

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

Catalog #: *ENZ-GEN511*

ENZ-GEN511-0012 – for 12 reactions

ENZ-GEN511-0024 – for 24 reactions

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Product Manual

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TABLE OF CONTENTS

Carefully note the handling and storage conditions of each kit component.

INTRODUCTION	2
BACKGROUND	4
MATERIALS SUPPLIED	5
ADDITIONAL MATERIALS SUPPLIED	6
STORAGE & STABILITY	6
SAFETY WARNINGS & PRECAUTIONS	7
PRINCIPLE OF THE ASSAY	7
PROCEDURE	9
TROUBLESHOOTING	16
CONTACT INFORMATION	18

INTRODUCTION

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 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, we first developed the RNA Bisulfite-Seq Kit (Illumina) (Cat. ENZ-GEN510) by utilizing its “Post-Bisulfite” technology and further refines its methylated RNA assay expertise with the development of the AMPINEXT™ 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.

BACKGROUND

Uses: The AMPINEXT™ 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 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.

MATERIALS SUPPLIED

Component	12 reactions (ENZ-GEN511-0012)	24 reactions (ENZ-GEN511-0024)	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	-20°C
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
Product Manual	1	1	RT

*Spin the solution down to the bottom before use.

**Always cap spin columns before placing them in the microcentrifuge.

ADDITIONAL MATERIALS NEEDED

The following are required but not provided:

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



Storage temp

STORAGE & STABILITY

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.

SAFETY WARNINGS & PRECAUTIONS



Important/ Warning

1. Wear appropriate personnel protective apparel. Avoid contact with clothes and exposed skin. In case of accidental skin exposure, flush with water immediately. Consult a physician if required.
2. Use a safety pipetting device for all pipetting. Never pipet by mouth.
3. Interpretation of the results is the sole responsibility of the user.

PRINCIPLE OF THE ASSAY

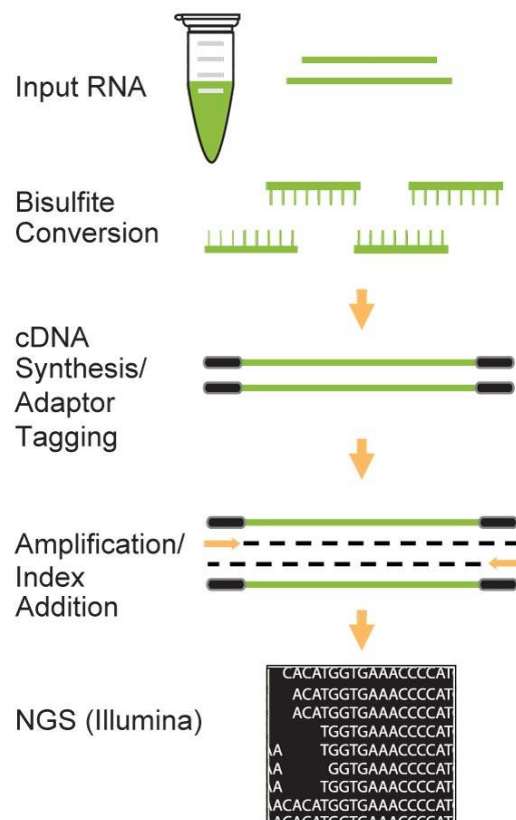


Fig 1: Workflow of the AMPINEXT™ 5-mC RNA Bisulfite-Seq Easy Kit (Illumina).

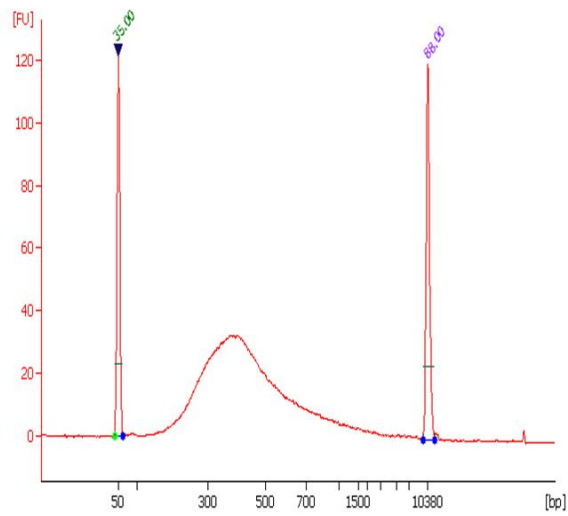


Fig 2: Size distribution of library fragments: Post-bisulfite cDNA library was prepared from 50 ng of input mouse RNA using the AMPINEXT™ 5-mC RNA Bisulfite-Seq Easy Kit (Illumina).

PROCEDURE

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:

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 1 ml 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.

- e. Insert each column into a new 1.5 ml tube. Add 12 μ l 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.

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

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

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 μ l of **RNA Digestion Buffer** and 1 μ l 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 μ l 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 μ l 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 μ l **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 μ l to a new 0.2 ml PCR tube.

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 μ l
5X Reaction Buffer	3.5 μ l
Adaptor-B (10 μM)	2 μ l
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 μ l 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 μ l 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 μ l 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 μ l **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 μ l 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:

Component	Size (μ l)
HiFi Master Mix (2X)	12.5 μ l
Primer U	1 μ l
Primer I	1 μ l
cDNA Library	10.5 μ l
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 AMPINEXT™ NGS Barcode (Index) Set-12 (Cat. No. ENZ-GEN507). 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	10 sec	Variable*
	55°C	20 sec	
	72°C	20 sec	
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 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 <u>diluted Desulphonation Solution</u> is added into every 1 ml of <u>90% ethanol</u> in Step 1d.
	Kit is not stored or handled properly.	Store all components of the kit according to the storage conditions specified in this Product Manual.
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 <u>90% ethanol</u> 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.

<p>Unexpected peak size: Presence of <150 bp adaptor dimers or presence of larger fragments than expected.</p>	<p>Improper ratio of MQ Binding Beads to cDNA volume during size selection.</p>	<p>Check if the correct volume of MQ Binding Beads is added to the cDNA solution accordingly. Proper ratios should remove the fragments of unexpected peak size.</p>
	<p>Insufficient tagging.</p>	<p>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.</p>
	<p>Over-amplification of library.</p>	<p>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.</p>



Product Manual

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