Enzo

Product Manual

AMPINEXT™ RNA Bisulfite-Seq Kit (Illumina)

Catalog #: *ENZ-GEN510*

ENZ-GEN510-0012 – for 12 reactions

ENZ-GEN510-0024 – for 24 reactions

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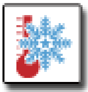

Carefully note the handling and storage conditions of each kit component.

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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 developed AMPINEXT™ RNA Bisulfite-Seq Kit (Illumina) by utilizing its "Post-Bisulfite" technology. The kit is specifically optimized and validated for RNA bisulfite conversion and converted RNA library preparation. As the first commercially available RNA bisulfite-seq product, this kit has the following advantages and features:

- **Fast 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.

- **High sensitivity, efficiency and flexibility:** Can be used for both non-barcoded (singleplexed) and barcoded (multiplexed) library preparation. Optimized RNA bisulfite method and enhanced adaptor ligation eliminates loss of fragments and selection bias, which enables input RNA to be as low as 5 ng.

- **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.

BACKGROUND

Uses: The AMPINEXT™ RNA Bisulfite-Seq Kit (Illumina) is designed to carry 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-1 µg. For an optimal reaction, the input RNA amount should be 200-500 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-GEN510-0012)	24 reactions (ENZ-GEN510-0024)	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 RT Reaction Buffer*	70 µl	140 µl	-20ºC
10 mM dNTP Mix*	15 µl	30 µl	-20ºC
0.1M DTT*	30 µl	60 µl	-20ºC
RNase Inhibitor*	15 µl	30 µl	-20ºC
Random Primer (50 µM)*	15 µl	30 µl	-20ºC
RT Enzyme Mix*	15 µl	30 µl	-20ºC
10X End Polishing Buffer*	30 µl	60 µl	-20°C
End Polishing Enzyme Mix*	13 µl	26 µl	-20°C
End Polishing Enhancer*	13 µl	26 µl	-20°C
2X Ligation Buffer*	250 µl	500 µl	-20°C
T4 DNA Ligase*	15 µl	30 µl	-20°C
Adaptors (50 µM)*	15 µl	30 µl	-20°C
MQ Binding Beads*	1.6 ml	3.2 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
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**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 on frozen ice packs at 4°C.

Upon receipt: (1) Store the following components at -20°C immediately: **5X RT Reaction Buffer**, **10 mM dNTP mix**, **0.1M DTT, RNase Inhibitor, Random Primer**, **RT Enzyme Mix, 10X End Polishing Buffer**, **End Polishing Enzyme Mix**, **End Polishing Enhancer, 2X Ligation Buffer**, **T4 DNA Ligase**, **Adaptors**, **2X HiFi PCR Master Mix**, **Primer U**, **Primer I**, and **Elution Buffer.** Store the following components at 4°C: **MQ Binding Beads**. Store all other components at room temperature.

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

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 1 µg). In this preparation, RNA is simultaneously bisulfite converted and fragmented to the appropriate length during the bisulfite process. cDNA is synthesized from the bisulfite-treated RNA and used for ligation with specific adaptors that are necessary for amplification and sequencing. The fragments are size selected and purified using **MQ Binding Beads**, which allows for quick and precise size selection of DNA. Size-selected DNA fragments are amplified using a high-fidelity PCR Mix, which ensures maximum yields from minimum amounts of starting material and provides highly accurate amplification of library DNA with low error rates and minimal bias.

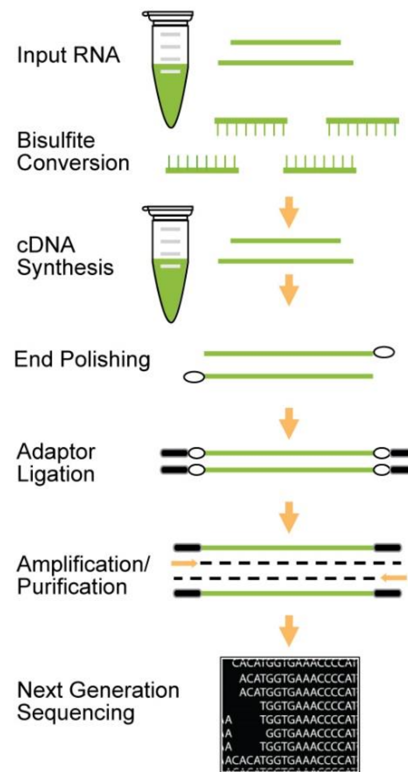

Fig 1: Workflow of the AMPINEXT™ RNA Bisulfite-Seq Kit.

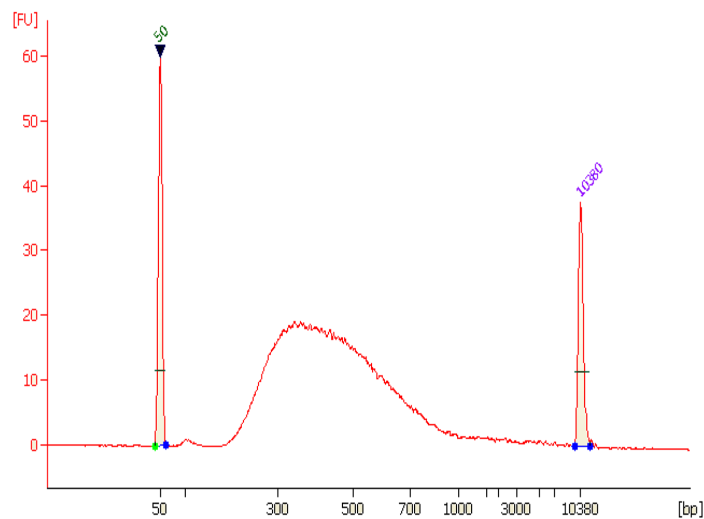

Fig 2: Size distribution of library fragments: Post-bisulfite cDNA library was prepared from 10 ng of input RNA using the AMPINEXT™ RNA Bisulfite-Seq Kit.

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 1 µg per reaction. An optimal amount is 200–500 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 min (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 min. 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 min.

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 min at room temperature, then centrifuge at 12,000 rpm for 1 min. 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 min. 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 min.

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 min to elute converted RNA.

f. 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. Add the following to a 0.2-ml PCR tube on ice:

Component	Amount
Bisulfite-converted RNA (200-500 ng)	10 µl
Random Primer (50 uM)	1 µl
10 mM dNTP Mix	1 µl

b. Heat in a thermocycler (no heated lid) at 65°C for 3 minutes. Place immediately on ice for at least 1 minute.

c. Add the following to the tube on ice:

5X RT Reaction Buffer	4 µl
0.1M DTT	2 µl
RNase Inhibitor	1 µl
RT Enzyme Mix	1 µl
Total Volume	**20 µl**

Vortex the sample briefly to mix and collect by brief centrifugation. Incubate as follows: 42˚C for 45 min followed by 80˚C for 5 min in a thermocycler (without heated lid).

Store the cDNA synthesis reaction at -20°C, or purify the cDNA and proceed directly to the next step for cDNA end polishing.

5. cDNA Purification

a. Resuspend **MQ Binding Beads** by vortex.

b. Add 40 µl of resuspended beads to the PCR tube of RT reaction. Mix thoroughly on a vortex mixer or by pipetting up and down at least 10 times.

c. Incubate for 5 minutes at room temperature to allow DNA 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 200 µl of freshly prepared *80% ethanol* to the tube. Incubate at room temperature for 1 min, and then carefully remove and discard the ethanol.

f. Repeat Step 5e two times for total of three washes.

g. Open the PCR tube cap and air dry beads for 10 minutes while the tube is on the magnetic stand.

h. Resuspend the beads in 12 µl **Elution Buffer** and incubate at room temperature for 2 minutes to release the DNA from the beads.

i. Capture the beads by placing the tube in the magnetic stand for 4 minutes or until the solution is completely clear.

j. Transfer 11 µl to a new 0.2 ml PCR tube.

6. cDNA End Polishing

a. Prepare end repair reaction in a 0.2 ml PCR tube according to Table 1:

Table 1. End Polishing Reaction

Component	Volume
cDNA	10 µl
10X End Polishing Buffer	1.5 µl
End Polishing Enzyme Mix	1 µl
End Polishing Enhancer	1 µl
Distilled Water	1.5 µl
Total volume	**15 µl**

b. Mix and incubate for 20 min at 25°C and 20 min at 72°C in a thermocycler (without heated lid).

7. Adaptor Ligation

a. Prepare a reaction mix for adaptor ligation according to Table 2. Add the following reagents to a 0.2 ml PCR tube containing end polished cDNA from Step 6.

Table 2. Adaptor Ligation

Component	Volume
End polished cDNA (from Step 6)	15 µl
2X Ligation Buffer	17 µl
T4 DNA Ligase	1 µl
Adaptors	1 µl
Total volume	**34 µl**

b. Mix and incubate for 15 min at 25°C in a thermocycler (without heated lid).

Note: (1) The pre-annealed adapters included in the kit are suitable for both non-barcoded (singleplexed) and barcoded (multiplexed) cDNA library preparation and fully compatible with Illumina platforms, such as MiSeq® or HiSeq™ sequencers. (2) If using adaptors from other suppliers (both single-end and barcode adaptors), make sure they are compatible with Illumina platforms and

add the correct amount (final concentration 1.5-2 µM, or according to the supplier's instruction).

8. Size Selection/Clean-up

Note: If the starting DNA amount is less than 500 ng, size selection is not recommended and alternatively, clean-up of ligated cDNA can be performed directly prior to PCR amplification according to step 8.1 of the protocol.

8.1 Clean-up of Ligated cDNA

a. Resuspend **MQ Binding Beads** by vortex.
b. Add 34 µl of resuspended beads to the PCR tube of ligation reaction. Mix thoroughly on a vortex mixer or by pipetting up and down at least 10 times.
c. Incubate for 5 minutes at room temperature to allow DNA 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 DNA.)*
e. Keep the PCR tube in the magnetic stand and add 200 µl of freshly prepared *80% ethanol* to the tube. Incubate at room temperature for 1 min, and then carefully remove and discard the ethanol.
f. Repeat Step e two times for total of three washes.
g. Open the PCR tube cap and air dry beads for 10 minutes while the tube is on the magnetic stand.
h. Resuspend the beads in 12 µl **Elution Buffer**, and incubate at room temperature for 2 minutes to release the DNA from the beads.
i. Capture the beads by placing the tube in the magnetic stand for 4 minutes or until the solution is completely clear.
j. Transfer 11 µl to a new 0.2 ml PCR tube.

8.2. Size selection of Ligated cDNA (Optional)

a. Resuspend MQ Binding Beads by vortex.
b. Add 14 µl of resuspended MQ Binding Beads to the tube of ligation reaction. Mix well by pipetting up and down at least 10 times.
c. Incubate for 5 minutes at room temperature.

d. Put the tube on an appropriate magnetic stand until the solution is clear (about 4 minutes). Carefully transfer the supernatant containing cDNA to a new tube (Caution: do not discard the supernatant). Discard the beads that contain the unwanted large fragments.
e. Add 10 µl resuspended beads to the supernatant, mix well and incubate for 5 minutes at room temperature.
f. 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.)
g. Keep the PCR tube in the magnetic stand and add 200 µl of freshly prepared 80% ethanol to the tube. Incubate at room temperature for 1 min, and then carefully remove and discard the ethanol.
h. Repeat Step g one time, for total of two washes.
i. Open the PCR tube cap and air dry beads for 10 minutes while the tube is on the magnetic stand.
j. Resuspend the beads in 12 µl Elution Buffer, and incubate at room temperature for 2 minutes to release the DNA from the beads.
k. Capture the beads by placing the tube in the magnetic stand for 4 minutes or until the solution is completely clear.
l. Transfer 11 µl to a new 0.2 ml PCR tube.

9. Library Amplification

a. Prepare the PCR Reactions

Thaw all reaction components including master mix, DNA/RNase free water, primer solution and cDNA 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 (µl)
HiFi Master Mix (2X)	12.5 µl
Primer U	1 µl
Primer I	1 µl
Adaptor Ligated cDNA	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 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 55°C 72°C	20 sec 20 sec 20 sec	Variable*
Final Extension	72°C	2 min	1

** PCR cycles may vary depending on the amount of input RNA. In general, use 10 PCR cycles for 500 ng, 11 PCR cycles for 200 ng, 12 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.*

10. Clean-up of Amplified Library DNA

a. Resuspend **MQ Binding Beads** by vortex.

b. Add 25 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 5 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 200 µl of freshly prepared *80% ethanol* to the tube. Incubate at room temperature for 1 min, and then carefully remove and discard the ethanol.

f. Repeat Step 10e two times for a total of three washes.

g. Open the PCR tube cap and air dry beads for 10 minutes while the tube is on the magnetic stand.

h. Resuspend the beads in 22 µl **Elution Buffer**, and incubate at room temperature for 2 minutes to release the DNA from the beads.
i. Capture the beads by placing the tube in the magnetic stand for 4 minutes or until the solution is completely clear.
j. Transfer 20 µl to a new 0.2 ml PCR tube.

Quality of the prepared library can be assessed using an Agilent Bioanalyzer or comparable method. Library fragments should have the correct size distribution (ex: 300 bps at peak size) without adaptors or adaptor-dimers. To check the size distribution, dilute library 5-fold with water and apply it to an Agilent high sensitivity chip. If there is presence of <150 bp adaptor dimers or of larger fragments than expected, they should be removed. To remove fragments below 150 bps, use 0.8X MQ Binding Beads according to steps a-l of section 8.1 “Clean-up of Ligated cDNA”. To remove fragments above 500 bps, follow steps a-l of section 8.2 “Size Selection of Ligated cDNA”.

The prepared cDNA library can be quantified with various DNA library quantification methods.

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., < 1ng or >1 μg).	Increase or decrease input RNA to within the correct range, or to the optimal range of 200-500 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 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 90% ethanol for RNA clean-up.
Little or no cDNA synthesis	Poor RNA quality (RNA is severely degraded).	Check if the sample RNA 260/280 ratio is between 1.9 - 2.0. Analyze RNA on a denaturing gel to verify RNA integrity.
	RT inhibitor is contained in RNA.	Common RT inhibitors such as SDS, EDTA and formamide can be removed by re-precipitation and clean-up of RNA with ethanol.
	Temperature is incorrect.	Check if the temperature is appropriate for cDNA synthesis
	Insufficient amount of starting RNA.	Increase the amount of starting RNA, especially for amplifying low-copy genes from total RNA.
	Kit is not stored or handled properly.	Store all components of the kit according to the storage conditions specified in this Product Manual.
Poor specificity in qPCR	Non-specific primers.	PCR primers were not appropriate or were incorrectly designed. Ensure the primers are specific for the target genes.

	Genomic DNA contamination.	Treat RNA with DNase I and re-purify.
Low yield of library	Insufficient amount of starting cDNA.	To obtain the best results, the amount of input cDNA should be >10 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: Presence of <150 bp adaptor dimers or presence of larger fragments than expected.	Improper ratio of **MQ Binding. Beads** to cDNA volume during size selection.	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
	Insufficient ligation.	Too much or too little input cDNA may cause insufficient ligation, which can shift the peak size of the fragment population to be shorter or larger than expected. Make sure that the ligation reaction 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.

Product Manual

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