SureSelect^{XT} Methyl-Seq combined with Post-Bisulfite Adaptor Tagging (PBAT) enables targeted shotgun bisulfite sequencing from low input DNA

Technical Note – Draft_B

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Abstract

DNA methylation is one of the most important epigenetic modifications involved in the regulation of gene expression. Whole-genome bisulfite sequencing (WGBS) is a powerful method that provides a genome-wide view of the 5-methylation status of cytosine residues at the single-nucleotide resolution. The cost to sequence the entire mammalian genome with enough read depth is still high, so focusing on targeted genomic regions is a cost effective alternative to WGBS for DNA methylation studies. The Agilent Technologies SureSelect^{XT} Methyl-Seq kit combines SureSelect target enrichment technology with shotgun bisulfite sequencing (SBS). This kit enables targeted DNA methylation studies on the most relevant regions for epigenetic research with unprecedented sequence coverage. To target applications that require less than 1–3 µg of input gDNA, this technical note describes a method to use Post-Bisulfite Adapter Tagging (PBAT) for targeted SBS analysis. This method, or SureSelect-PBAT, enables an input of 1 µg of gDNA using an amplification-free template preparation, an input of 100 ng of gDNA with 3 cycles of PCR amplification, or a minimum input of 30 ng of gDNA with 4 cycles of PCR amplification to produce high-quality data. The competing product has a 5–6 day protocol as compared to the SureSelect-PBAT method of only 3 days. SureSelect-PBAT provides an effective solution for samples with limited amount of gDNA to advance DNA methylation research.

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Introduction

Post-Bisulfite Adaptor Tagging

Post-Bisulfite Adaptor Tagging (PBAT) is a method originally developed for whole-genome bisulfite sequencing (WGBS)[1]. Bisulfite treatment selectively changes unmethylated cytosine to uracil, while methylated cytosine remains unconverted. The methylation status of each cytosine can be determined by reading the sequence of the target region after bisulfite treatment; this procedure is called bisulfite sequencing (BS)[2]. WGBS is a large-scale version of BS, where shotgun sequencing is performed on bisulfite treated gDNA. While WGBS has increasingly been used in DNA methylation studies, one of the major drawbacks for this type of template preparation was the lack of a low sample input protocol. Conventional protocols require 5 µg of starting DNA and several cycles of PCR amplification[3, 4], which is undesirable for two reasons: (1) the requirement for large amounts of starting material prevents this application from being used with samples of limited quantity and (2) extensive PCR amplification cycles causes sequencing biases and skews the quantitative accuracy of the assay. Therefore, a method for template preparation from limited sample input amounts for WGBS is in high demand. For WGBS, sample DNA is tagged with specific sequences at both ends. This is achieved by first tagging the adaptor sequences to the sample DNA, followed by bisulfite treatment. Unfortunately, bisulfite treatment is known to induce DNA fragmentation, which causes the degradation of the basic template structure and leads to a severe loss of sample. To avoid the bisulfite-induced degradation of adaptor-tagged DNA, we devised the PBAT strategy wherein adaptor tagging is performed after bisulfite treatment. For effective tagging of single-stranded bisulfite treated DNA, the PBAT method employs two rounds of random primer extension, where the first round is on the bisulfite-treated DNA and the second round is on the first strand DNA immobilized on beads. This results in a highly sensitive, amplification-free preparation of sequencing template for WGBS from as little as 30 ng of mammalian gDNA. PBAT was successfully applied to mouse WGBS, where enough starting material can be obtained from 400–1,000 germinal vesicle-stage oocytes[5] or a few thousand primordial germ cells[6]. More recently, it has enabled WGBS of mouse zygotes and human oocytes and blastcysts[7, 8].

SureSelect Target Enrichment System

The Agilent Technologies SureSelect Target Enrichment System provides efficient enrichment of genomic areas of interest, which allows for the substantial reduction in sequencing costs due to the ability to sequence multiple samples per study. The Agilent SureSelect^{XT} Methyl-Seq protocol combines the SureSelect Target Enrichment System with shotgun bisulfite sequencing (SBS) to allow for a cost effective, genome-scale quantitative analysis of DNA methylation at the single-nucleotide resolution. The current portfolio of SureSelect^{XT} Methyl-Seq systems includes kits for human, mouse and rat models and are comprised of carefully selected, comprehensive designs focusing on genomic regions specific to epigenetic control (Table 1).

Table 1. Design content for SureSelect^{XT} Methyl-Seq Systems

SureSelect^{XT} Methyl-Seq System	Design Content
Human Methyl-Seq (84 Mb Design)	• CpG islands • Cancer, tissue specific DMRs • Gencode promoters • DMRs or regulatory features in: ◦ CpG islands, shores and shelves ±4kb ◦ DNase I hypersensitive sites ◦ Refseq genes ◦ Ensembl regulatory features
Mouse Methyl-Seq (109 Mb Design) *Early Access*	• CpG islands • Tissue specific DMRs • Ensembl Regulatory features in: ◦ CpG islands, shores and shelves ± 4kb ◦ DNase I hypersensitive sites ◦ Histone Modifications ◦ Transcription Factor Binding Sites (TFBS) ◦ Polymerase Binding Sites • Open Regulatory Annotation (ORegAnno): ◦ Promoters ◦ Enhancers ◦ TFBS ◦ Regulatory Polymorphisms
Rat Methyl-Seq (97 Mb Design) *Early Access*	• Gene promoters • CpG islands, shores ± 1kb • Tissue specific DMRs

PBAT enhances the sensitivity and speed of SureSelect^{XT} Methyl-Seq

Since conventional protocols are designed to perform bisulfite treatment after completing both adaptor-tagging and target enrichment, the bisulfite-induced loss of template is inevitable. To compensate for this loss, up to 5 µg of input gDNA and PCR amplification are required. By combining the PBAT strategy with SureSelect^{XT} Methyl-Seq (SureSelect-PBAT), as shown in Figure 1, a significant decrease in the required amount of starting input gDNA is achieved. SureSelect-PBAT requires 1 µg of gDNA for a PCR-free preparation of the template and a minimum of 30 ng of gDNA with 4 amplification cycles for targeted SBS[9]. A recommended input of 100 ng of gDNA with 3 amplification cycles provides a balanced performance. By using SureSelect-PBAT, the template preparation workflow for targeted SBS has been reduced to 3 days, which is an overall improvement of 2–3 days as compared to a competitor's protocol. (Figure 2A and 2B).

This method has been shown to exhibit high consistency between varied sample inputs for the SureSelect-PBAT workflow and with WGBS. SureSelect-PBAT was evaluated on the methylation levels of CpG sites that were covered by 20 or more reads along with the methylation status of imprinted genes. Based on plotted methylation levels (moving averages; window size, 500 bp; step size, 250 bp), a high consistency among 5 SureSelect-PBAT libraries from 30 ng to 3 µg input (R^2 >0.955) and with WGBS data (R^2 = 0.951) was reported[9].

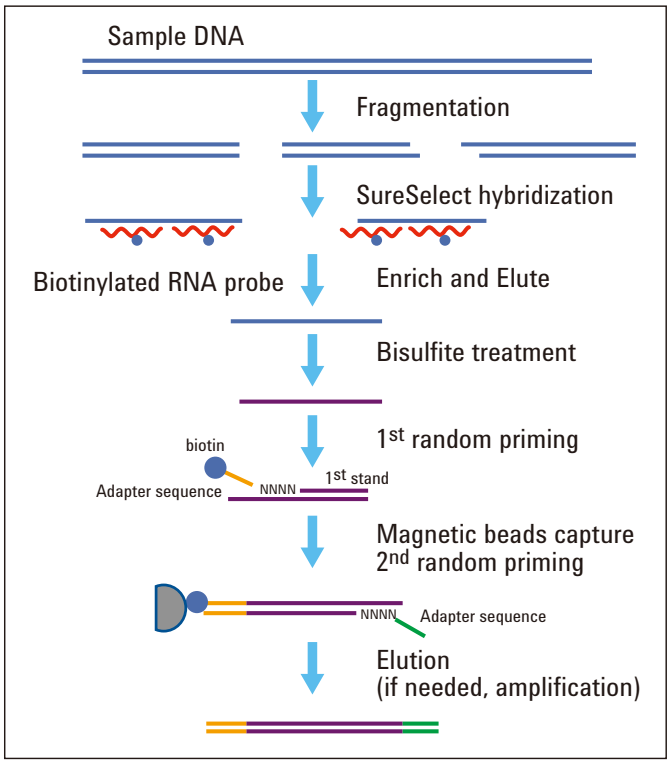

Figure 1 SureSelect-PBAT workflow

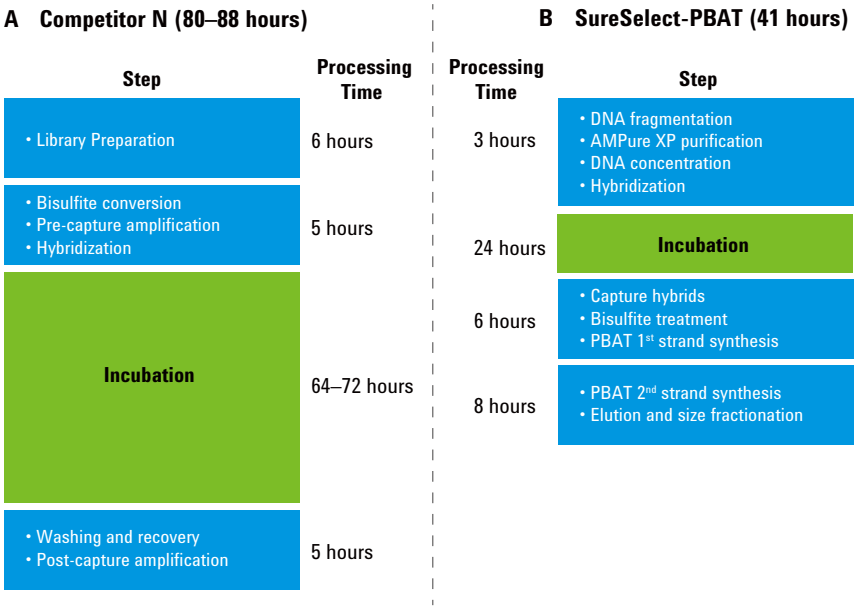

Figure 2 Workflow comparisons between (A) the 5-6 day protocol from competitor N and (B) the 3 day SureSelect-PBAT protocol.

Protocol

1. Before you begin

NOTE *The SureSelect-PBAT method requires a certain amount of input DNA from the SureSelect hybridization for adapter priming. Sufficient yields are obtained from the Human Methyl-Seq (84 Mb), Mouse Methyl-Seq (109 Mb) and Rat Methyl-Seq captures (97 Mb) after SureSelect hybridization to continue with post-bisulfite adapter tagging (PBAT). However, smaller capture sizes may not generate sufficient yield after hybridization to continue with PBAT. Currently, we have not validated the lower limits of target size for SureSelect-PBAT.*

The quality of starting gDNA may affect the final yield and need for optimization. When high quality gDNA is provided, 30 ng is the minimum input amount for the SureSelect-PBAT protocol. Otherwise, we recommend 100 ng of gDNA as the starting input amount for samples.

Procedural Notes

1. Prolonged exposure of SureSelect Elution Buffer to air can decrease product performance by altering the pH of the solution. Keep the Elution Buffer container tightly sealed when not in use.
2. Required Reagents are listed in Table 2.
3. Required Equipment is listed in Table 3 (page 6).

Table 2. Required reagents

Description	Vendor and part number
Select one:	
SureSelect^{XT} Human Methyl-Seq Capture Library	Agilent
16 reactions	5190-4661
96 reactions	5190-4662
SureSelect^{XT} Mouse Methyl-Seq Capture Library (Early Access)	
16 reactions	931052
SureSelect^{XT} Rat Methyl-Seq Capture Library (Early Access)	
16 reaction	931143
96 reaction	931144
SureSelect Target Enrichment Kit	Agilent
16 reactions	930671
96 reactions	930672
EZ-DNA Methylation Gold Kit	Zymo Research
50 reactions	D5005
200 reactions	D5006
Dynabeads MyOne Streptavidin T1	Life Technologies
2 mL	65601
10 mL	65602
100 mL	65603
Agencourt AMPure XP	Beckman Coulter
5 mL	A63880
60 mL	A63881
450 mL	A63882
Qubit dsDNA BR Assay Kit	Life Technologies
100 assays, 2-1000 ng	Q32850
500 assays, 2-1000 ng	Q32853
KAPA Library Quantification Kit for Illumina	KAPA KK4824
KAPA Library Amplification Kit	KAPA KK2611
Conventional 10x PCR Buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 15 mM $MgCl_2$) 5 mL	Sigma Aldrich P2192 or equivalent

Table 2. Required reagents *continued*

Description	Vendor and part number
Klenow Fragment (3′→5′ exo-) Be sure to use the enzyme provided at 10-fold higher concentration (i.e., #M0212M; 50,000 U/mL) rather than the conventional ones (i.e., #M0212S and #M0212L; 5,000 U/mL)	NEB M0212M
Bst DNA Polymerase Large Fragment 1,600 units at 8,000 units/mL	NEB M0275S
Exonuclease I 3,000 units at 20,000 units/mL	NEB M0293S
Phusion Hot Start II High-Fidelity DNA Polymerase 100 units at 2 units/µL	Finnzymes F-549S
2.5 mM dNTPs 1 mL	Life Technologies R725-01
10 mM Tris-acetate, pH 8.0	
10 mM Tris-HCl, pH 7.5	
2x BW(Li) Solution (50 mL) • LiCl* 6.3 g • 1 M Tris-HCl, pH 8.0 0.5 mL • 500 mM EDTA 0.1 mL *The process of dissolution of LiCl is exothermic. To avoid bumping of the solution, dissolve LiCl in 40 mL of double-distilled water (ddH2O) at a time. After complete dissolution of LiCl, add Tris and EDTA, and adjust the volume to 50 mL with ddH2O.	
10 M NaOH stock solution	Sigma Aldrich 656054 Fluka
Hybridization Buffer A (50 mL) • 5 M NaCl, 9 mL (final 900 mM) • 1 M Tris-HCl, pH 7.4, 9 mL (final 180 mM) • ddH_2O 32 mL	
100% Ethanol, molecular biology grade	Sigma Aldrich E7023
Nuclease-free Water (not DEPC-treated)	Ambion AM9930
1x Low TE Buffer (10mM Tris-HCl, pH8.0, 0.1 mM EDTA)	Life Technologies 4389764
Formamide, deionized	Wako Pure Chemical Industries 066-02301 or equivalent
Mineral oil LIGHT WHITE	Wako Pure Chemical Industries 194836 or equivalent
PhiX Control Kit v3 (10 lanes)	Illumina FC-110-3001
Oligonucleotides (OPC grade)* Bio-PEA2-W4N4 (100 µM) PE-reverse-N4 (100 µM) For single-end sequencing PBAT-PE-iX-W4N4 (100 µM each) For pair-end and index sequencing Primer 3 (100 µM) PBAT-PE-Seq (100 µM) PBAT-PE-Idx (100 µM) PE-forward primer (100 µM) PE-reverse primer (100 µM)	Operon or equivalent

**Please see Tables 23 and 24 in the appendix for detailed information about the oligo sequences.*

Table 3. Required equipment

Description	Vendor and Part Number
SureCycler 8800 Thermal Cycler	Agilent G8800A
96 well plate module for SureCycler 8800 Thermal Cycler	Agilent G8810A
SureCycler 8800 Compatible 96-well plates 8 well strip tubes	Agilent 410088 410092
Tube cap strips, domed	Agilent 410096
Optical strip caps	Agilent 401425
Covaris Sample Preparation System, E-series or S-series	Covaris
Covaris sample holders 96 microTUBE plate (E-series instruments only) microTUBE for individual sample processing	Covaris 520078 520045
DNA LoBind Tubes, 1.5-mL PCR clean, 250 pieces	Eppendorf 22431021 or equivalent
Centrifuge	Eppendorf model 5804 or equivalent
Qubit Fluorometer	Life Technologies Q32857
Qubit assay tubes	Life Technologies Q32856
Magnetic separator DynaMag-2 magnet (for samples processed in tube)	Life Technologies 123-21D
Magnetic stand DynaMag-96 side magnet (for PCR strips and 96 well PCR plates) NGS MagnaStand (for PCR strips)	Life Technologies 123-31D Nippon Genetics FG-SSMAG2
Nutator plate mixer	BD Diagnostics 421105 or equivalent
Multichannel pipette	Pipetman or equivalent
P10, P20, P200 and P1000 pipettes	Pipetman P10, P20, P200, P1000 or equivalent
Vacuum concentrator	Savant SpeedVac or equivalent
Ice bucket	Any major vendor
Powder-free gloves	Any major vendor
Sterile, nuclease-free aerosol barrier pipette tips	Any major vendor
Timer	Any major vendor
Vortex mixer	Any major vendor
Heat block (qty of 2) suitable for incubation temperatures of 65 °C and 99 °C	Any major vendor
Parafilm	Any major vendor
Water bath for incubation temperature at 65 °C	Any major vendor

2. Sample Preparation

Step 1. Estimation of DNA concentration

NOTE *Accurate estimation of DNA concentration is critical. Avoid direct quantification using the OD260 value, as various materials other than DNA absorb light at 260 nm, leading to an overestimation of DNA concentration.*

1. Measure DNA concentration of sample DNA with Qubit dsDNA BR Assay Kit and Qubit fluorometer according to the manufacturer's instruction.

Step 2. Shear DNA

1. Set up the Covaris instrument.
   a. Check that the water in the Covaris tank is filled with fresh deionized water to fill line level 12 on the graduated fill line label.
   b. Check that the water covers the visible glass part of the tube.
   c. Set the chiller temperature between 2 °C to 5 °C to ensure that the temperature reading in the water bath displays 5 °C.
   d. *Optional*. Supplement the circulated water chiller with ethylene glycol to 20% volume to prevent freezing.
   e. On the instrument control panel, push the Degas button. Degas the instrument for least 30 minutes before use. Refer to the Covaris instrument user guide.
2. Dilute 30 ng – 3 µg of high-quality gDNA with 1X Low TE Buffer in a 1.5-mL LoBind Tube to a total volume of 130 µL.
3. Place a Covaris microTube into the loading and unloading station. Keep the cap on the tube.
4. Use a tapered pipette tip to slowly transfer the 130 µL of DNA sample through the pre-split septa. Be careful not to introduce a bubble into the bottom of the tube.
5. Secure the microTube in the tube holder and shear the DNA with the settings in Table 4. The target peak is 500 to 600 bp.

Table 4 Shearing settings for Covaris instruments (for use with SonoLab 7 or newer)

Setting	Value
Peak Power	140
Duty factor	10%
Cycles per Burst	200
Duration Time	55 seconds

6. Put the Covaris microTube back into the loading and unloading station.
7. While keeping the snap-cap on, insert a pipette tip through the pre-split septa and then slowly remove the sheared DNA.
8. Transfer each 130-µL sheared DNA sample to a fresh 1.5 mL tube.

Step 3. Purify sample using AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes.
2. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color. Do not freeze.
3. Add 240 µL of homogenous AMPure XP beads to each 130 µL DNA sample. Pipette up and down to mix.
4. Incubate the samples for 15 minutes at room temperature.
5. Put the tubes or plate into the appropriate magnetic separation device. Wait for the solution to clear (approximately 3 to 5 minutes).
6. Keep the samples in the magnetic separation device. Carefully remove and discard the cleared solution from each tube or well. Do not touch the beads while removing the solution.
7. Continue to keep the samples in the magnetic separation device while you add 500 µL of 75% ethanol to each tube.
8. Wait for 1 minute to allow any disturbed beads to settle and then remove the ethanol.
9. Dry the samples on a thermal cycler, set to hold samples at 37 °C, for 5 minutes or until the residual ethanol completely evaporates. Do not over dry the bead pellet. Elution efficiency is significantly decreased when the bead pellet is excessively dried.
10. Add 20 µL of nuclease-free water to each sample.
11. Mix well on a vortex mixer and briefly spin the tubes or plate to collect the liquid.
12. Incubate for 2 minutes at room temperature.
13. Put the tubes or plate into the magnetic separation device and leave for 2 to 3 minutes, until the solution is clear.
14. Transfer the cleared supernatant (approximately 20 µL) to a fresh 1.5 mL LoBind tube.

3. Hybridization

Step 1. Hybridize the library

1. Use all of the sheared DNA for hybridization.
2. Use a vacuum concentrator to concentrate the sample at ≤45 °C.
   a Add the entire volume of sheared (20 µL) DNA to an Eppendorf tube. Poke one or more holes in the lid with a narrow gauge needle. You can also break off the cap, cover with parafilm, and poke a hole in the parafilm.
   b Evaporate the solvent using a vacuum concentrator on low heat (less than 45 °C).
   c Reconstitute with nuclease-free water to a final volume of 7 µL. Pipette up and down along the sides of the tube for optimal recovery.
   d Mix well on a vortex mixer and spin in a centrifuge for 1 minute.
3. Before proceeding to the next steps, prepare 2 heat blocks set at 99 °C and 65 °C
4. Mix the components in Table 5 at room temperature to prepare the DNA sample mix.
5. Mix the components in Table 6 at room temperature to prepare the hybridization buffer mix.
6. Mix the components in Table 7 at room temperature to prepare the capture library mix.
7. Add 80 µL of mineral oil into the three tubes prepared in steps 4–6.
   a Add 80 µL of mineral oil into the DNA sample mix prepared in step 4.
   b Add 80 µL of mineral oil into the hybridization buffer mix prepared in step 5.
   c Add 80 µL of mineral oil into the capture library mix prepared in step 6.
   d Spin all three tubes in a centrifuge and confirm the separation of 2 layers.
8. Put the DNA sample mix from Step 7a in a heat block and incubate at 99 °C for 10 min.
9. Next, incubate the DNA sample mix from step 8 for 5 min at 65 °C.
10. Shortly after step 9, incubate the hybridization buffer mix from step 7b and the capture library mix from step 7c at 65 °C for 10 min.
11. While maintaining the tube at 65 °C load the entire volume of the hybridization buffer mix, including mineral oil into the capture library mix. Mix well by slowly pipetting up and down 8 to 10 times.
12. While maintaining the tube at 65 °C, load the entire volume of the hybridization buffer and the capture library mix, including mineral oil, into the DNA sample mix. Mix well by slowly pipetting up and down 8 to 10 times.
13. Incubate the hybridization mixture for 24 hours at 65 °C.

Table 5 DNA sample mix

Reagent	Volume for 1 reaction
Fragmented and purified DNA from previous step	7 µL
Formamide, deionized	3 µL

Table 6 Hybridization buffer mix

Reagent	Volume for 1 reaction
SureSelect Hyb #1	7.5 µL
SureSelect Hyb #2	0.3 µL
SureSelect Hyb #3	3.0 µL
SureSelect Hyb #4	3.0 µL

Table 7 Capture library mix

Reagent	Volume for 1 reaction
SureSelect Methyl-Seq Capture Library	5.0 µL
SureSelect RNase Block	0.5 µL
Nuclease-free water	1.0 µL

Step 2. Prepare streptavidin beads

1. Pre-warm SureSelect Wash Buffer #2 at 65 °C in a circulating water bath. Each sample needs 1500 µL + extra volume of Wash Buffer #2.
2. Vigorously re-suspend the Dynabeads MyOne Streptavidin T1 magnetic beads on a vortex mixer, as magnetic beads will settle during storage.
3. For each hybridization, add 50 µL Dynal magnetic beads to a 1.5-mL microfuge LoBind tube.
4. Wash the beads:
   a Add 200 µL of SureSelect Binding Buffer.
   b Mix the beads using a vortexer for 5 seconds.
   c Put the tubes into a magnetic separation device, and allow the solution to clear.
   d Remove and discard the supernatant.
   e Repeat steps a through d for a total of 3 washes.

Step 3. Capture hybrids using streptavidin beads

1. While keeping the tubes at 65 °C, add 200 µL of the binding buffer into the hybridization mix and mix well by pipetting.
2. Transfer the entire solution from step 1 to the tube containing beads and invert the tube 3 to 5 times to mix.
3. Incubate the hybrid-capture/ bead solution on a Nutator, or equivalent, for 30 minutes at room temperature. Make sure the sample is properly mixing in the tube.
4. Briefly centrifuge the tube.
5. Separate the beads from the buffer using a magnetic separation device and remove the supernatant.
6. Re-suspend the beads in 500 µL of SureSelect Wash Buffer #1 by vortexing for 5 seconds.
7. Separate the beads and buffer on a magnetic separation device and remove the supernatant.
8. Wash the beads:
   a Re-suspend the beads in 500 µL of 65 °C pre-warmed SureSelect Wash Buffer #2 and vortex for 5 seconds.
   b Incubate the samples for 10 minutes at 65 °C in a recirculating water bath, heat block or equivalent. Do not use a tissue incubator, as it cannot properly maintain temperature.
   c Briefly centrifuge the tube.
   d Separate the beads and buffer on a magnetic separation device and remove the supernatant.
   e Repeat steps a through d for a total of 3 washes. Make sure all of the wash buffer has been removed.
   f Re-suspend the beads in 20 µL of SureSelect Elution Buffer and vortex for 5 seconds.
9. Incubate the samples for 20 minutes at room temperature.
10. During the 20-minute incubation, prepare the EZ DNA Methylation-Gold Kit CT Conversion Reagent as described in steps 1 and 2 on page 10.
11. Separate the beads and buffer on a magnetic separation device.
12. Use a pipette to transfer the supernatant to a new 1.5-mL microfuge tube.
13. The supernatant contains the captured DNA and the beads can now be discarded.
14. Proceed immediately to “Bisulfite Conversion”.

4. Bisulfite Conversion

This chapter describes the necessary steps for bisulfite treatment of the captured DNA in order to differentiate methylated and unmethylated DNA segments. When treated with bisulfite, unmethylated cytosine residues in the library are converted to uracil residues. Methylated cytosine residues remain unmodified, resulting in a primary sequence difference that may be detected and quantified in subsequent NGS analysis.

Step 1. Modify captured DNA by bisulfite conversion

NOTE *In this step, use the EZ DNA Methylation-Gold Kit from Zymo Research to modify unmethylated cytosine residues in the captured DNA to uracil residues using bisulfite conversion. The treated DNA is then desulphonated using a Zymo-Spin IC column and additional reagents from the EZ DNA Methylation-Gold Kit.*

1. Re-suspend one vial of solid CT Conversion Reagent by adding 900 µL of nuclease-free water, 300 µL of M-Dilution Buffer, and 50 µL of M-Dissolving Buffer to the vial.
2. Mix for 10 minutes with frequent vortexing at room temperature.
3. To each 20-µL captured library sample, add 130 µL of the CT Conversion Reagent from step 2.
4. Vortex briefly, then briefly spin in a centrifuge.
5. Transfer 50 µL of the mixture to each of the three PCR tubes.
6. Place the tubes in a thermal cycler and incubate the bisulfite conversion reactions at 64 °C for 2.5 hours.

   NOTE *For precise control of the reaction time, include a 4 °C hold as shown in Table 8. After completing the bisulfite conversion step at 64 °C, proceed immediately to desulphonation in step 7. Do not keep the sample at 4 °C for an extended period of time.*

Table 8 Thermal cycler program for bisulfite conversion

Step	Temperature*	Time
Step 1	64 °C	2.5 hours
Step 2	4 °C	Hold

** The bisulfite conversion protocol provided with the Zymo Research kit includes an initial 98 °C incubation step. This step is omitted in the SureSelect-PBAT protocol.*

7. Desulphonate the sample using a Zymo-Spin IC column. Use one column for each 150-µL DNA sample after recombining the three 50 µL bisulfite conversion reactions for each DNA library.

   NOTE *Before starting the desulphonation procedure, make sure that the M-Wash buffer provided with the EZ DNA Methylation-Gold Kit has been prepared to contain 80% ethanol, according to the kit instructions.*

   a Add 600 µL of M-Binding Buffer to a Zymo-Spin IC column and place the column in a collection tube.

   b Load the 150-µL bisulfite-converted DNA sample onto the column.

   c Cap the column and mix well by inverting the column five times. Centrifuge for 60 seconds at 13,000 rpm. Discard the flow-through and then place the column back in the same collection tube.

   d Wash the column by adding 100 µL of prepared M-Wash Buffer. Centrifuge for 60 seconds at 13,000 rpm. Discard the flow-through and then place the column back in the same collection tube.

   e Add 200 µL of M-Desulphonation Buffer to the column. Incubate at room temperature for 15 minutes.

   f Centrifuge for 60 seconds at 13,000 rpm. Discard the flow-through and then place the column back in the same collection tube.

   g Add 200 µL of prepared M-Wash Buffer to the column. Centrifuge for 60 seconds at 13,000 rpm. Discard the flow-through and then place the column back in the same collection tube.

   h Add another 200 µL of prepared M-Wash Buffer to the column. Centrifuge for 60 seconds at 13,000 rpm.

   i Place the column in a fresh 1.5 mL tube.

   j Allow the column to sit at room temperature for 2 minutes.

   k Add 20 µL of M-Elution Buffer to the column and incubate at room temperature for 2 minutes.

   l Centrifuge for 60 seconds at 13,000 rpm. Retain the combined 20-µL flow-through for further processing.

5. First-strand synthesis

Step 1. First-strand synthesis

1. Prepare the First-strand synthesis reaction mix as follows:

Table 9 First-strand synthesis reaction mix

Reagent	Volume for 1 reaction
10x NEBuffer 2 *	5 µL
2.5 mM dNTPs	5 µL
Nuclease-free water	16 µL
Bio-PEA2-W4N4 (100 µM)	4 µL
Bisulfite-treated sample DNA	20 µL

**10x NEBuffer 2 is included in Klenow Fragment (NEB M0212M)*

2. Place the tube on a thermal cycler and run the first strand synthesis program as shown in Table 10. After 5 min of starting step 2 (i.e., incubation step at 4 °C) on the program outlined in Table 10, pause the program and remove the tube from the thermal cycler. Add 1.5 µL of Klenow Fragment (3'→5' exo–) to the first-strand synthesis solution and mix well.
3. Place the tube back on the thermal cycler and resume the program in Table 10 to complete the first-strand synthesis reaction.

   NOTE *You can stop here by either leaving the tube at 4 °C or storing at –20 °C until use. The bisulfite-treated DNA is now double-stranded and excessive primers in the solution serve as carrier DNA to prevent the adsorption of template DNA to the tube wall.*

Step 2. Removal of excess primers

1. Transfer the solution of the first strand reaction (~50 µL) into a new 1.5-mL tube and add 50 µL of AMPure XP beads. Mix well and spin the tube briefly.

   NOTE *The DNA solution to AMPure XP bead ratio at this step is 1:1. This ensures effective removal of DNA fragments that are shorter than 200 bp (i.e. primers and primer dimers) and retains the products of the first-strand synthesis, which are longer than 200 bp.*
2. Let the tube stand at room temperature for 10 min.
3. Place the tube on a magnetic separation device and wait for the beads to be collected. Then remove the supernatant carefully so as not to aspirate the beads.
4. Add 200 µL of 75% ethanol to wash the beads and then remove the supernatant.
5. Add 45 µL of 10 mM Tris-acetate (pH 8.0) and vortex the tube well to disperse the beads. Following a brief centrifugation, place the tube on the magnetic separation device and wait for the beads to be collected.
6. Transfer the supernatant into a new 1.5-mL tube. Add 5 µL of 10x PCR Buffer and 50 µL of AMPure XP beads to the supernatant. Then mix well and spin briefly.

   NOTE *Addition of 10x PCR Buffer at this step increases the reproducibility and yield of AMPure XP-based purification of DNA.*
7. Let the tube stand at room temperature for 10 min.
8. Place the tube on the magnetic separation device and wait for the beads to be collected. Then, remove the supernatant carefully so as not to aspirate the beads.
9. Add 200 µL of 75% ethanol to wash the beads and then remove the supernatant.
10. Add 51 µL of 10 mM Tris-acetate (pH 8.0) and vortex the tube well to disperse the beads. After a brief centrifugation, place the tube on the magnetic separation device and wait for the beads to be collected.
11. Transfer the supernatant in a new 1.5 mL tube.

Table 10 Thermal cycler program for first-strand synthesis

Step	Temperature	Time
Step 1	94 °C	5 min
Step 2	4 °C	20 min After 5 min from starting step 2, pause and add 1.5 µL Klenow Fragment (3'→5' exo–)
Step 3	Gradual increment from 4 °C to 37 °C at a rate of +1 °C /min *	
Step 4	37 °C	90 min
Step 5	70 °C	10 min
Step 6	4 °C	Hold

** If your thermal cycler cannot generate a temperature ramp of +1 °C/min, you may use a two-step PCR cycling program with an increment of temperature by 1 °C for every step. If the first cycle of the program is set at 4.0 °C for 30 sec followed by 4.5 °C for 30 sec, the temperature will reach 37 °C after 33 cycles with the intended rate of +1 °C/min.*

Step 3. Capturing biotinylated DNA on streptavidin beads

1. Take 20 µL of a well-dispersed suspension of Dynabeads MyOne Streptavidin T1 beads into a 1.5 mL tube. Place the tube on a magnetic separation device to collect the beads.
2. Remove the supernatant and add 50 µL of 2x BW(Li) buffer to suspend the beads.
3. Add the suspension of beads to the eluted DNA obtained in step 2 above.
4. Incubate the tube at room temperature for 30 min by gently rotating the tube.
5. Place the tube on a magnetic separation device to collect the beads and then remove the supernatant.
6. Add 180 µL of 2× BW(Li) buffer to the beads, vortex well and spin the tube briefly.
7. Place the tube on a magnetic separation device to collect the beads and then remove the supernatant.
8. Prepare 0.1 M NaOH from 10 N NaOH. Suspend the beads in 180 µL of 0.1 M NaOH solution and vortex well. Prepare 0.1 M NaOH from 10 N NaOH just prior to use. Incubate at room temperature for 2 min and spin briefly.
9. Place the tube on a magnetic separation device to collect the beads and then remove the supernatant.
10. Repeat steps 8 and 9 once again.
11. Add 180 µL of 2× BW(Li) buffer to the beads, vortex well and spin the tube briefly.
12. Place the tube on a magnetic separation device to collect the beads and then remove the supernatant.
13. Add 180 µL of 10 mM Tris-HCl (pH 7.5) to the beads, vortex well and spin the tube briefly.

6. Second-strand synthesis

Step 1. Second-strand synthesis

1. Place the tube on a magnetic separation device to collect the beads and remove the supernatant.
2. Prepare the second-strand synthesis reaction mix as follows and add to the beads.

Table 11 Second-strand synthesis reaction mix

Reagent	Volume for 1 reaction
10x NEBuffer 2	5 µL
2.5 mM dNTPs	5 µL
Nuclease-free water	36 µL
PE-reverse-N4 (100 µM) for single-end sequencing, or unique PBAT-PE-iX-W4N4 (100 µM) for paired-end and index sequencing	4 µL

3. Suspend the beads by vortexing and transfer the bead suspension into a new 0.2 mL tube.
4. Place the tube on the thermal cycler and run the second strand synthesis program as shown in Table 12. After 5 min of starting step 2 (i.e. incubation step at 4 °C) on the program as outlined in Table 12, pause the program and remove the tube from the thermal cycler. Add 1.5 µL of Klenow Fragment (3'→5' exo–) to the second-strand synthesis solution and mix well.
5. Place the tube back on the thermal cycler and resume the program in Table 12 to complete the second-strand synthesis reaction.

Table 12 Thermal cycler program for Second-strand synthesis

Step	Temperature	Time
Step 1	94 °C	5 min
Step 2	4 °C	20 min After 5 min from starting step 2, pause and add 1.5 µL Klenow Fragment (3'→5' exo–)
Step 3	Gradual increment from 4 °C to 37 °C at a rate of +1 °C/min *	
Step 4	37 °C	30 min
Step 5	70 °C	10 min
Step 6	4 °C	Hold

** If your thermal cycler cannot generate a temperature ramp of +1 °C/min, you may use a two-step PCR cycling program with an increment of temperature by 1 °C for every step. If the first cycle of the program is set at 4.0 °C for 30 sec followed by 4.5 °C for 30 sec, the temperature will reach to 37 °C after 33 cycles with the intended rate of +1 °C/min.*

Step 2. Chase reaction

1. Place the tube on a magnetic separation device to collect the beads and remove the supernatant.
2. Prepare the chase reaction mix as follows and add to the beads.

Table 13 Chase reaction mix

Reagent	Volume for 1 reaction
10x ThermoPol Buffer *	5 µL
2.5 mM dNTPs	5 µL
Nuclease-free water	40 µL
Bst DNA Polymerase Large Fragment	1 µL

**10x ThermoPol buffer is included in Bst DNA Polymerase Large Fragment box.*

3. Incubate the reaction at 65 °C for 30 min.

Step 3. Elution/extension of template DNA

1. Place the tube on a magnetic separation device to collect the beads and remove the supernatant.
2. Prepare the elution/extension reaction mix as follows and add to the beads.

Table 14 Elution/extension reaction mix

Reagent	Volume for 1 reaction
5x Phusion HF Buffer *	10 µL
2.5 mM dNTPs	5 µL
Nuclease-free water	35 µL
Primer 3 (100 µM)	0.4 µL
Phusion Hot Start II High-Fidelity DNA Polymerase	1 µL

**5x Phusion HF Buffer is included in Phusion Hot Start II High-Fidelity DNA Polymerase box.*

NOTE *This step not only enables the precise selection of double-stranded DNA by SPRI beads, but also synthesizes the sequence required for bridge PCR.*

3. Place the tube on a thermal cycler and run the Elution / extension reaction program shown in Table 15.

Table 15 Thermal cycler program for Elution/extension reaction

Step	Temperature	Time
Step 1	94 °C	5 min
Step 2	55 °C	15 min
Step 3	68 °C	30 min
Step 4	4 °C	Hold

4. Place the tube on a magnetic separation device to collect the beads and transfer the supernatant into a new 1.5 mL tube.
5. Add 1 µL of Exonuclease I to the supernatant, mix well, and incubate the tube at 37 °C for 15 min followed by heat-inactivation at 70 °C for 10 min.

Step 4. Purify sample using AMPure XP beads

1. Add 50 µL of AMPure XP beads to the eluted DNA (50 µL), mix well and spin briefly.
2. Let the tube stand at room temperature for 10 min.
3. Place the tube on a magnetic separation device and wait for the beads to separate. Then, remove the supernatant carefully so as not to aspirate the beads.
4. Add 200 µL of 75% ethanol to wash the beads and then remove the supernatant.
5. Add 45 µL of 10 mM Tris-acetate (pH 8.0) and vortex the tube well to disperse the beads. After a brief centrifugation, place the tube on a magnetic separation device and wait for the beads to be collected.
6. Transfer the supernatant into a new 1.5-mL tube. Add 5 µL of 10x PCR Buffer and 50 µL of AMPure XP beads to the supernatant. Mix well and spin briefly.
7. Let the tube stand at room temperature for 10 min.
8. Place the tube on a magnetic separation device and wait for the beads to be collected. Then, remove the supernatant carefully so as not to aspirate the beads.
9. Add 200 µL of 75% ethanol to wash the beads and remove the supernatant.
10. Add 21 µL of 10 mM Tris-acetate (pH 8.0) and vortex the tube well to disperse the beads. Following a brief centrifugation, place the tube on a magnetic separation device and wait for the beads to be collected.
11. Transfer the supernatant to a new 1.5-mL tube.

7. qPCR quantitation of template DNA

Determine the exact molar concentration of template DNA using an appropriate qPCR assay. For a reliable quantitation using qPCR, prepare six serial dilutions of the DNA standard (in duplicate), a no-template control (NTC) reaction in duplicate and 1/1000 fold dilution of the DNA libraries.

NOTE *The product contains not only intact sequencing templates but also several-fold greater amounts of byproducts. It is thus essential to determine the correct concentration of the template DNA by qPCR and not by fluorometry. Use Library Quantification Kits for Illumina (KAPA biosystems) according to the manufacturer's instruction for quantification. In addition, the byproducts also make it impossible to directly examine the size of template DNA by electrophoresis.*

8. PCR amplify the captured libraries

Step 1. Determine the number of PCR cycles

1. Based on the template DNA concentration as determined by qPCR, determine the required number of PCR cycles by referring to Table 16.

$$\text{The required multiplying factor} = \frac{\text{(desired concentration x desired volume x rate for template DNA x number of lanes)}}{\text{(template DNA concentration x template DNA volume)}}$$

Where:

- Desired concentration: the desired final concentration for clustering (pM)
- Desired volume: the desired final volume for clustering (µL), see Table 16
- Rate for template DNA: Net template DNA amount minus phiX DNA amount, see Table 17
- Number of lanes: the number of sequencing lanes
- Template DNA concentration: calculated by qPCR quantitation (pM)
- Template DNA volume: the remaining template DNA volume after qPCR (µL)

Table 16 Final volume and % template DNA in each sequencer mode

Sequencer - mode	Final volume	Rate for template DNA
HiSeq – High Throughput Mode	120 µL	0.9 – 1.0
HiSeq – Rapid Mode	135 µL	0.5
MiSeq	600 µL	0.7

2. If the required multiplying factor is below 1.0, there is no need to amplify the template DNA. If, on the other hand, the required multiplying factor is higher than or equal to 1.0, it is necessary to determine the number of PCR cycles using the following formula.

   The required number of PCR cycles = Log2 (the required multiplying factor) + additional cycles*

   **If there is template DNA loss during the purification step after PCR amplification or if the sequencing fails, additional PCR cycles (up to 2) may be necessary.*

3. Prepare the appropriate volume of the PCR reaction mixture for each template DNA as shown in Table 17. Vortex the mixture well and keep on ice.

Table 17 Preparation of PCR reaction mix for each template DNA

Reagent	Volume for 1 Reaction
2x KAPA HiFi HotStart ready Mix	25 µL
Remaining template DNA volume after qPCR	x µL
100 µM PE-forward primer	0.2 µL
100 µM PE-reverse primer	0.2 µL
Nuclease-free water	(24.6 – x) µL

4. Place the plate in a thermal cycler and run the PCR amplification program as shown in Table 18.

Step 2. Purify the amplified libraries using AMPure XP beads

1. Add 50 µL of AMPure XP beads to the amplified DNA library (50 µL), mix well and spin briefly.
2. Let the tube stand at room temperature for 10 min.
3. Place the tube on a magnetic separation device and wait for the beads to be separated. Then, remove the supernatant carefully so as not to aspirate the beads.
4. Add 200 µL of 75% ethanol to wash the beads and then remove the supernatant.
5. Add 45 µL of 10 mM Tris-acetate (pH 8.0) and vortex the tube well to disperse the beads. After a brief centrifugation, place the tube on the magnetic separation device and wait for the beads to be collected.
6. Transfer the supernatant into a new PCR tube. Add 5 µL of 10x PCR Buffer and 50 µL of AMPure XP beads to the supernatant. Mix well and spin briefly.
7. Let the tube stand at room temperature for 10 min.
8. Place the tube on the magnetic separation device and wait for the beads to be collected. Then, remove the supernatant carefully so as not to aspirate the beads.
9. Add 200 µL of 75% ethanol to wash the beads and remove the supernatant.
10. Add 20 µL of 10 mM Tris-acetate (pH 8.0), and vortex the tube well to disperse the beads. Following a brief centrifugation, place the tube on a magnetic separation device and wait for the beads to be collected.
11. Transfer the supernatant to a new 1.5 mL tube.

Table 18 Thermal cycler program for PCR amplification

Segment	Number of Cycles	Temperature	Time
1	1	98 °C	1 min
2	Number of cycles as determined during step 1-2	98 °C	1 min
		55 °C	1 min
		72 °C	1 min
3	1	4 °C	Hold

9. qPCR quantitation of final template DNA

Determine the exact molar concentration of template DNA using an appropriate qPCR assay. For a reliable quantitation using qPCR, prepare six serial dilutions of the DNA standard (in duplicate), a no-template control (NTC) reaction in duplicate and 1/1000 fold dilution of the DNA libraries.

10. Prepare sequencing

NOTE *For paired-end and index sequencing, be sure to add PBAT-PE-Seq and PBAT-PE-Idx to the Illumina primer mixes for read 2 and index, respectively, at a final concentration of 0.5 µM.*

NOTE *Bisulfite-converted DNA has an extremely biased base composition. To enable correct color normalization of fluorescent signals, each flow cell must include a control lane for the phiX control template. Alternatively, the phiX control can be added to the sample at the concentration specified for each sequencer mode.*

NOTE *Please refer to the latest Illumina protocol for sequencing details.*

Calculation of template volume required for sequencing

Calculate the volume of template required in cluster generation with the following equation and parameters as shown in Table 19.

For a single lane of Illumina HiSeq 2000 or HiSeq 2500 in high output mode using cBot

1. Dispense 100 µL, plus extra volume for pipetting loss, of Hybridization Buffer A to a new 1.5-mL tube and place it on ice.
2. Prepare 2 M NaOH by diluting a 10 M NaOH solution.
3. Denature sequencing templates as shown in Table 20.
4. Let the tube stand at room temperature for 5 min.
5. Add 100 µL of Hybridization Buffer A to the denatured template, mix well and place the tube on ice.
6. Start cluster generation according to the manufacturer's instruction.

Table 19 Calculation of template volume

Platform	HiSeq 2000 or HiSeq 2500 *high output mode*	Hi Seq 2500 *rapid run mode with cBot*	HiSeq 2500 *rapid run mode without cBot* or MiSeq
Target concentration of denatured template (pM)		10*	
Target volume of template (µL)	120	70	480
Molar concentration of template (pM) determined in Section 9		y	
Volume of template required = x (µL)	$\frac{120 \times 10}{y}$	$\frac{70 \times 10}{y}$	$\frac{480 \times 10}{y}$

** For the target concentration, 10 pM is a good point to start optimization*

Table 20 Template denaturing mixture for HiSeq high output mode

Reagent	Volume
Template DNA solution	x µL
Nuclease-free water	(19 – x) µL
2 M NaOH	1 µL

For a single lane of Illumina HiSeq 2500 in rapid run mode using cBot

1. Dispense 58 µL, plus extra volume for pipetting loss, of Hybridization Buffer A to a new 1.5 mL tube and place it on ice.
2. Prepare 2 M NaOH by diluting 10 M NaOH solution.
3. Denature sequencing templates as shown in Table 21.
4. Let the tube stand at room temperature for 5 min.
5. Add 58 µL of the ice-cold Hybridization Buffer A to the denatured template, mix well and place the tube on ice.
6. Add 70 µL of ice-cold 8 pM denatured phiX control to the tube and mix well.
7. Start cluster generation according to the manufacturer's instruction.

For Illumina HiSeq 2500 in rapid run mode without cBot or Illumina MiSeq

1. Dispense 400 µL, plus extra volume for pipetting loss, of Hybridization Buffer A to a new 1.5-mL tube and put it on ice.
2. Prepare 2 M NaOH by diluting 10 M NaOH solution.
3. Denature sequencing templates as shown in Table 22.
4. Let the tube stand at room temperature for 5 min.
5. Add 400 µL of ice-cold Hybridization Buffer A, mix well and place the tube on ice.
6. Add 120 µL of ice-cold 8 pM denatured phiX control to the tube and mix well.
7. Start run according to the manufacturer's instruction.

Table 21 Template denaturing mixture for HiSeq rapid run mode using cBot

Reagent	Volume
Template DNA solution	x µL
Nuclease-free water	(11 – x) µL
2 M NaOH	0.6 µL

Table 22 Template denaturing mixture for HiSeq rapid run mode without cBot or MiSeq

Reagent	Volume
Template DNA solution	x µL
Nuclease-free water	(76 – x) µL
2 M NaOH	4 µL

11. Data Analysis

PBAT data analysis requires alignments that are the exact opposite of 'directional' mode of Bismark. Refer to the following web-site for further information.

http://www.bioinformatics.babraham.ac.uk/projects/bismark/Bismark_User_Guide_v0.7.12.pdf

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13. Appendix

Table 23. Oligo sequences (OPC grade)

Oligo Number	Oligo Name	Sequence
1	Bio-PEA2-W4N4 (100 µM)	5′-biotin-ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT WWW WNN NN-3′
2	PE-reverse-N4 (100 µM)— *For single-end sequencing*	5′-CAA GCA GAA GAC GGC ATA CGA GAT NNN N-3′
3	PBAT-PE-iX-W4N4 (100 µM each)— *For pair-end and index sequencing*	5′-CAA GCA GAA GAC GGC ATA CGA GAT XXX XXX GTA AAA CGA CGG CCA GCA GGA AAC AGC TAT GAC WWW WNN NN-3′
4	Primer 3 (100 µM)	5′-AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC T-3′
5	PBAT-PE-Seq (100 µM)	5′-GTA AAA CGA CGG CCA GCA GGA AAC AGC TAT GAC-3′
6	PBAT-PE-Idx (100 µM)	5′-GTC ATA GCT GTT TCC TGC TGG CCG TCG TTT TAC-3′
7	PE-forward primer (100 µM)	5′-AAT GAT ACG GCG ACC ACC GAG ATC TAC AC-3′
8	PE-reverse primer (100 µM)	5′-CAA GCA GAA GAC GGC ATA CGA GAT-3′

NOTE *Replace the underlined hexamer (oligo 3, Table 23) with each of the following index sequences (Table 24), which are complementary to those used in the TruSeq DNA LT Sample Prep Kit. The index numbers 17, 24 and 26 are not used.*

Table 24. Index sequences for underlined hexamer in oligo 3

Index #	Sequence	Index #	Sequence	Index #	Sequence
1	CGTGAT	9	CTGATC	18	GCGGAC
2	ACATCG	10	AAGCTA	19	TTTCAC
3	GCCTAA	11	GTAGCC	20	GGCCAC
4	TGGTCA	12	TACAAG	21	CGAAAC
5	CACTGT	13	TTGACT	22	CGTACG
6	ATTGGC	14	GGAACT	23	CCACTC
7	GATCTG	15	TGACAT	25	ATCAGT
8	TCAAGT	16	GGACGG	27	AGGAAT

www.agilent.com/genomics/methyl-seq

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