EpiNext™ Bisulfite Sequencing Kit (Illumina)

Base Catalog # P-1056

PLEASE READ THIS ENTIRE USER GUIDE BEFORE USE

Uses: The EpiNext™ Bisulfite Sequencing Kit (Illumina) is designed to carry out bisulfite conversion, followed by a "post-bisulfite" library preparation process for Illumina platform-based bisulfite sequencing, all in one kit. Intended applications include whole genome bisulfite sequencing, oxidative bisulfite sequencing, reduced representative bisulfite sequencing, and various other bisulfite-based next generation sequencing techniques. The optimized protocol and components of the kit allow the DNA to be bisulfite converted and fragmented simultaneously followed by quick non-barcoded (singleplexed) and barcoded (mutilplexed) library construction using sub-nanogram quantities of bisulfite converted DNA.

Starting Material and Input amount: Starting materials can be genomic DNA isolated from various tissue/cell samples such as fresh and frozen tissues, cultured cells from a flask or microplate, microdissection samples, FFPE tissues, plasma/serum, and body fluid samples, etc. DNA enriched from various enrichment reactions such as ChIP, MeDIP/hMeDIP, or exon capture may also be used as starting materials. DNA should be without any previous restriction digestion step. Plasmid DNA can be used for bisulfite treatment with or without previous linearization, as the kit allows for DNA denaturation status to remain during the entire DNA bisulfite conversion process and direct ligation of adaptors to bisulfite DNA. Input amount of DNA can be from 0.5 ng to 1 ug. For optimal preparation, the input amount should be 100 ng to 200 ng.

Precautions: To avoid cross-contamination, carefully pipette the sample or solution into the tube/vials. Use aerosol-barrier pipette tips and always change pipette tips between liquid transfers. 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-1056-12	24 reactions Cat. #P-1056-24	Storage Upon Receipt
Modification Buffer	3 ml	6 ml	RT
Modification Powder	2 vials	4 vials	RT
DNA Binding Solution	6 ml	12 ml	RT
Desulphonation Solution	70 µl	140 µl	RT
Elution Solution	0.5 ml	1 ml	RT
F-Spin Column	15	30	RT
F-Collection Tube	15	30	RT
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*	1000 µl	2000 µl	-20°C
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** Spin the solution down to the bottom prior to use.*

SHIPPING & STORAGE

The kit is shipped on frozen ice packs at 4°C.

Upon receipt: Store the following components at -20°C immediately: **5X Conversion Buffer**, **Conversion Enzyme Mix**, **Conversion Primer**, **10X End Repair Buffer**, **End Repair Enzyme Mix**, **10X dA-Tailing Buffer**, **Klenow Fragment (3'-5' exo$^-$)**, **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.

MATERIALS REQUIRED BUT NOT SUPPLIED

- ☐ 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
- ☐ DNA sample

GENERAL PRODUCT INFORMATION

Quality Control: Each lot of EpiNext™ Bisulfite Sequencing 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. Thus, only use the User Guide that was supplied with the kit when using that kit.

Usage Limitation: The EpiNext™ Bisulfite Sequencing Kit (Illumina) is for research use only and is not intended for diagnostic or therapeutic application.

Intellectual Property: The EpiNext™Bisulfite Sequencing Kit (Illumina) and methods of use contain proprietary technologies by Epigentek.

A BRIEF OVERVIEW

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 targets used for therapeutics.

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 µg) 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, Epigentek offers the EpiNext™ Bisulfite Sequencing Kit (Illumina). The kit has the following features:

- **Innovative method** - Allows for simultaneous bisulfite conversion and size-appropriate DNA fragmentation. The bisulfite DNA can be directly ligated to adaptors thereby eliminating the possibility of breaking adaptor-ligated fragments, which often occurs with traditional WGBS and RRBS methods.
- **Fast and streamlined procedure** - the procedure from DNA bisulfte treatment to PCR amplification can be finished within the same day (<8 h). Gel-free size selection/purification saves time and prevents handling errors, as well as loss of valuable samples.
- **Complete conversion** - The innovation reagent composition converts unmethylated cytosine into uracil at a level greater than 99.9%, with negligible inappropriate- or error-conversions of methylcytosine to thymine (<0.1%).
- **High sensitivity, efficiency and flexibility**- Adaptor ligation after bisulfite treatment eliminates loss of fragments and selection bias, which enables input DNA to be as low as 0.5 ng. The kit can be used for both non-barcoded (singleplexed) and barcoded (multiplexed) DNA library preparation.
- **Extremely convenient** - the kit contains all the required components for each step of the DNA library preparation process, which are sufficient for bisulfite conversion, ligation, clean-up, size selection, and library amplification, thereby allowing the bisulfite DNA library preparation to be streamlined for the most reliable and consistent results.
- **Minimal bias** - Ultra HiFi amplification enables achievement of reproducibly high yields of DNA libraries with minimal sequence bias and low error rates.
- **Broad sample suitability** - Starting materials can be genomic DNA isolated from various tissue/cell samples such as fresh and frozen tissues, cultured cells from a flask or microplate, microdissection samples, paraffin-embedded tissues, biopsy samples, embryonic cells, plasma/serum samples, and body fluid samples, etc. DNA enriched from various enrichment reactions such as ChIP, MeDIP/hMeDIP, or exon capture may also be used as starting materials.

PRINCIPLE & PROCEDURE

This kit includes all reagents required for a successful preparation of a DNA library by directly using bisulfite-converted DNA generated from a small amount of input DNA (500 pg to 500 ng). In this preparation, DNA is bisulfite converted and fragmented to the appropriate length simultaneously during the bisulfite process. The bisulfite-treated DNA, which is in single stranded form, is then converted to dsDNA and directly used for ligation with BisDNA-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.

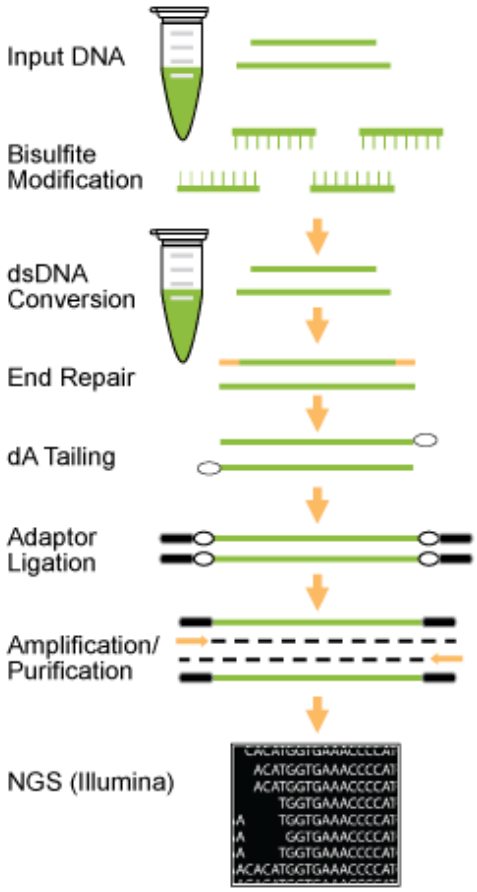

Fig 1. Workflow of the EpiNext™ Bisulfite Sequencing Kit

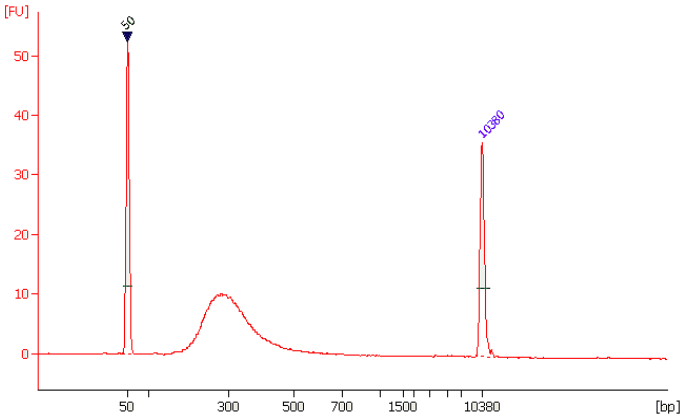

Fig 2. Size distribution of library fragments: Post-bisulfite DNA library was prepared from 10 ng of input DNA using the EpiNext™ Bisulfite Sequencing Kit.

ASSAY PROTOCOL

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

Starting Materials

Input DNA Amount: DNA amount can range from 500 pg to 1 µg per reaction. An optimal amount is 100 ng to 200 ng per reaction. Starting DNA may be in water or in a buffer such as TE. DNA should be of high quality and relatively free of RNA. RNAse I can be used to remove RNA.

DNA Isolation: You can use your method of choice for DNA isolation. Epigentek offers a series of genomic DNA isolation kits for your convenience.

DNA Storage: Isolated genomic DNA can be stored at 4°C or -20°C until use.

1. Bisulfite DNA Modification

a. Add 1 ml of **Modification Buffer** to 1 vial of **Modification Powder** to generate **Modification Solution**. Mix by inverting and shaking the vial repeatedly for 3-4 min (trace amount of undissolved **Modification Powder** may remain, which is normal as **Modification Powder** is saturated in solution).

b. For each 0.2 ml PCR tube, add 150 µl of the mixed **Modification Solution** followed by adding 1-5 µl of sample DNA.

Note: *Check if the sample DNA volume is large and the concentration is lower than 5 ng/µl. If so, it is recommended to concentrate DNA using Epigentek's DNA Concentrator Kit (Cat. No. P-1006) prior to bisulfite treatment.*

*Prepared **Modification 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.*

c. Tightly close the PCR tubes and place them in a thermocycler with heated lid. Program and run the thermocycler according to the following:

95°C 5 min
65°C 30 min
95°C 5 min
65°C 30 min
95°C 5 min
65°C 60 min
Hold 18-20°C up to 6 h

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

2. Modified DNA Clean-Up

a. Add 250 µl of **DNA Binding Solution** to each column. Then transfer the samples from each PCR tube (from Step 1) to each column containing the **DNA Binding Solution**. Centrifuge at 12,000 rpm for 45 sec. Remove columns from collection tubes and discard the flowthrough. Place columns back into collection tubes.

b. Add 250 µl of 90% ethanol to each column. Centrifuge at 12,000 rpm for 45 sec.

c. Prepare final **Desulphonation Buffer** by adding 30 µl of **Desulphonation Solution** to every 1 ml of 90% ethanol, and mix. Add 100 µl of the final **Desulphonation Buffer** (**Desulphonation Solution** and 90% ethanol mixture) to each column. Allow columns to sit for 15 min at room temperature, then centrifuge at 12,000 rpm for 45 sec. Remove columns from collection tubes and discard the flowthrough. Place columns back into collection tubes.

d. Add 250 µl of 90% ethanol to each column. Centrifuge at 12,000 rpm for 45 sec. Remove columns from collection tubes and discard the flowthrough. Place columns back into collection tubes. Add 250 µl of 90% ethanol to each column again and centrifuge at 12,000 rpm for 45 sec.

e. Insert each column into a new 1.5 ml tube. Add 10 µl of **Elution Solution** directly to each column's filter membrane. Centrifuge at 12,000 rpm for 60 sec to elute converted DNA.

Modified DNA is now ready to use for post-bisulfite DNA library preparation, or storage at or below –20°C for up to 3 months. The peak size of converted DNA is 250-300 bps.

Note: *To ensure the DNA is properly modified, we recommend checking the bisulfite-treated DNA by real time methylation-specific PCR (MS-PCR). For your convenience and the best results, Epigentek provides Methylamp™ MS-qPCR Fast Kit (Cat# P-1028) for real time MS-PCR. Both positive primers (b-actin, component of kit Cat. No. P-1028) and negative primers (GAPDH, component of kit Cat, No. P-1029) are also separately available for checking conversion efficiency.*

3. dsDNA Conversion Reaction

a. Prepare dsDNA Conversion reaction in a 0.2 ml PCR tube according to Table 1:

Table 1. dsDNA Conversion

Component	Volume
Bisulfite DNA*	10 µl (100-200 ng input DNA)
5X Conversion Buffer	4 µl
Conversion Primer	2 µl
Distilled water	3 µl
Total Volume	***19 µl***

b. Mix and incubate for 5 min at 95°C in a thermocycler followed by 5 min at 4°C or on ice.
c. Add 1 µl of **Conversion Enzyme Mix** to the reaction tube. Mix and incubate for 60 min at 37°C in a thermocycler.

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

4. Clean Up of Converted dsDNA

a. Resuspend **MQ Binding Beads** by vortex.
b. 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.
c. Incubate for 10 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 2 minutes). Carefully remove and discard the supernatant. *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 4e one time for a total of two washes.
g. Open the cap of the PCR tube 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 2 minutes or until the solution is completely clear.
j. Transfer clear solution to a new 0.2 ml PCR tube for end repair reaction.

5. DNA End Repairing

a. Prepare end repair reaction in 0.2 ml PCR tube according to Table 2:

Table 2. End Repair Reaction

Component	Volume
Converted dsDNA (from Step 4)	11-12 µl
10X End Repair Buffer	2 µl
End Repair Enzyme Mix	1 µl
Distilled Water	5-6 µl
Total Volume	***20 µl***

b. Mix and incubate for 30 min at 20°C in a thermocycler.

6. Clean-up of End Repaired DNA

a. Resuspend **MQ Binding Beads** by vortex.
b. Add 36 µl 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.
c. Incubate for 10 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 2 minutes). Carefully remove and discard the supernatant. *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 90% ethanol to the tube. Incubate at room temperature for 1 min, and then carefully remove and discard the ethanol.
f. Repeat Step 6e one time for a total of two washes.
g. Open the cap of the PCR tube 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 2 minutes or until the solution is completely clear.
j. Transfer clear solution to a new 0.2 ml PCR tube for dA-tailing reaction.

7. DNA dA-Tailing

a. Prepare the reaction mix for dA-tailing according to Table 3. Add the following reagents to a 0.2 ml PCR tube containing end repaired DNA from step 6.

Table 3. dA-Tailing Reaction

Component	Volume
End repaired DNA (from Step 6)	12 µl
10X dA-tailing Buffer	1.5 µl
Klenow Fragment (3'-5' exo$^-$)	1 µl
Distilled Water	0.5 µl
Total Volume	***15 µl***

b. Mix and incubate for 30 min at 37°C followed by 10 min at 75°C in a thermocycler (without heated lid).

8. Adaptor Ligation

a. Prepare a reaction mix for adaptor ligation according to Table 4. Add the following reagents to a 0.2 ml PCR tube containing end repaired/dA-tailed DNA from Step 7.

Table 4. Adaptor Ligation

Component	Volume
End repaired/dA-tailed DNA (from Step 7)	15 µl
2X Ligation Buffer	17 µl
T4 DNA Ligase	1 µl
Adaptors	1 µl
Total Volume	***34 µl***

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

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 µM, or according to the supplier's instruction).*

9. Size Selection/Clean-up

9.1. Size Selection of Ligated DNA (Optional)

Note*: 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 the Step 9.2 protocol – "Clean-Up of Ligated DNA".*

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 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.
e. Add 10 µl of 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 2 minutes). Carefully remove and discard the supernatant. *Be careful not to disturb or discard the beads that contain DNA.*
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 9.1g one time, for a 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 clear solution to a new 0.2 ml PCR tube for PCR amplification.

9.2. Clean-Up of Ligated DNA

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 2 minutes). Carefully remove and discard the supernatant. *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 9.2e 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 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 2 minutes or until the solution is completely clear.
j. Transfer 11 µl clear solution to a new 0.2 ml PCR tube for PCR amplification.

10. Library Amplification

a. 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	Size (µl)
HiFi Master Mix (2X)	12.5 µl
Primer U	1 µl
Primer I	1 µl
Adaptor Ligated DNA	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 **Primer I** with one of the12 different barcodes (indexes) contained in the EpiNext™ NGS Barcode (Index) Set-12 (Cat. No. P-1060). You can also add user-defined barcodes (Illumina compatible) instead of **Primer I**.*

b. Program the PCR Reactions:

Place the reaction plate in the PCR 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 input DNA amount. In general, use 10 PCR cycles for 500 ng, 14 cycles for 50 ng, 18 cycles for 5 ng and 22 cycles for 1 ng DNA input. Further optimization of PCR cycle number may be required.*

11. 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 DNA 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 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 11e 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 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 2 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 other comparable methods. 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** (e.g., dilute amplified library DNA to 20 µl with TE and then add 20 µl of **MQ Binding Beads**) according to sub-steps a through j of Step 11 – "Clean-up of Amplified Library DNA". To remove fragments above 500 bps, follow sub-steps a through l of Step 9.1 – "Size Selection of Ligated DNA".

The prepared DNA library can be quantified using various DNA library quantification methods.

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

TROUBLESHOOTING

Problem	Possible Cause	Suggestion
DNA is poorly modified	Poor DNA quality (DNA is severely degraded).	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.
	Too little DNA or too much DNA (i.e., < 500 pg or >1 µg).	Increase or decrease input DNA to within the correct range, or to the optimal amount of 100-200 ng.
	Temperature or thermocycling condition is incorrect.	Check for appropriate temperature or thermocycling conditions.
	Insufficient DNA clean-up.	Ensure that 30 µl of **Desulphonation Solution** is added into every 1 ml of 90% ethanol in Step 2c.
Elute contains little or no DNA	Poor input DNA quality (degraded).	Check if DNA is degraded by running a gel.
	DNA Binding Solution is not added into the sample.	Ensure that **DNA Binding Solution** is added in Step 2a.
	Concentration of ethanol solution used for DNA clean-up is not correct.	Use 90% ethanol for DNA clean-up.
	Sample is not completely passed through the filter membrane of column.	Centrifuge for 1 min at 12,000 rpm or until the entire sample has passed through the filter membrane.
Low yield of library	Insufficient amount of bisulfite DNA.	To obtain the best results, the optimized amount of input DNA for bisulfite treatment should be 100-200 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 Adaptor Ligation, Size Selection and Amplification.
	Improper storage of the kit.	Ensure that the kit has not exceeded the expiration. Standard shelf life, when stored properly, is 6 months from date of receipt.
Unexpected peak size of Agilent® Bioanalyzer® trace: Presence of <150 bp adaptor dimers or presence of larger fragments than expected.	Improper ratio **MQ Binding Beads** to DNA volume in size selection.	Check if the correct volume of **MQ Binding Beads** is added to DNA solution accordingly. Proper ratios should remove the fragments with unexpected peak size. Ex: use 0.8X **MQ Binding Beads** to remove fragments below 150 bps or to remove fragments above 500 bps follow the protocol according to steps a-l of **9.1 Size Selection.**
	Insufficient ligation.	Too much and too little input DNA may cause insufficient ligation, which can shift

		peak size of fragment population to be shorter or larger than expected. Make sure that ligation reaction is properly processed with 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.

RELATED PRODUCTS

DNA Isolation and Cleanup

P-1003 FitAmp™ General Tissue Section DNA Isolation Kit
P-1004 FitAmp™ Plasma/Serum DNA Isolation Kit
P-1006 DNA Concentrator Kit
P-1007 FitAmp™ Gel DNA Isolation Kit
P-1009 FitAmp™ Paraffin Tissue Section DNA Isolation Kit
P-1017 FitAmp™ Urine DNA Isolation Kit
P-1018 FitAmp™ Blood and Cultured Cell DNA Extraction Kit

DNA Bisulfite Conversion

P-1001 Methylamp™ DNA Modification KIt
P-1026 BisulFlash™ DNA Modification Kit

DNA Library Prep

P-1051 EpiNext™ DNA Library Preparation Kit (Illumina)
P-1053 EpiNext™ High-Sensitivity DNA Library Preparation Kit (Illumina)
P-1055 EpiNext™ Post-Bisulfite DNA Library Prep Kit (Illumina)

NGS Barcode

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