Product Manual

AMPINEXT™ High-Sensitivity Bisulfite-Seq Kit (Illumina)

Catalog #: *ENZ-GEN508*

ENZ-GEN508-0012 – for 12 reactions

ENZ-GEN508-0024 – for 24 reactions

For the latest product information, including support documentation, visit us online:
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Product Manual

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

INTRODUCTION

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, we offer the AMPINEXT™ High-Sensitivity Bisulfite-Seq Kit (Illumina). This 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 currently used WGBS and RRBS methods.
- **Fast and streamlined procedure:** The procedure from DNA bisulfite treatment to ready-to-use library DNA can be completed within 6 hours and 30 minutes.

- **Complete conversion:** Completely converts unmethylated cytosine into uracil (>99%) with negligible inappropriate- or error-conversions of methylcytosine to thymine.
- **High sensitivity and efficiency:** Innovative adaptor ligation of bisulfite DNA eliminates loss of fragments and selection bias, which enables input DNA to be as low as 0.2 ng, making it ideal for methylation profiling of precious, limited samples. The kit can be used for both non-barcoded (singleplexed) and barcoded (multiplexed) DNA library preparation.
- **Extremely convenient:** The kit contains all required components for each step of DNA library preparation, 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.
- **Board sample suitability:** Starting materials can be genomic DNA isolated from various tissue/cell samples such as fresh and frozen tissue, cultured cells from a flask or microplate, microdissection samples, paraffin-embedded tissue, biopsy, 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 be also used as starting material.

BACKGROUND

Uses: The AMPINEXT™ High-Sensitivity Bisulfite-Seq Kit is designed to prepare bisulfite-converted DNA libraries for Illumina platform-based bisulfite sequencing including whole genome bisulfite sequencing, oxidative bisulfite sequencing, reduced representation bisulfite sequencing, and other bisulfite-next generation sequencing. The optimized protocol and components of the kit allow subnanogram DNA to be used for simultaneous bisulfite conversion and fragmentation followed by non-barcoded (singleplexed) and barcoded (multiplexed) library construction in less than 7 hours.

Starting Material and Input amount: Starting materials can be genomic DNA isolated from various tissue/cell samples such as fresh and frozen tissue, cultured cells from a flask or microplate, microdissection samples, FFPE tissue, 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 material. 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 0.2 ng to 200 ng. For optimal preparation, the input amount should be 10-50 ng.

MATERIALS SUPPLIED

Components	12 reactions (ENZ-GEN508 - 0012)	24 reactions (ENZ-GEN508 - 0024)	Storage Upon Receipt
Modification Buffer	3 ml	6 ml	RT
Modification Powder	2 vials	4 vials	RT
DNA Binding Solution	5 ml	10 ml	RT
Desulphonation Solution	50 µl	100 µl	RT
Elution Solution	0.5 ml	1 ml	RT
F-Spin Column	15	30	RT
F-Collection Tube	15	30	RT
5X Reaction Buffer*	100 µl	200 µl	-20°C
Reaction Enzyme Mix*	50 µl	100 µl	-20°C
Adaptor-F (10 µM) *	28 µl	56 µl	-20°C
Adaptor-S (10 µM) *	28 µl	56 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*	500 µl	1000 µl	-20°C
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** Spin the solution down to the bottom prior to use.*

ADDITIONAL MATERIALS NEEDED

The following are required but not provided:

- ☐ Vortex mixer
- ☐ Agilent® Bioanalyzer® or comparable method to assess the quality of the DNA library
- ☐ Thermocycler
- ☐ Centrifuge including desktop centrifuge (up to 14,000 rpm)
- ☐ 96-well format magnetic stand
- ☐ Pipettes and pipette tips
- ☐ PCR tubes or plates
- ☐ 1.5 ml microcentrifuge tubes
- ☐ 100% Ethanol
- ☐ Distilled or Deionized water
- ☐ DNA 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 Reaction Buffer, Reaction Enzyme Mix**, **Adaptor-F**, **Adaptor-S**, **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.

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 successfully preparing a library directly using bisulfite DNA generated from a tiny amount of input DNA. In this preparation, DNA is simultaneously bisulfite converted and fragmented to the appropriate length during the bisulfite process. The bisulfite-treated DNA, which is in single stranded form, is then simultaneously converted to dsDNA and adaptor ligated. The ligated fragments are size selected and purified using **MQ Binding Beads**, followed by amplification with 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 minimum bias.

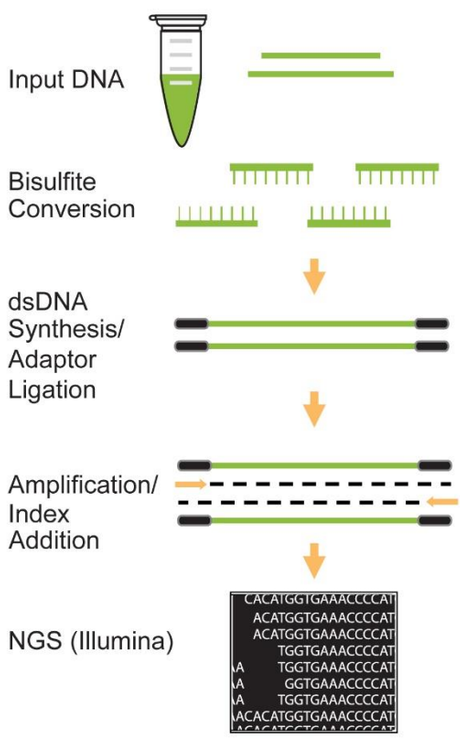

Fig 1. Workflow of the AMPINEXT™ High-Sensitivity Bisulfite-Seq Kit

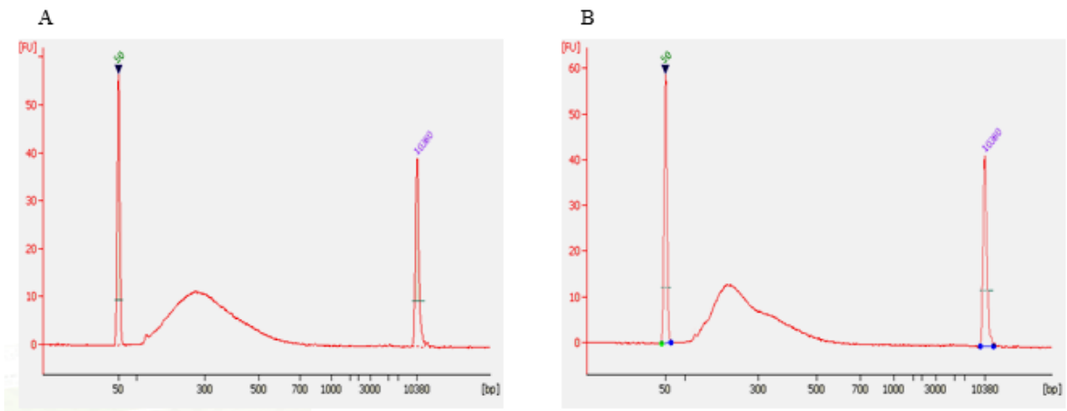

Fig 2. Size distribution of library fragments. Post-bisulfite DNA libraries were prepared from human placenta DNA using the AMPINEXT™ High-Sensitivity Bisulfite-Seq Kit: A: 10 ng; B: 50 ng.

PROCEDURE

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.

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 0.2 ng to 200 ng per reaction. An optimal amount is 10 ng-50 ng per reaction. Starting DNA may be in water or in a buffer such as TE. DNA should be 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. we offer 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.

DNA Fragment Purification: Use Magnetic Separator for DNA fragment purification with **MQ Binding Beads**.

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 (a 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 1-10 µl of sample DNA (20-100 ng).

Note: Check if the sample DNA volume is large and if the concentration is less than 5 ng/µl. If so, it is recommended to concentrate DNA 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 90 min

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

2. Converted DNA Clean-Up

a. Add 300 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 10-15 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 12.5 µl of **Elution Solution** directly to each column's filter membrane. Centrifuge at 12,000 rpm for 60 sec to elute converted DNA.

Converted 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 150-300 bps.

***Note**: If the amount of input DNA was > 10 ng, you may ensure the DNA is properly converted. We recommend checking the bisulfite-treated DNA by real time methylation-specific PCR (MS-PCR).*

3. dsDNA Conversion

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

Table 1. dsDNA Conversion

Component	Volume
Converted DNA (from Step 2) *	12 µl (10-50 ng input DNA)
5X Reaction Buffer	4 µl
Adaptor-F (10 µM)	2 µl
Reaction Enzyme Mix	2 µl
Total volume	**20 µl**

***** *If converted DNA volume is less than 12 µl, add distilled water to make the total volume 20 µl.*

b. Mix and incubate for 55 min at 37°C in a thermocycler without heated lid (make sure to set lid temperature to 25°C).

4. Clean-Up of dsDNA

***Note**: To ensure the correct ratio of **MQ Binding Beads** to sample solution during DNA clean up , make sure that any bead solution stuck on the outside of the pipette tip is removed before adding beads into the sample vial.*

a. Resuspend **MQ Binding Beads** by vortex.
b. Add exactly 24 µl of resuspended beads to the PCR tube of dsDNA synthesis 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 150 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 4e two times for a total of three washes.
g. Open the cap of the PCR tube and air dry beads for 2-3 minutes while the tube is on the magnetic stand.
h. Resuspend the beads in 10.5 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 10 µl of clear solution to a new 0.2 ml PCR tube for library synthesis.

5. Library Synthesis

a. Prepare library synthesis reaction in a 0.2 ml PCR tube according to Table 2:

Table 2. Library Synthesis

Component	Volume
dsDNA (from Step 4)	10 µl
5X Reaction Buffer	3.5 µl
Adaptor-S (10 µM)	2 µl
Total volume	**15.5 µl**

b. Mix and incubate for 2 min 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 min. Add 2 µl of **Reaction Enzyme Mix** and then incubate at 37°C for 60 min in a thermocycler without heated lid.

6. Clean-Up of Synthesized Library

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

b. Add exactly 21 µl 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 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. Remove tube from magnet. Add 150 µl of freshly prepared *90% ethanol* to the tube to resuspend the beads. Put the PCR tube back in the magnetic stand. Incubate at room temperature for 1 min, and then carefully remove and discard the ethanol.

f. Keep the PCR tube in the magnetic stand and add 150 µl of freshly prepared *90% ethanol* to the tube. Incubate at room temperature for 1 min, and then carefully remove and discard the ethanol.

g. Repeat Step 6f one more time for a total of three washes.

h. Open the cap of the PCR tube and air dry beads for 2-3 minutes while the tube is on the magnetic stand.

i. Resuspend the beads in 11 µl **Elution Buffer**, and incubate at room temperature for 2 minutes to release the DNA from the beads.

j. Capture the beads by placing the tube in the magnetic stand for 2 minutes or until the solution is completely clear.

k. Transfer 10.5 µl of clear solution to a new 0.2 ml PCR tube for library amplification and indexing.

7. Library Amplification and Indexing

a. Prepare the PCR Reactions:

Thaw all reaction components including master mix and primer solution. 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 Table 3:

Table 3. Library Amplification and Indexing

Component	Size (µl)
HiFi Master Mix (2X)	12.5 µl
Primer U	1 µl
Primer I (or barcode index)	1 µl
Synthesized library DNA (from Step 6)	10.5 µl
Total Volume	**25 µl**

Important Note: (1) *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 AMPINEXT™ NGS Barcode (Index) Set-12 (Cat. No. ENZ-GEN107) to generate each indexed library. You can also add user-defined barcodes (Illumina compatible) instead of **Primer I**. (2) Each indexed library can be combined in equal amounts to form multiplexed libraries for sequencing. (3) The amount of indexed library can be quantified using qPCR, Qubit or Picogreen assays.*

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	10 sec 20 sec 20 sec	22*
Final Extension	72°C	2 min	1

** 22 cycles is for 10 ng of input DNA. PCR cycles may vary depending on the input DNA amount. In general, use 19 cycles for 50 ng, 24 cycles for 1 ng, and 26 cycles for 0.2 ng of input DNA. Further optimization of PCR cycle number may be required.*

8. Clean-Up of Amplified Library

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

b. Add exactly 20 µl (0.8X) of resuspended beads to the amplified library. 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 2-3 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 150 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. Remove tube from magnet. Add 150 µl of freshly prepared *90% ethanol* to the tube to resuspend the beads. Put the PCR tube back in the magnetic stand. Incubate at room temperature for 1 min, and then carefully remove and discard the ethanol.

g. Repeat Step 8f one more time for a total of three washes.

h. Open the PCR tube cap and air dry beads for 2-3 minutes while the tube is on the magnetic stand.

i. Resuspend the beads in 10 µl **Elution Buffer**, and incubate at room temperature for 2 minutes to release the DNA from the beads.

j. Capture the beads by placing the tube in the magnetic stand for 2-3 minutes or until the solution is completely clear.

k. Transfer 10 µl to a new 0.2 ml PCR tube for immediate use or store at -20°C until ready to use for sequencing.

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.*

TROUBLESHOOTING

Problem	Possible Cause	Suggestion
DNA is poorly converted	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 gel. Ensure that RNA is removed by RNase treatment.
	Too little DNA or too much DNA (i.e., <5 pg or >200 ng).	Increase or decrease input DNA to within the correct range, or to the optimal amount of 10-50 ng.
	Temperature or thermal cycling condition is incorrect.	Check for appropriate temperature or thermal cycling 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 10-50 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 dsDNA Conversion, Library Synthesis and Library Amplification and Indexing.
	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.
Presence of <150 bp adaptor dimers	Improper ratio **of MQ Binding Beads** to DNA volume in size selection.	Check if the correct volume of **MQ Binding Beads** is added to DNA solution accordingly. Use 0.8X **MQ Binding Beads** to remove fragments below 150 bps.
	Insufficient ligation.	Too little input DNA and too much adaptors, may cause insufficient ligation and adaptor dimers. Make sure that ligation reaction is properly processed with the proper amount of input DNA and adaptors.
	Over-amplification of library.	PCR artifacts from over-amplification of library may cause increased adaptor dimers. Make sure to use proper PCR cycles to avoid this problem.

NOTES

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