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Post-bisulfite adaptor tagging for PCR-free whole-genome bisulfite sequencing

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Summary

Post-bisulfite adaptor tagging (PBAT) is a highly efficient procedure to construct libraries for whole-genome bisulfite sequencing (WGBS). PBAT attaches adaptors to bisulfite-converted genomic DNA to circumvent bisulfite-induced degradation of library DNA inherent to conventional WGBS protocols. Consequently, it enables PCR-free WGBS from nanogram quantities of mammalian DNA, thereby serving as an invaluable tool for methylomics.

Key words: Methylome, Next generation sequencing, PCR-free, Single-nucleotide resolution

Running Head: PBAT: post-bisulfite adaptor tagging

1. Introduction

The current gold standard for single-base resolution methylome analysis is whole-genome bisulfite sequencing (WGBS). WGBS was first applied to the *Arabidopsis* methylome [1,2] and then applied to a variety of organisms. While its power is remarkable, it has a practical drawback in terms of the amount of input DNA: it typically requires a few micrograms of DNA (*i.e.*, approximately a million mammalian diploid cells). This number of cells is difficult or sometimes even prohibitive for various biologically interesting samples, such as mammalian oocytes, early embryonic tissues, and tissue stem cells. Extensive PCR amplification to compensate limitation of input DNA not only exacerbates biased genomic representation, but makes the estimate of methylation level inaccurate. This is due to the bisulfite conversion that induces sequences differences in the methylated and unmethylated alleles of the same locus leading to differential amplification.

To expand the range of samples suitable for WGBS, a novel protocol that requires a much smaller amount of input DNA than the conventional ones is required. Although bisulfite treatment induces DNA fragmentation, the conventional as well as tagmentation-based protocols [3] ***(****see also* **Chapter 5*****)*** include the step for bisulfite-treatment of adaptor-tagged DNAs that results in the degradation and low yield of DNA for the generation of the library (**Fig. 1A**). To circumvent this adverse effect, we conceived a novel principle termed Post-Bisulfite Adaptor Tagging (PBAT), in which adaptor tagging follows bisulfite

treatment, in contrast to the other protocols [4] (**Fig. 1B**). Since it is difficult to efficiently ligate adaptors to bisulfite-treated, denatured DNAs, we developed a simple adaptor-tagging protocol using two rounds of random primer extension (**Fig. 1C**).

The random priming-based PBAT protocol can generate a PCR-free library from as little as 125 pg of DNA. It typically allows generating a WGBS library of sufficient quality and diversity to achieve ~30-fold, PCR-free coverage of the mammalian genomes from ~30 ng of DNA. We and others have successfully applied PBAT to various samples ranging from plants, fungi and animals, especially those with limited amounts of DNA (*see also* **Chapter 8**). For instance, PBAT has realized PCR-free, mouse WGBS from only 1,000 oocytes and a few thousand flow-sorted primordial germ cells [5,6]. We have also applied PBAT to target-enriched genomic DNA, thereby achieving a highly efficient targeted methylome sequencing [7]. In contrast to conventional PCR-assisted WGBS, PBAT can preferentially cover GC-rich genic regions and CpG islands. We expect that the PBAT protocol described below will help readers conduct various novel WGBS applications.

2. Materials

2.1. Reagents

1. Qubit dsDNA BR Assay Kit.
2. Qubit dsDNA HS Assay Kit.

3. Qubit ssDNA Assay Kit.

4. Agencourt AMPure XP beads.

5. 10x PCR buffer: 100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$. pH 8.3.

6. RNA 6000 Pico Kit (Agilent).

7. High Sensitivity DNA Kit (Agilent).

8. 10 mM Tris-Hacetate, pH 8.0.

9. 10 mM Tris-HCl, pH 7.5.

10. Klenow fragment (3′→5′ exo^-): high concentration (50,000 units/ml) (New England Biolabs, NEB, *see* **Note 1**).

11. Bst DNA polymerase large fragment (80,000 units/ml) (NEB).

12. Exonuclease I (20,000 units/ml).

13. Phusion Hot Start II High-Fidelity DNA Polymerase (2 units/μl).

14. EZ DNA Methylation-Gold Kit (ZYMO Research).

15. Dynabeads M-280 Streptavidin.

16. 2x BW solution: dissolve 6.3 g LiCl in 40 ml of double-distilled water (ddH_2O), after LiCl has completely dissolved, add 0.5 ml of 1 M Tris-HCl, pH 8.0 and 0.1 ml of 0.5 M EDTA, and adjust the volume to 50 ml with ddH_2O (*see* **Note 2**).

17. 0.1 M NaOH (*see* **Note 3**).

18. KAPA Library Quantification Kit for Illumina (KAPA Biosystems).

19. Hybridization Buffer A: combine 9 ml of 5 M NaCl and 9 ml of 1 M Tris-HCl, pH 7.4, and bring to a final volume of 50 ml with ddH_2O.

2.2 Oligonucleotides

1. Bio-PEA2-N4: 100 μM

   5′-biotin-ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT NNN N-3′.

2. PE-reverse-N4: 100 μM

   5′-CAA GCA GAA GAC GGC ATA CGA GAT NNN N-3′.

3. PBAT-PE-iX-N4: 100 μM each

   5′-CAA GCA GAA GAC GGC ATA CGA GAT XXX XXX GTA AAA CGA CGG CCA GCA GGA AAC AGC TAT GAC NNN N-3′.

   Replace the underlined hexamer with one of the following index sequences, which are complementary to those used in the Illumina's TruSeq DNA LT Sample Prep Kit, so that the same index numbers as those in the kit can be used. The index numbers 17, 24 and 26 are reserved by Illumina for unknown reasons.

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

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:

   5′-GTA AAA CGA CGG CCA GCA GGA AAC AGC TAT GAC-3′.

6. PBAT-PE-Idx:

   5′-GTC ATA GCT GTT TCC TGC TGG CCG TCG TTT TAC-3′.

2.3. Plastic disposables

1. Microcentrifuge and PCR tubes: In all the steps, use of low-retention 1.5 and 0.2 ml tubes is recommended.
2. Pippette tips: use of low-retention tips is recommended for dispensing streptavidin-coated magnetic beads.

2.4. Equipment

1. DynaMag-2 Magnet (LifeTechnologies, or equivalent).
2. SPRIPlate 96R Magnet Plate (Beckman Coulter, or equivalent).
3. High-speed refrigerated microcentrifuge.
4. Agilent Bioanalyzer 2100.
5. Qubit Fluorometer or Qubit 2.0 Fluorometer.
6. TOMY PMC-060 Capsulefuge (or equivalent).
7. Thermal cycler.
8. StepOnePlus Real-Time PCR System (Applied Biosystems or equivalent).

3. Methods

3.1. Bisulfite Treatment (Day 1)

1. Measure the concentration of the DNA sample with the Qubit dsDNA BR Assay Kit and Qubit Fluorometer according to the manufacturer's instructions (*see* **Note 4**).
2. Add 900 µl of ddH_2O, 50 µl of M-Dissolving Buffer, and 300 µl of M-Dilution Buffer to one tube of CT Conversion Reagent from the EZ DNA Methylation-Gold Kit. Dissolve the material by rotating the tube of CT Conversion Reagent for 10 min. at room temperature.

3. Mix the following components well: 130 μl of CT conversion reagent, (20 − x) μl ddH_2O, and x μl of sample DNA (*see* **Notes 5, 6**).

4. Divide the solution into three 50 μl aliquots in 0.2 ml tubes.

5. Place the tubes on a thermal cycler, and start the following program: 98°C for 10 min., 64°C for 150 min., followed by hold at at 4°C.

6. Place a Zymo-Spin IC Column in a Collection Tube and add 600 μl of M-Binding Buffer to the column.

7. Add the sample from Step 5 to the M-Binding Buffer in the column. Close the cap and mix by inverting several times.

8. Centrifuge at full speed (≥10,000 x *g*) for 30 s.

9. Reload the flow-through onto the same column again (*see* **Note 7**).

10. Centrifuge at full speed (≥10,000 x *g*) for 30 s. Discard the flow-through.

11. Add 100 μl of M-Wash Buffer prepared with ethanol to the column, and centrifuge at full speed for 30 s. Discard the flow-through.

12. Add 200 μl of M-Desulphonation Buffer to the column and let the column stand at room temperature for 15 min.

13. Centrifuge at full speed for 30 s. Discard the flow-through.

14. Add 200 μl of M-Wash Buffer with ethanol to the column and centrifuge at full speed for 30 s. Discard the flow-through.

15. Repeat the wash in Step 14 one more time and then transfer the spin column to a new clean 1.5 ml tube.

16. Add 22 µl of M-Elution Buffer directly to the column matrix and let the column stand at room temperature for 2 min. Centrifuge at full speed for 30 s to elute the DNA (*see* **Notes 8, 9**).

3.2. First-strand synthesis (Day 1)

1. Prepare the first-strand synthesis reaction mix as follows: Add 5 µl of 10x NEB Buffer 2, 5 µl of 2.5 mM dNTPs, 16 µl of ddH_2O, 4 µl of 100 µM primer Bio-PEA2-N4 and 20 µl of bisulfite-treated sample DNA from **Section 3.1**.
2. Place the tube on a thermal cycler and start the following program: 94°C for 5 min., 4°C for 20 min., gradual increase from 4°C to 37°C at a rate of +1°C /min., 37°C for 90 min., 70°C for 10 min., followed by hold at at 4°C (*see* **Note 10**).
3. After 5 min. of incubation at 4°C (i.e., the second step of the program), 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 mix and mix well.
4. Place the tube on the thermal cycler again and resume the program to complete the first-strand synthesis reaction (*see* **Note 11**).

3.3. Removal of Excess Primers (Day 2)

1. Transfer the solution of the first-strand reaction (~50 μl) into a new 1.5 ml tube, add 50 μl of AMPure XP beads, mix well, and spin the tube briefly (*see* **Note 12**).
2. Let the tube stand at room temperature for 10 min.
3. Place the tube on a magnetic stand and wait for the beads to be collected. Then, remove the supernatant carefully and make sure 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-HAcetate buffer and vortex the tube well to suspend the beads. After a brief centrifugation, place the tube on the magnetic stand 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 (*see* **Note 13**).
7. Let the tube stand at room temperature for 10 min.
8. Place the tube on the magnetic stand and wait for the beads to be collected. Then remove the supernatant carefully 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-HAcetate buffer and vortex the tube well to suspend the beads. After a brief centrifugation, place the tube on the magnetic stand and wait for the beads to be collected.

11. Transfer the supernatant in a new 1.5 ml tube.

12. Use 1 µl of the supernatant to measure the DNA concentration using the Qubit dsDNA HS kit (*see* **Note 14**).

3.4. Capturing Biotinylated DNA on Streptavidin Beads (Day 2)

1. Take 20 µl of well-dispersed suspension of Dynabeads M280 Streptavidin beads into a 1.5 ml tube. Place the tube on a magnet stand to collect the beads.

2. Remove the supernatant, and add 50 µl of 2x BW buffer to suspend the beads.

3. Add the suspension of beads to the product obtained in **Section 3.3**.

4. Incubate the tube at room temperature for 30 min. while gently rotating the tube.

5. Place the tube on the magnet stand to collect the beads and then remove the supernatant.

6. Add 180 µl of 2x BW buffer to the beads, vortex well, and spin the tube briefly.
7. Place the tube on the magnet stand to collect the beads and then remove the supernatant.
8. Suspend the beads in 180 µl of 0.1 M NaOH, vortex well, incubate at room temperature for 2 min., and spin briefly.
9. Place the tube on the magnet stand to collect the beads and then remove the supernatant.
10. Repeat steps 8 and 9.
11. Add 180 µl of 2x BW buffer to the beads, vortex well, and spin the tube briefly.
12. Place the tube on the magnet stand to collect the beads and then remove the supernatant.
13. Add 180 µl of 10 mM Tris-HCl to the beads, vortex well, and spin the tube briefly.

3.5. Second-strand Synthesis *(Day 2)*

1. Place the tube on a magnet stand to collect the beads and remove the supernatant.

2. Prepare the second-strand synthesis reaction mix as follows and add to the beads: 5 µl of 10x NEB Buffer 2, 5 µl of 2.5 mM dNTPs, 36 µl of ddH_2O, 4 µl of 100 µM PE-reverse-N4, for single-end sequencing, or PBAT-PE-iX-N4 for paired-end and index sequencing.

3. Suspend the beads by vortexing, and transfer the beads suspension into a new 0.2 ml tube.

4. Place the tube on a thermal cycler and start the following program: 94°C for 5 min., 4°C for 20 min., gradual increase from 4°C to 37°C at a rate of +1°C/min., 37°C for 30 min., 70°C for 10 min., followed by hold at at 4°C (*see* **Note 10**).

5. After 5 min. of the incubation at 4°C (i.e., the second step of the program), 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.

6. Place the tube on the thermal cycler again and resume the program to complete the second-strand synthesis reaction.

3.6. Chase reaction *(Day 2)*

1. Place the tube on a magnet stand to collect the beads and remove the supernatant.

2. Prepare the chase reaction mix as follows and add to the beads: 5 µl of 10x ThermoPol Buffer (provided with the Bst polymerase large fragment), 5 µl of 2.5 mM dNTPs, 40 µl of ddH_2O and 1 µl of Bst DNA polymerase large fragment.

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

3.7. Elution/extension of Template DNA (Day 2) (*see* **Note 15**)

1. Place the tube on a magnet stand to collect the beads and remove the supernatant.

2. Prepare the elution/extension reaction mix as follows and add to the beads: 10 µl 5x Phusion HS buffer, 5 µl of 2.5 mM dNTPs, 35 µl of ddH_2O, 0.4 µl of 100 µM Primer 3 and 1 µl of Phusion Hot Start High-fidelity DNA polymerase

3. Start the following program: 94°C for 5 min., 55°C for 15 min., 68°C for 30 min., followed by hold at at 4°C.

4. Place the tube on the magnet stand 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 30 min. followed by heat inactivation at 70°C for 10 min.

6. Use 1 μl of eluted DNA with the Qubit dsDNA HS Kit to measure the concentration of DNA.

3.8. Size fractionation *(Day 2)*

1. Add 50 μl of AMPure XP beads to the eluted DNA (50 μl), mix well, and spin briefly.
2. Place the tube on a magnetic stand and wait for the beads to be separated. Then, remove the supernatant carefully not to aspirate the beads.
3. Add 200 μl of 75% ethanol to wash the beads and then remove the supernatant.
4. Add 45 μl of 10 mM Tris-HAcetate buffer and vortex the tube well to suspend the beads. After a brief centrifugation, place the tube on the magnetic stand and wait for the beads to be collected.
5. Transfer the supernatant to a new 1.5 ml tube.
6. 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 stand and wait for the beads to be collected. Then, remove the supernatant carefully not to aspirate the beads.

9. Add 200 µl of 75% ethanol to wash the beads, and then remove the supernatant.

10. Add 22 µl of 10 mM Tris-HAcetate buffer and vortex well to disperse the beads. After a brief centrifugation, place the tube on the magnetic stand and wait for the beads to be collected.

11. Transfer the supernatant to a new 1.5 ml tube.

12. Use 1 µl of the supernatant to measure the concentration of DNA using the Qubit dsDNA HS kit (*see* **Note 16**).

13. Subsequently, determine the exact molar concentration of template DNA using an appropriate qPCR assay (see **Note 17**).

3.9. Illumina sequencing

Here, we provide guidance about sequencing PBAT libraries on Illumina HiSeq2000, HiSeq2500 and MiSeq instruments.

3.9.1. Calculation of template volume required for sequencing

1. Calculate the volume of template required in cluster generation using the following equation and parameters (*see* **Note 18**).

Platform	HiSeq 2000	HiSeq 2500 in rapid mode with cBot	HiSeq 2500 in rapid mode without cBot or MiSeq
Target concentration of denatured template (pM)	10*	10*	10*
Target volume of template (µl)	120	70	480
Molar concentration of template (pM)	Y	Y	Y
Volume of template required = x (µl)	$\frac{120 \times 10}{y}$	$\frac{70 \times 10}{y}$	$\frac{480 \times 10}{y}$

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

3.9.2 Running a Single Lane of Illumina HiSeq2000

1. Dispense 100 µl plus extra volume of hybridization buffer A to a new 1.5 ml tube and place it on ice.
2. Prepare a 2 M NaOH by diluting a 10 M NaOH solution.
3. Denature sequencing templates by combining x µl of template DNA solution, (19 − x) µl of ddH_2O and 1 µl of 2 M NaOH.

4. Let the tube stand at room temperature for 5 min.

5. Add 100 μl of the ice-cooled hybridization buffer A to the denatured template, mix well, and place the tube on ice.

6. Start cluster generation according to the manufacturer's instructions (*see* **Note 19**).

3.9.2 Running a Single Lane of Illumina HiSeq2500 in Rapid Mode with cBot

1. Dispense 58 μl plus extra volume of hybridization buffer A to a new 1.5 ml tube and place it on ice.

2. Prepare a 2 M NaOH by diluting a 10 m NaOH solution.

3. Denature sequencing templates by combining x μl of template DNA solution, (11 − x) μl of ddH_2O and 0.6 μl of 2 M NaOH.

4. Let the tube stand at room temperature for 5 min.

5. Add 58 μl of the ice-cooled 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 (*see* **Note 20**).

7. Start cluster generation according to the manufacturer's instruction.

3.9.3 Running a Single Lane of Illumina HiSeq2500 in Rapid Mode Without cBot or Illumina MiSeq

1. Dispense 400 μl plus extra volume of hybridization buffer A to a new 1.5 ml tube and put it on ice.
2. Prepare a 2 M NaOH by diluting a 10 M NaOH solution.
3. Denature sequencing templates by combining x μl of template DNA solution, (76 − x) μl of ddH_2O and 4 μl of 2 M NaOH.
4. Let the tube stand at room temperature for 5 min.
5. Add 400 μl of the 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 (*see* **Note 20**).
7. Start run according to the manufacturer's instruction (see **Note 21**).

4. Notes

1. Be sure to use high concentration enzymes (i.e., 50,000 U/ml).
2. Dissolving LiCl is an exothermic process. To avoid bumping of the solution, dissolve LiCl in 40 ml of ddH_2O once completely. Then, add Tris and EDTA, and adjust the volume to 50 ml with ddH_2O.

3. Dilute from 10 M NaOH stock before use.

4. Accurate estimation of DNA concentration is critical. We routinely use the Qubit dsDNA BR Assay Kit for the purpose. Avoid measuring at an optical density of 260 nm, because various materials other than DNA absorb light at 260 nm and often result in an overestimation of DNA concentration. The size of input DNA seems to be less critical, as it does not affect the yield of library (**Fig 2**).

5. We routinely start with 100 ng of DNA, because this amount is easy to handle. However, note that the maximum efficiency of template preparation is achieved with ~1 ng of DNA as a starting material. Thus, the bisulfite-treated DNA may be divided into several aliquots before first-strand synthesis to further increase the efficiency of template preparation. All reagents used in this step are provided in EZ DNA methylation kit.

6. Use freshly prepared CT conversion reagent to ensure high yield and efficient bisulfite conversion.

7. Because we occasionally encountered “shunts” in the column through which the solution flows to have minimal contact with the resin, the column should be carefully inspected before use. Reloading of the flow-through increases the contact of the solution with the resin.

8. The elution volume (22 μl) includes 1 μl for determination of yield by the Qubit ssDNA Assay kit and 1 μl for QC with the Agilent Bioanalyzer using the RNA 6000 Pico Kit. Typically, the yield of DNA is between 30% and 70% of the input. The typical size range of denatured DNA is 100~1,000 nt with a peak around 600 nt (Fig 3). When the starting amount of DNA is <30 ng, both platforms will fail to detect the eluted DNA. Thus, omit these QC steps and reduce the elution volume in Step 17 to 20 μl.

9. *Do not stop here.* Proceed immediately to the first-strand synthesis step, because the bisulfite-treated DNA is labile.

10. 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 as 4.0°C for 30 sec followed by 4.5°C for 30 sec, then the temperature will reach to 37°C after 33 cycles with the intended rate of +1°C/min.

11. You can stop here either by leaving the tube at 4°C or storing it at −20°C until use. This is presumably because the bisulfite-treated DNAs are now double-stranded and excessive primers in the solution serve as a carrier DNA to prevent the adsorption of template DNA to the tube wall.

12. At this mixing ratio (i.e., DNA solution:AMPure XP of 1:1), DNA fragments less than 200 bp are effectively removed in the supernatant. While primers and primer dimers are less than 100 nt, the products of the first-strand synthesis are larger than 200 bp.

13. Addition of 10x PCR Buffer at this step increases the reproducibility and yield of AMPure XP-based purification of DNA.

14. Typical yields at this step are between 40% and 60% of the input. When the starting amount of DNA is <30 ng, the kit will fail to quantify the DNA. Thus, omit this QC step and reduce the elution volume in Step 10 to 50 μl.

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

16. Typical yield at this step is between 20% and 40% of the input DNA. When the starting amount of DNA is <30 ng, the kit will fail to quantify DNA. Thus, omit this QC step and reduce the elution volume in Step 9 to 21 μl.

17. Note that the product obtained in **Section 3.8** contains not only intact sequencing templates but also several-fold greater amounts of by-products. Thus, it is essential to determine the correct concentration of the template DNA by qPCR but not by fluorometry. We perform quantification using Library Quantification Kits for Illumina (KAPA biosystems) according to the

manufacturer's instruction, because it is easy to use and highly reproducible. Typical mass yield at this step is calculated to be 2~8% relative to the starting amount of DNA. If the starting DNA is 100 ng, this number corresponds to ~20 fmol of template, which is sufficient for 20 lanes or 4 runs of sequencing on a Illumina HiSeq 2000 or MiSeq, respectively. In addition, the by-products make it impossible to examine the size of template DNA directly by electrophoresis. Accordingly, we analyze the size of qPCR product. Because the size distribution of PCR-amplified templates becomes unreliable after the PCR reaches the plateau, we run the amplified product ona 6% polyacrylamide/7M urea gel in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA). As shown in Fig 4, the typical size is between 200 bp and 500 bp with a peak around 300 bp.

18. Be sure to add PBAT-PE-Seq and PBAT-PE-Idx to the Illumina primer mix at the final concentration of 0.5 μM each for paired-end and index sequencing.

19. Since bisulfite-converted DNA has an extremely biased base composition, each flow cell must include a control lane for the phiX control template to enable correct normalization of fluorescent signals.

20. For the same reason as described in **Note 19**, the phiX control template must be added to the sample. Follow the instruction provided by Illumina for the preparation of denatured phiX control template.

21. We usually use 101 cycles for sequencing. The reads are adequately processed and used for mapping. While a number of bisulfite mappers have been made available, it often happens that unique characteristics of PBAT reads not only necessitates appropriate preprocessing of the data but hampers their efficient mapping. We recommend a popular bisulfite mapper, bismark, which has an option "pbat" (http://www.bioinformatics.babraham.ac.uk/projects/bismark/). We also provide BMap that uses an algorithm termed adaptive seeds for highly efficient mapping of PBAT reads (http://itolab.med.kyushu-u.ac.jp/BMap/).

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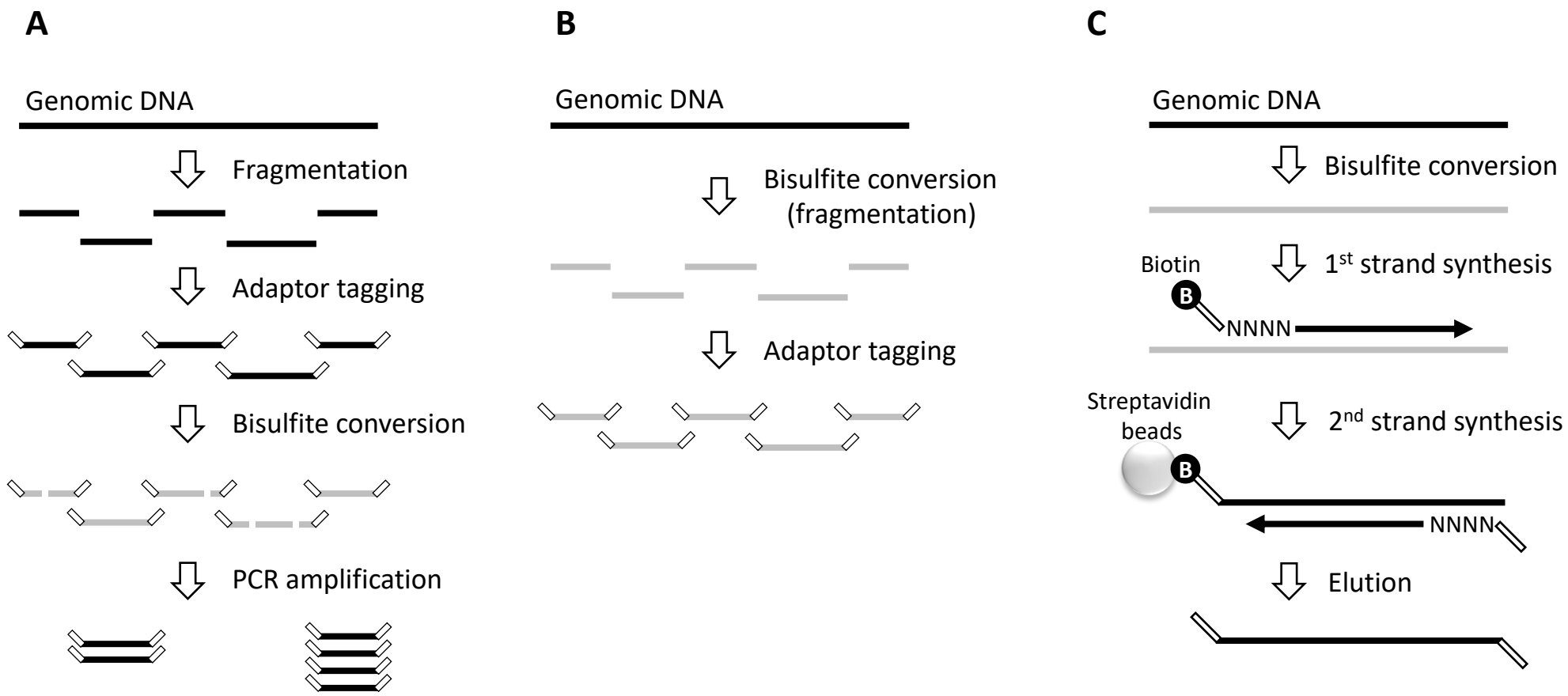
Figure Legends

Fig. 1. Principle of PBAT. (A) Conventional WGBS protocols. Bisulfite treatment follows adaptor-tagging and degrades adaptor-tagged library DNAs. (B) PBAT strategy. Adaptor-tagging follows bisulfite treatment to circumvent bisulfite-induced fragmentation of adaptor-tagged library DNAs. (C) Random priming-mediated PBAT. Two rounds of random primer extension on bisulfite-converted genomic DNA generate directionally adaptor-tagged library DNAs.

Fig. 2. Effect of input DNA size. (A) Agarose gel electrophoresis of DNAs variously fragmented using the Covaris S220 acoustic sonicator. (B) Yields of libraries generated from 100 ng of the variously fragmented DNA shown in (A).

Fig. 3. Typical size distribution of bisulfite-treated DNA. Bisulfite-treated genomic DNA was separated on an Agilent Bioanalyzer 2100 using the RNA 6000 pico kit.

Fig. 4. Typical size distribution of sequencing templates. Amplification products of qPCR to quantify libraries generated from the indicated amount of input DNA were separated on 6% Novex TBE-Urea gel and stained with SYBR Gold.


A
Genomic DNA
Fragmentation
Adaptor tagging
Bisulfite conversion
PCR amplification
B
Genomic DNA
Bisulfite conversion
(fragmentation)
Adaptor tagging
C
Genomic DNA
Bisulfite conversion
Biotin
1st strand synthesis
B
NNNN
Streptavidin
beads
2nd strand synthesis
B
NNNN
Elution

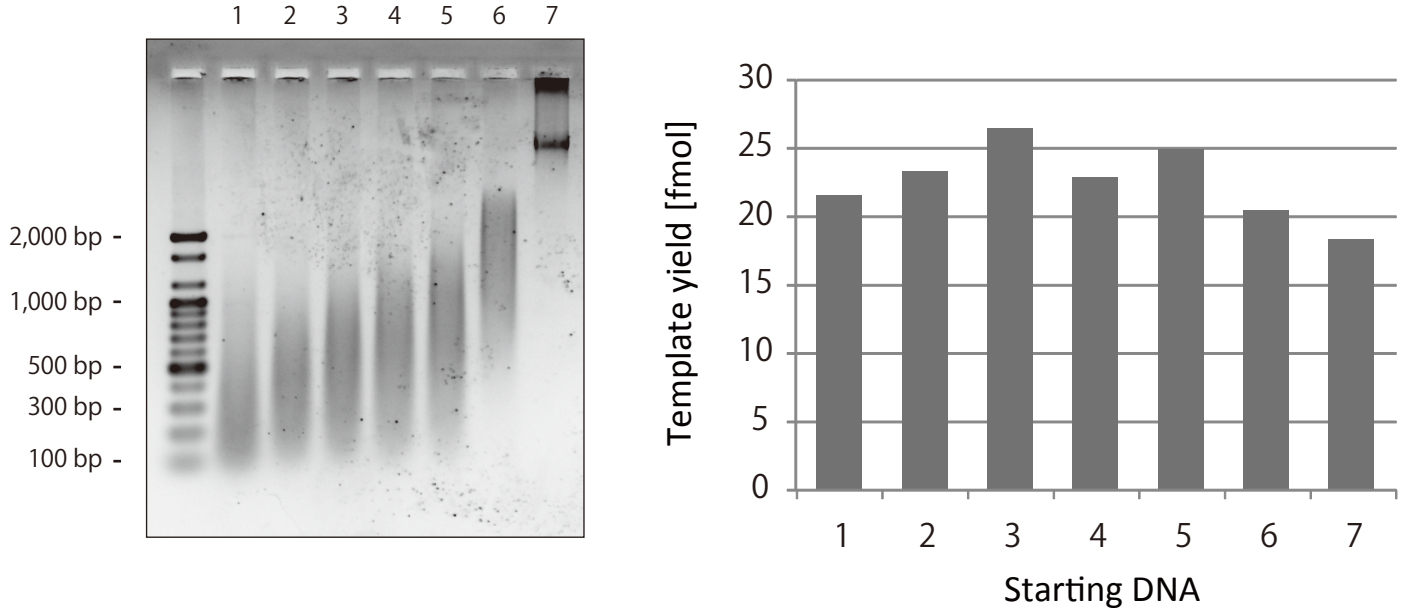

1 2 3 4 5 6 7
2,000 bp
1,000 bp
500 bp
300 bp
100 bp
Template yield [fmol]
30
25
20
15
10
5
0
1 2 3 4 5 6 7
Starting DNA

Figure 2

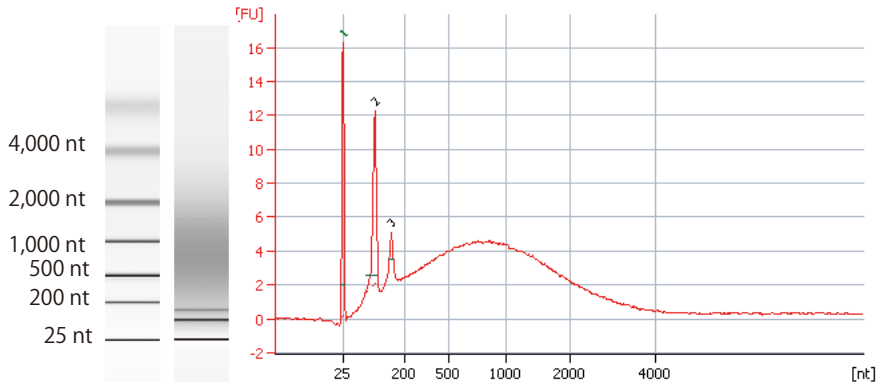

4,000 nt
2,000 nt
1,000 nt
500 nt
200 nt
25 nt
[FU]
16
14
12
10
8
6
4
2
0
-2
25
200
500
1000
2000
4000
[nt]

Fig 3

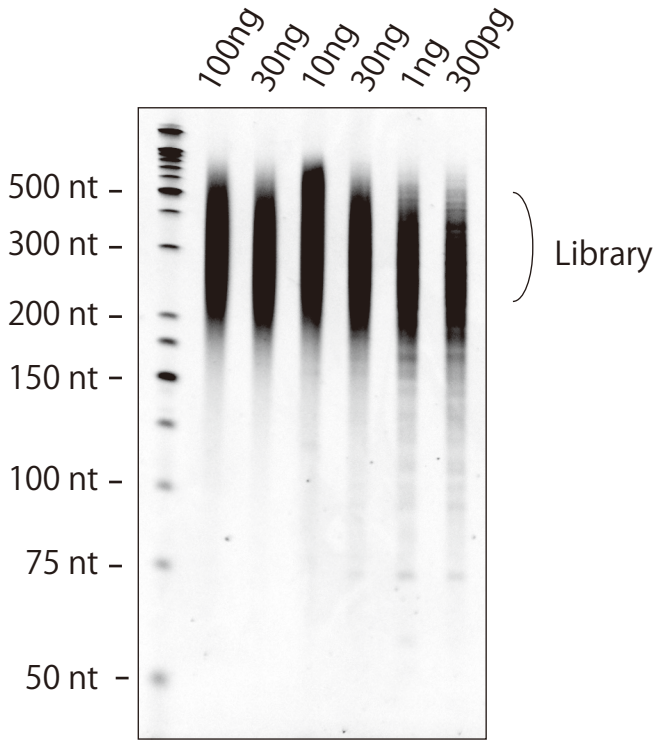

100ng
30ng
10ng
30ng
1ng
300pg
500 nt
300 nt
200 nt
150 nt
100 nt
75 nt
50 nt
Library

Fig 4