

ab185906 – Post-Bisulfite DNA Library Preparation Kit (For Illumina[®])

Instructions for Use

For the preparation of a DNA library for various Illumina[®] platform-based bisulfite sequencing (bisulfite-seq) assays.

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

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1. BACKGROUND

DNA methylation occurs by the covalent addition of a methyl group (CH_3) at the 5-carbon of the cytosine ring, resulting in 5-methylcytosine (5-mC). DNA methylation is essential in regulating gene expression in nearly all biological processes including development, growth, and differentiation. Alterations in DNA methylation have been demonstrated to cause a change in gene expression. For example, hypermethylation leads to gene silencing or decreased gene expression while hypomethylation activates genes or increases gene expression. Aberrant DNA methylation is also associated with pathogenesis of diseases such as cancer, autoimmune disorders, and schizophrenia. Thus genome-wide analysis of DNA methylation could provide valuable information for discovering epigenetic markers used for disease diagnosis and potential therapeutic targets.

Several methods such as whole genome bisulfite sequencing (WGBS) or reduced representation bisulfite sequencing (RRBS) are currently used for genome-wide DNA methylation analysis. These methods convert unmethylated cytosine to uracil while 5-mC remains unmodified by the bisulfite treatment. This allows epigenetic differences to be interpreted as genetic differences, which can then be detected by sequencing at single-base resolution and on a genome-wide scale. However, traditional methods to achieve this still do not have practical use because (1) such methods require large amounts of DNA ($>1 \mu\text{g}$) as input material, which is difficult to prepare from limited biological samples such as tumor biopsy samples, early embryos, embryonic tissues and circulating DNA; (2) such methods require that DNA is first sheared and then ligated to adaptors followed by bisulfite conversion (post-ligation bisulfite conversion). This procedure causes most of the DNA fragments contained in the adaptor-DNA fragment constructs to be broken, and thereby form mono-tagged templates that will be removed during library enrichment. Thus, incomplete coverage and bias occur when performing whole genome bisulfite sequencing; and (3) such methods are time-consuming (2 days). To overcome the weaknesses of these methods,

Abcam offers the Post-Bisulfite DNA Library Preparation Kit (For Illumina®). The kit has the following features:

This kit has the following features:

- Allows bisulfite-converted DNA to be used directly for ligation, thereby eliminating the possibility of breaking adapter-ligated fragments, which can often occur in currently used WGBS and RRBS methods.
- Fast 5-hour procedure, from input starting material to library amplification.
- Gel-free size selection/purification saves time and prevents handling errors, as well as loss of valuable samples.
- High sensitivity, efficiency and flexibility-- direct ligation of adapter to bisulfite-converted DNA fragments reduces loss of fragments and selection bias, which enables pre-bisulfite input DNA to be as low as 1 ng. The kit can be used for both non-barcoded (singleplexed) and barcoded (multiplexed) DNA library preparation.
- Comprehensive set of components to accommodate each step of DNA library preparation -- ligation, clean-up, size selection, and library amplification - for convenience, consistency, and reliability.
- Ultra HiFi amplification enables achievement of reproducibly high yields of DNA libraries with minimal sequence bias and low error rates.

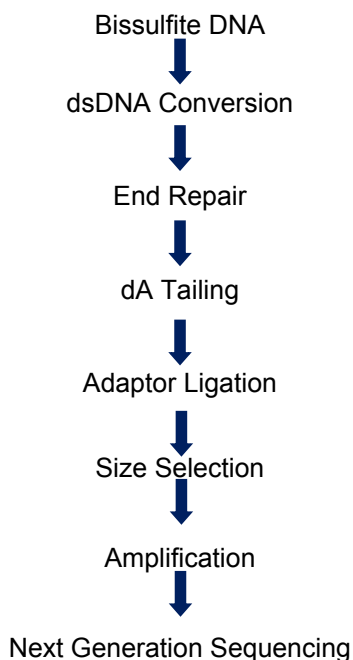
The Post-Bisulfite DNA Library Preparation Kit (For Illumina®) is suitable for preparing a DNA library for various Illumina® platform-based bisulfite sequencing (bisulfite-seq) assays such as whole genome bisulfite sequencing (WGBS), oxidative bisulfite sequencing (oxBs-seq), reduced representative bisulfite sequencing (RRBS), and other bisulfite-next generation sequencing applications. The optimized protocol and components of the kit allow both non-barcoded (singleplexed) and barcoded (multiplexed) DNA libraries to be quickly constructed using sub-nanogram input concentrations of DNA since the DNA is first bisulfite-converted and then used for library preparation.

With this kit, bisulfite-treated DNA (which is in single-stranded form), is converted to double-stranded DNA and directly used for ligation with BisDNA-specific adapters which are necessary for amplification and

sequencing. The fragments are then size selected and purified with MQ beads, which allows for quick and precise size selections of DNA. Size-selected DNA fragments are then amplified with a high- fidelity PCR Mix, ensuring maximum yields from minimum amounts of starting material and providing a highly accurate amplification of library DNA with low error rates and minimal bias.

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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	12 Tests	24 Tests	Storage Condition (Before Preparation)
5X Conversion Buffer	50 µL	100 µL	-20°C
Conversion Enzyme Mix	15 µL	30 µL	-20°C
Conversion Primer	26 µL	52 µL	-20°C
10X End Repair Buffer	40 µL	80 µL	-20°C
End Repair Enzyme Mix	25 µL	50 µL	-20°C
10X dA-Tailing Buffer	40 µL	80 µL	-20°C
Klenow Fragment (3'-5' exo-)	15 µL	30 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	1.8 mL	3.6 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	1 mL	2 mL	-20°C

6. MATERIALS REQUIRED, NOT SUPPLIED

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

- Vortex Mixer
- 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
- Bisulfite-treated DNA sample

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 tests. A ‘test’ simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**

9. REAGENT PREPARATION

All reagents are supplied ready to use.

10. SAMPLE PREPARATION

- 10.1 **Amount of Input DNA used for bisulfite treatment:** Input amount of pre-bisulfite DNA can range from 500 pg to 1 µg per reaction, which could yield bisulfite-treated DNA ranging from at least 50 pg to 200 ng. An optimal pre-bisulfite DNA amount is 100 ng to 200 ng per reaction. Input DNA should be of high quality and relatively free of RNA. RNase I can be used to remove RNA.
- 10.2 **Bisulfite-treated DNA:** Bisulfite DNA can be generated with home-brew bisulfite conversion protocols or commercially available kits. However most of these protocols/kits cannot generate fragments of appropriate length for library preparation use. The peak size of bisulfite-treated DNA fragments should be 200-400 bps in length.
- 10.3 **Validation of bisulfite DNA quality:** To ensure that the bisulfite-treated DNA is suitable for direct ligation, we recommend checking the bisulfite-treated DNA by real time methylation-specific PCR (MSP).

11. ASSAY PROCEDURE

11.1 dsDNA Conversion Reaction

11.1.1 Prepare dsDNA Conversion reaction in a 0.2 mL PCR tube according to Table below:

Component	Sample (µL)
Bisulfite DNA*	10 (100-200 ng)
5X Conversion Buffer	4
Conversion Primer	2
Distilled Water	3
Total Volume	19

Note: The optimal amount of input DNA should be 100 ng to 200 ng and eluted volume after bisulfite treatment should be < 20 µL.

11.1.2 Mix and incubate for 5 min at 95°C in a thermocycler (without heated lid) followed by 5 min at 4°C or on ice.

11.1.3 Add 1 µL of Conversion Enzyme Mix to the reaction tube. Mix and incubate for 60 min at 37°C in a thermocycler (without heated lid).

11.2 Clean-Up of Converted dsDNA

11.2.1 Resuspend MQ Binding Beads by vortex.

11.2.2 Add 36 µL of resuspended beads to the PCR tube of dsDNA conversion reaction. Mix thoroughly on a vortex mixer or by pipetting up and down at least 10 times.

11.2.3 Incubate for 10 minutes at room temperature to allow DNA to bind to the beads.

11.2.4 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 DNA.

- 11.2.5 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.
- 11.2.6 Repeat Step 11.2.5 one time for a total of two washes.
- 11.2.7 Open the cap of the PCR tube and air dry beads for 10 minutes while the tube is on the magnetic stand.
- 11.2.8 Resuspend the beads in 12 μL Elution Buffer, and incubate at room temperature for 2 minutes to release the DNA from the beads.
- 11.2.9 Capture the beads by placing the tube in the magnetic stand for 2 minutes or until the solution is completely clear.
- 11.2.10 Transfer the clear solution to a new 0.2 mL PCR tube for the end repair reaction.

11.3 DNA End Repairing

- 11.3.1 Prepare DNA End Repairing reaction in a 0.2 mL PCR tube according to Table below:

Component	Sample (μL)
Converted dsDNA (from Step 11.2)	11-12
10X End Repair Buffer	2
End Repair Enzyme Mix	2
Distilled Water	5-6
Total Volume	20

- 11.3.2 Mix and incubate for 30 min at 20°C in a thermal cycler

11.4 Clean-Up of End Repaired DNA

- 11.4.1 Resuspend MQ Binding Beads by vortex.
- 11.4.2 Add 36 μ L of resuspended beads to the PCR tube of end repair reaction. Mix thoroughly on a vortex mixer or by pipetting up and down at least 10 times.
- 11.4.3 Incubate for 10 minutes at room temperature to allow DNA to bind to the beads.
- 11.4.4 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 DNA.
- 11.4.5 Keep the PCR tube in the magnetic stand and add 200 μ L of freshly prepared 80% ethanol to the tube. Incubate at room temperature for 1 min, and then carefully remove and discard the ethanol.
- 11.4.6 Repeat Step 11.4.5 one time for a total of two washes.
- 11.4.7 Open the cap of the PCR tube and air dry beads for 10 minutes while the tube is on the magnetic stand.
- 11.4.8 Resuspend the beads in 12 μ L Elution Buffer, and incubate at room temperature for 2 minutes to release the DNA from the beads.
- 11.4.9 Capture the beads by placing the tube in the magnetic stand for 2 minutes or until the solution is completely clear.
- 11.4.10 Transfer the clear solution to a new 0.2 mL PCR tube for the dA tailing reaction.

11.5 DNA dA-Tailing

11.5.1 Prepare the dA-Tailing reaction in a 0.2 mL PCR tube according to Table below:

Component	Sample (µL)
End repaired DNA (from Step 11.4)	12
10X dA-tailing Buffer	1.5
Klenow Fragment (3'-5' exo-)	1
Distilled Water	0.5
Total Volume	15

11.5.2 Mix and incubate for 30 min at 37°C followed by 10 min at 75°C in a thermocycler.

11.6 Adaptor Ligation

11.6.1 Prepare the Adaptor Ligation reaction in a 0.2 mL PCR tube according to Table below:

Component	Sample (µL)
End repaired/dA-tailing DNA (Step 11.5)	15
2X Ligation Buffer	17
T4 DNA Ligase	1
Adaptors	1
Total Volume	34

11.6.2 Mix and incubate for 10 min at 25°C in a thermocycler (without heated lid).

11.6.3 Note: (1) The pre-annealed adaptors included in the kit are suitable for both non-barcoded (singleplexed) and barcoded

(multiplexed) DNA library preparation and are 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 μ M, or according to the supplier's instruction).

11.7 Size Selection of Ligated DNA (Optional)

If the starting DNA amount is less than 200 ng, size selection is not recommended and alternatively, clean-up of ligated DNA can be performed prior to PCR amplification according to 11.8. protocol – “Clean-Up of Ligated DNA”

- 11.7.1 Resuspend MQ Binding Beads by vortex.
- 11.7.2 Add 14 μ L of resuspended MQ Binding Beads to the tube of ligation reaction. Mix well by pipetting up and down at least 10 times.
- 11.7.3 Incubate for 5 minutes at room temperature.
- 11.7.4 Put the tube on an appropriate magnetic stand until the solution is clear (about 2 minutes). Carefully transfer the supernatant containing DNA to a new tube (Caution: Do not discard the supernatant). Discard the beads that contain the unwanted large fragments.
- 11.7.5 Add 10 μ L of re-suspended beads to the supernatant, mix well and incubate for 5 minutes at room temperature.
- 11.7.6 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 DNA.
- 11.7.7 Keep the PCR tube in the magnetic stand and add 200 μ L of freshly prepared 80% ethanol to the tube. Incubate at room temperature for 1 min, and then carefully remove and discard the ethanol.
- 11.7.8 Repeat Step 11.7.7 one time, for a total of two washes.

- 11.7.9 Open the PCR tube cap and air dry beads for 10 minutes while the tube is on the magnetic stand.
- 11.7.10 Resuspend the beads in 12 μ L Elution Buffer, and incubate at room temperature for 2 minutes to release the DNA from the beads.
- 11.7.11 Capture the beads by placing the tube in the magnetic stand for 2 minutes or until the solution is completely clear.
- 11.7.12 Transfer clear solution to a new 0.2 mL PCR tube for PCR amplification.

11.8 Clean-Up of Ligated DNA

- 11.8.1 Resuspend MQ Binding Beads by vortex.
- 11.8.2 Add 34 μ L 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.
- 11.8.3 Incubate for 5 minutes at room temperature to allow DNA to bind to beads.
- 11.8.4 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 DNA.
- 11.8.5 Keep the PCR tube in the magnetic stand and add 200 μ L of freshly prepared 80% ethanol to the tube. Incubate at room temperature for 1 min, and then carefully remove and discard the ethanol.
- 11.8.6 Repeat Step 11.8.5 two times for a total of three washes.
- 11.8.7 Open the PCR tube cap and air dry beads for 10 minutes while the tube is on the magnetic stand.
- 11.8.8 Resuspend the beads in 12 μ L Elution Buffer, and incubate at room temperature for 2 minutes to release the DNA from the beads.
- 11.8.9 Capture the beads by placing the tube in the magnetic stand for 2 minutes or until the solution is completely clear.
- 11.8.10 Transfer 11 μ L of clear solution to a new 0.2 mL PCR tube for PCR amplification.

11.9 Library Amplification

11.9.1 Prepare the PCR Reactions:

Thaw all reaction components including master mix, DNA/RNA free water, primer solution and DNA 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	Sample (µL)
HiFi PCR Master Mix (2X)	12.5
Primer U	1
Primer I	1
Adaptor Ligated DNA	10.5
Total Volume	25

Important Note: Use of Primer I included in the kit will generate a singleplexed library. For multiplexed library preparation, replace Primer I with user-defined barcodes (Illumina® compatible) instead of Primer I.

11.9.2 Program the PCR Reactions:

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

Cycle Step	Temp (°C)	Time (seconds)	Cycle #
Activation	98	30	1
Cycling	98	20	Variable*
	55	20	
	72	20	
Final Extension	72	120	1

Note: PCR cycles may vary depending on the input DNA amount. In general, use 12 PCR cycles for 200 ng, 13 cycles for 100 ng,

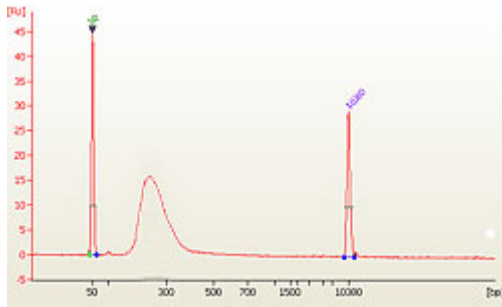
15 cycles for 50 ng, 17 cycles for 10 ng, and 22 cycles for 1 ng DNA input. Further optimization of PCR cycle number may be required by the end user.

11.10 Clean-up Amplified Library DNA

- 11.10.1 Resuspend MQ Binding Beads by vortex.
- 11.10.2 Add 25 μ L 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.
- 11.10.3 Incubate for 5 minutes at room temperature to allow DNA to bind to the beads.
- 11.10.4 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 DNA.
- 11.10.5 Keep the PCR tube in the magnetic stand and add 200 μ L of freshly prepared 80% ethanol to the tube. Incubate at room temperature for 1 min, and then carefully remove and discard the ethanol.
- 11.10.6 Repeat Step 11.10.5 two times for total of three washes.
- 11.10.7 Open the PCR tube cap and air dry beads for 10 minutes while the tube is on the magnetic stand.
- 11.10.8 Resuspend the beads in 12 μ L Elution Buffer, and incubate at room temperature for 2 minutes to release the DNA from the beads.
- 11.10.9 Capture the beads by placing the tube in the magnetic stand for 2 minutes or until the solution is completely clear.
- 11.10.10 Transfer 12 μ L of clear solution to a new 0.2 mL PCR tube.

12. ANALYSIS

Typical Results



Size distribution of library fragments as demonstrated by a post-bisulfite DNA library constructed using ab185906 Post-Bisulfite DNA Library Preparation Kit (For Illumina®) from 10 ng of input DNA.

13. TROUBLESHOOTING

Problem	Cause	Solution
Low yield of library	Insufficient amount of bisulfite DNA	To obtain the best results, the amount of input DNA for bisulfite treatment should be 100-200 ng
	Insufficient purity of bisulfite DNA	Check if the sample DNA 260/280 ratio is between 1.8-1.9 and if DNA is degraded by running a gel. Ensure that RNA is removed by RNase treatment
	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 Adaptor Ligation, Size Selection and Amplification
	Improper storage of the kit	Ensure that the kit has not exceeded the expiration date. Standard shelf life, when properly stored, is 6 months from date of receipt

RESOURCES

Unexpected peak size trace: Presence of <150 bp adaptor dimers or presence of larger fragments than expected	Improper ratio of MQ Binding Beads to DNA volume during size selection	Check if the correct volume of MQ Binding Beads was added to the DNA solution accordingly. Proper ratios should remove fragments of unexpected peak size
	Insufficient ligation	Too much and too little input DNA 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 using the proper amount of input DNA
	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.

14. NOTES

RESOURCES

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