ab185911 – RNA Bisulfite Conversion Kit

Instructions for Use

For bisulfite conversion of RNA isolated from various tissue or cell samples

This product is for research use only and is not intended for diagnostic use.

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1. BACKGROUND

DNA cytosine methylation occurs by the covalent addition of a methyl group at the 5-carbon of the cytosine ring by DNA methyltransferases, resulting in 5-methylcytosine (5-mC). 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 covered 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 the 5-mC modification in eukaryotic RNAs. 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 RT-PCR amplification, cloning, and sequencing yields reliable information about RNA cytosine methylation states. By treating RNA with bisulfite, cytosine residues are deaminated to uracil while leaving 5-methylcytosine intact.

Abcam offers the RNA Bisulfite Conversion Kit to effectively and efficiently prepare converted RNA for use in various downstream analyses. The kit is specifically optimized and validated for bisulfite conversion of RNA has the following advantages and features:

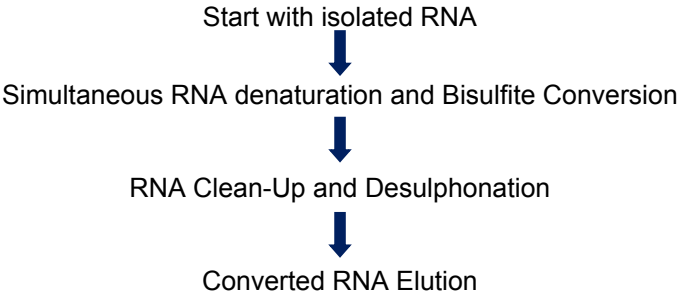
- The entire procedure can be finished within 3 hours.
- Completely converts unmethylated RNA cytosine into uracil (>99.9%) with no or negligible inappropriate/error conversion of methylcytosine to

thymine (<0.1%) when the indicated range of input sample RNA is used.
- Powerful protection against RNA degradation, with over 90% of RNA loss prevented.
- Included control primers are specific against bisulfite-converted RNA and can be used to test whether the bisulfite conversion has been properly achieved.
- Low amount of input RNA can be used for bisulfite conversion with as low as 5 ng per reaction.
- Simple, reliable, and consistent reaction conditions.

The RNA Bisulfite Conversion Kit (ab185911) is suitable for various downstream RNA methylation analyses including methylation specific RT-PCR, MS-HRM, and bisulfite-sequencing including pyrosequencing and deep-sequencing from various tissue or cell samples.

The RNA Bisulfite Conversion Kit (ab185911) contains all reagents required for fast bisulfite conversion on a RNA sample. The unique conversion mix solution contains powerful RNA protection reagents to prevent chemical and thermophilic degradation, thus leading to an accelerated conversion of all cytosines to uracil with negligible methylcytosine deamination. The non-toxic RNA capture solution enables RNA to tightly bind to the column filter, so that converted RNA cleaning can be carried out on the column to effectively remove residual bisulfite and salts.

2. ASSAY SUMMARY



3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit as given in the table upon receipt.

Observe the storage conditions for individual prepared components in sections 9 & 10.

For maximum recovery of the products, centrifuge the original vial prior to opening the cap.

5. MATERIALS SUPPLIED

Item	50 Reactions	Storage Condition (Before Preparation)
Conversion Buffer	8 mL	RT
Conversion Powder	5 vials	RT
NA Binding Solution	13 mL	RT
F-Spin Column	50	RT
F-Collection Tube	50	RT
Desulphonation Solution	300 µL	RT
Control Primer-F	10 µL	–20°C
Control Primer-R	10 µL	–20°C
Elution Buffer	1.2 mL	RT

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

- Adjustable pipette or multiple-channel pipette
- Multiple-channel pipette reservoirs
- Thermocycler with heated lid*
- 0.2 mL PCR tubes
- 100% Ethanol
- Distilled water
- RNA sample
- Desktop centrifuge (up to 14,000 rpm)

**Since the bisulfite reaction is not overlaid with mineral oil, only thermal cyclers with heated lids are suitable for this procedure*

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures
- Do not use kit or components if it has exceeded the expiration date on the kit labels
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted
- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.

8. TECHNICAL HINTS

- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Complete removal of all solutions and buffers during wash steps.
- **This kit is sold based on number of Reaction. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**

9. REAGENT PREPARATION

9.1 **Conversion Solution**

Add 1.4 mL of Conversion Buffer and 40 µL of Desulfonation 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).

Note: *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.*

9.2 **70% Ethanol**

Add 3 mL of distilled water to 7 mL of Ethanol.

9.3 **90% Ethanol**

Add 1 mL of distilled water to 9 mL of Ethanol.

9.4 **Desulfonation Solution**

Dilute Desulfonation Solution at a 1:11 ratio by adding 5 µL of Desulfonation Solution to 55 µL of distilled water. Next, add 2 µL of Diluted Desulfonation Solution to every 1 mL of 90% ethanol, and mix.

10. SAMPLE PREPARATION

Input Amount: The amount of RNA for each bisulfite reaction can be 5 ng to 1 µg. For an optimal reaction, the input RNA amount should be 200 ng to 500 ng. When using the EpiSeeker RNA Bisulfite Conversion Kit (ab185911) for methylation-specific RT-PCR with very small amounts of input RNA (<10 ng), the number of PCR cycles should be greater than 45. The yield of RNA purified after bisulfite conversion depends on the amount of input RNA, nature of RNA, and source of the starting material.

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

11. ASSAY PROCEDURE

11.1 RNA Bisulfite Conversion

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

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

11.2 Converted RNA Clean-Up

11.2.1 Add 250 µL of NA Binding Solution to each column. Then transfer the samples from each PCR tube 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.

11.2.2 Add 200 µL of 70% ethanol solution to each column. Centrifuge at 12,000 rpm for 1 min.

11.2.3 Add 200 µL of the working desulfonation buffer (Desulfonation Solution and 90% ethanol mixture) 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.

11.2.4 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 20 µL of 90% ethanol to each column again and centrifuge at 12,000 rpm for 1 min.

11.2.5 Insert each column into a new 1.5 ml tube. Add 10 - 20 µL of Elution Buffer directly to each column's filter membrane. Centrifuge at 12,000 rpm for 1 min to elute converted RNA.

11.2.6 Converted RNA is now ready for use, or storage at or below -20°C for up to 2 months. As the bisulfite-treated RNA is not stable, we recommend performing cDNA synthesis from bisulfite-treated RNA before next application or storage. Data Analysis Section 12 for cDNA synthesis.

12. ANALYSIS

cDNA Synthesis

You can use your method of choice for cDNA synthesis. For your convenience, Abcam offers Hi-Fi cDNA Synthesis Kit (ab185916) which is optimized and validated for synthesis of cDNA from bisulfite RNA.

12.1 Add the following in a 0.2 mL PCR tube on ice:

Component	Volume (µL)
Bisulfite-converted RNA (200-500 ng)	10
Random primer (50 µM)	1
10 mM dNTP mix	1

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

12.2 Add the following to the tube from Step 12.1 held on ice.

Component	Volume (µL)
5X RT Reaction Buffer	4
0.1 M DTT	2
RNAse inhibtor	1
RT Enzyme mix	1
Total Volume	20

Vortex the sample briefly to mix and collect by centrifugation Incubate as follows: 42℃ for 45 min followed by 80℃ for 5 min (no heated lid).

Store the cDNA synthesis reaction at -20°C, or proceed directly to next application such as methylation specific PCR or bisulfite-sequencing.

13. TROUBLESHOOTING

Problem	Cause	Solution
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 amount 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 Desulfonation Solution is added into every 1 mLof 90% ethanol
	Kit is not stored or handled properly	Store all components of the kit at room temperature
Eluate contains little or no RNA	The standard Poor input RNA quality (degraded)	Ensure a sufficient Check if RNA is degraded
	NA Binding Solution is not added into the sample	Ensure that NA Binding Solution is added
	Incorrect conc. of ethanol solution used for RNA cleanup	Use 90% ethanol for RNA clean-up

Poor results in downstream methylation-specific qRT-PCR	Little or no PCR product even in positive control	Ensure that cDNA synthesis is properly performed
		Ensure that all PCR components were added and that suitable PCR program is used (PCR cycle should be >40)
		PCR primers and probes were not appropriate or were incorrectly designed. Ensure the primer and probes are suitable for MS-PCR
	Significant non-specific PCR products	Failed bisulfite conversion. Ensure that all steps of the bisulfite treatment and cleanup protocol were followed and that input RNA amount is within the recommended range
		Primers and probes are not specific for converted RNA and target genes. Check the primer and probe design

14. NOTES

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