the reagents are properly added and incubation temperature and time are correct at each reaction step including End Polishing, Adaptor Ligation, Size Selection and Library Amplification.
	Improper storage of the kit.	Ensure that the kit has not exceeded the expiration date. Standard shelf life, when stored properly, is 6 months from date of receipt.
Unexpected peak size	Improper ratio of **MQ Binding**	Check if the correct volume of **MQ**

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of Agilent Bioanalyzer trace: Presence of <150 bp adaptor dimers or presence of larger fragments than expected.	**Beads** to cDNA volume during size selection.	**Binding Beads** is added to the cDNA solution accordingly. Proper ratios should remove the fragments of unexpected peak size.
	Insufficient tagging.	Too much or too little input cDNA may cause insufficient tagging, which can shift the peak size of the fragment population to be shorter or larger than expected. Make sure that the tagging reaction (at cDNA synthesis and library synthesis steps) is properly processed with the proper amount of input cDNA.
	Over-amplification of library.	PCR artifacts from over-amplification of the library may cause the fragment population to shift higher than expected. Make sure to use proper PCR cycles to avoid this problem.

RELATED PRODUCTS

DNA Bisulfite Modification

P-1001 Methylamp™ DNA Modification Kit
P-1010 Methylamp™ One-Step DNA Modification Kit
P-1016 Methylamp™ Whole Cell Bisulfite Modification Kit
P-1026 BisulFlash™ DNA Modification Kit

RNA Bisulfite Conversion

P-9003 Methylamp™ RNA Bisulfite Conversion Kit

Methylation Analysis

P-1028 Methylamp™ MS-qPCR Fast Kit
P-1056A EpiNext™ High-Sensitivity Bisulfite-Seq Kit (Illumina)

NGS Barcode

P-1060 EpiNext™ NGS Barcode (Index) Set-12

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