Reagents

- Qubit dsDNA BR Assay Kit (LifeTechnologies, # Q32850)
- Qubit dsDNA HS Assay Kit (LifeTechnologies, # Q32851)
- Qubit ssDNA Assay Kit (LifeTechnologies, # Q10212)
- Agencourt AMPure XP (Beckman Coulter, # A63880)
- Conventional 10× PCR Buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 15 mM $MgCl_2$)
- Agilent RNA 6000 Pico Kit (Agilent, #5067-1513)
- 10 mM Tris-acetate, pH 8.0
- 10 mM Tris-HCl, pH 7.5
- Klenow Fragment (3′→5′ exo$^-$) (NEB, #M0212M)

  Be sure to use the enzyme provided at 10-fold higher concentration (i.e., #M0212M; 50,000 U/mL) than the conventional ones (i.e., #M0212S and #M0212L; 5,000 U/mL)
- *Bst* DNA Polymerase Large Fragment (NEB, #M0275S)
- Exonuclease I (NEB, #M0293S)
- Phusion Hot Start High-Fidelity DNA Polymerase (Finnzyme, #F-540S)
- EZ DNA Methylation-Gold Kit (ZYMO Research, #D5005)
- Dynabeads M-280 Streptavidin (LifeTechnologies, #112-05D)
- 2× 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 (ddH_2O) at a time. After complete dissolution of LiCl, add Tris and EDTA, and adjust the volume to 50 mL with ddH_2O.*
- 0.1 N NaOH Solution

  Dilute 10 N NaOH stock solution before use.
- KAPA Library Quantification Kit for Illumina (KAPA, # KK4824 or qPCR platform specific ones)
- 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

Oligonucleotides (OPC grade)

- Bio-PEA2-N4 (100 µM)

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

- PE-reverse-N4 (100 µM)

  5′-CAA GCA GAA GAC GGC ATA CGA GAT NNN N-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 each of the following index sequences, which are complementary to those used in the adaptors used in 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

- 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′

- PBAT-PE-Seq (100 µM)

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

- PBAT-PE-Idx (100 µM)

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

Plastic disposables

- Tubes
  - Use low-retention 1.5- and 0.2-mL tubes in all the steps.
- Tips
  - Use low-retention tips for dispensing streptavidin-coated magnetic beads.

Equipment

- DynaMag-2 Magnet (LifeTechnologies) or its equivalent
- SPRIPlate 96R Magnet Plate (Beckman Coulter) or its equivalent
- TOMY MX-301 High-Speed Refrigerated Microcentrifuge or its equivalent
- Agilent Bioanalyzer 2100
- Qubit Fluorometer or Qubit 2.0 Fluorometer (Life Technologies)
- TOMY PMC-060 Capsulefuges or its equivalent
- ABI9700 thermal cycler or its equivalent
- StepOnePlus Real-Time PCR System (Applied Biosystems) or its equivalent
- XCell SureLock Mini-Cell (Life Technologies, # EI0001) or its equivalent
- Power supply for electrophoresis

Programs for thermal cycling

- Program 1 (for bisulfite treatment, see Section II)
    1. 98°C for 10 min
    2. 64°C for 150 min
    3. Soaking at 4°C
- Program 2 (for first-strand synthesis, see Section III)
    1. 94°C for 5 min
    2. 4°C for 20 min
    3. Gradual increment from 4°C to 37°C at a rate of +1°C/min*
    4. 37°C for 90 min
    5. 70°C for 10 min
    6. Soaking at 4°C
- Program 3 (for second-strand synthesis, see Section VI)
    1. 94°C for 5 min
    2. 4°C for 20 min
    3. Gradual increment from 4°C to 37°C at a rate of +1°C/min*
    4. 37°C for 30 min
    5. 70°C for 10 min
    6. Soaking at 4°C
- Program 4 (for elution, see Section VII)
    1. 94°C for 5 min
    2. 55°C for 15 min
    3. 68°C for 30 min
    4. Soaking at 4°C

**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.*

I. Estimation of DNA concentration (Day 1)

Accurate estimation of DNA concentration is critical. We routinely use Qubit dsDNA BR Assay Kit for the purpose. Do not measure OD260, because 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.

II. Bisulfite treatment (Day 1)

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.

1. 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.
   Use freshly prepared CT conversion reagent to ensure high yield and efficient bisulfite conversion.
2. Dissolve the material by rotating the tube of CT Conversion Reagent for 10 min at room temperature.
3. Mix the following components well.

CT Conversion Reagent	130 µL
ddH_2O	(20 − x) µL
Sample DNA	x µL

4. Divide the solution into three 50-µL aliquots in 0.2-mL tubes.
5. Place the tubes on a thermal cycler, and start Program 1.
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 × g) for 30 sec.
9. Reload the flow-through onto the same column again.

We occasionally encountered "shunts" in the column, through which the solution flows with minimal contact with the resin. Thus, we recommend to carefully inspect the column before use. Reloading of the flow-through would help the solution to have enough contact with the resin.

10. Centrifuge at full speed (≥10,000 × g) for 30 sec.
11. Discard the flow-through.
12. Add 100 µL of M-Wash Buffer prepared with ethanol to the column, and centrifuge at full speed for 30 sec. Discard the flow-through.
13. Add 200 µL of M-Desulphonation Buffer to the column, and let the column stand at room temperature for 15 min.
14. Centrifuge at full speed for 30 sec. Discard the flow-through.
15. Add 200 µL of M-Wash Buffer with ethanol to the column, and centrifuge at full speed for 30 sec. Discard the flow-through.
16. Repeat the wash in Step 15 once more, and transfer the spin column to a new 1.5-mL tube.
17. 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 sec to elute the DNA.

The elusion volume (22 µL) includes 1 µL for determination of yield by Qubit ssDNA Assay Kit and 1 µL for quality check (QC) with Agilent Bioanalyzer with RNA 6000 Pico Kit. Typically, the yield of DNA is between 30% and 70% of the input, and the size range of denatured DNA is 100~1,000 nt with a peak around 600 nt. 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 16 to 20 µL.

Never stop here. Proceed immediately to the first-strand synthesis step, because the bisulfite-treated DNA is labile.

III. First-strand synthesis (Day 1)

1. Prepare the first-strand synthesis reaction mix as follows.

10× NEBuffer 2	5 µL
2.5 mM dNTPs	5 µL
ddH_2O	16 µL
Bio-PEA2-N4 (100 µM)	4 µL
Bisulfite-treated sample DNA	20 µL

2. Place the tube on a thermal cycler and start Program 2.

3. After 5 min from starting step 2 of Program 2 (*i.e.*, incubation step at 4°C), 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.
4. Place the tube back on the thermal cycler and resume the Program 2 to complete the first-strand synthesis reaction.

You can stop here by either 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.

IV. 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, mix well, and spin the tube briefly.

   At this mixing ratio (i.e., DNA solution:AMPure XP = 1:1), DNA fragments shorter than 200 bp are effectively removed in the supernatant. While primers and primer dimers are shorter than 100 nt, the products of the first-strand synthesis are longer than 200 bp.
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 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 stand and wait for the beads to be collected.
6. Transfer the supernatant into a new 1.5-mL tube. Add 5 µL of 10× PCR Buffer and 50 µL of AMPure XP to the supernatant. Then mix well and spin briefly.

   Addition of 10× 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 stand 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 stand and wait for the beads to be collected.
11. Save the supernatant in a new 1.5-mL tube.
12. Use 1 µL of the supernatant to measure DNA concentration by the Qubit dsDNA HS Kit.

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

V. 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 the magnet stand 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 product obtained in Section IV.
4. Incubate the tube at room temperature for 30 min with 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 2× BW(Li) 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 N NaOH solution, 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 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 the magnet stand 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.

VI. Second-strand synthesis (Day 2)

1. Place the tube on the 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.

10× NEBuffer 2	5 µL
2.5 mM dNTPs	5 µL
ddH_2O	36 µL
PE-reverse-N4 (100 µM) for single-end sequencing, or PBAT-PE-iX-N4 (100 µM) for paired-end and index sequencing	4 µL

3. Suspend the beads by vortexing and transfer the beads suspension into a new 0.2-mL tube.
4. Place the tube on the thermal cycler and start Program 3.
5. After 5 min of starting step 2 of Program 3 (*i.e.* incubation step at 4°C), 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 back on the thermal cycler and resume the Program 3 to complete the second-strand synthesis reaction.

VII. 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 added to the beads.

10× ThermoPol Buffer	5 µL
2.5 mM dNTPs	5 µL
ddH_2O	40 µL
Bst DNA Polymerase Large Fragment	1 µL

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

VIII. Elution/extension of template DNA (Day 2)

1. Place the tube on the magnet stand to collect the beads and remove the supernatant.
2. Prepare the elution/extension reaction as follows and added to the beads.

5× Phusion HS Buffer	10.0 µL
2.5 mM dNTPs	5 µL
ddH_2O	35 µL
Primer 3 (100 µM)	0.4 µL
Phusion Hot Start High-Fidelity DNA Polymerase	1 µL

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. Start Program 4
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 and Qubit dsDNA HS Kit to measure the concentration of DNA.

IX. Size fractionation (Day 2)

1. Add 50 µL of AMPure XP to the eluted DNA (50 µL), mix well, and spin briefly.
2. Place the tube on the magnetic stand and wait for the beads to be separated. Then, remove the supernatant carefully so as 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-acetate (pH 8.0) and vortex the tube well to disperse 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 into a new 1.5-mL tube. Add 5 µL of 10× PCR Buffer and 50 µL of AMPure XP to the supernatant. Mix well and spin briefly.
6. Let the tube stand at room temperature for 10 min.
7. Place the tube on the magnetic stand and wait for the beads to be collected. Then, remove the supernatant carefully so as not to aspirate the beads.
8. Add 200 µL of 75% ethanol to wash the beads and remove the supernatant.

9. Add 22 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 stand and wait for the beads to be collected.
10. Transfer the supernatant to a new 1.5-mL tube.
11. Use 1 µL of the supernatant to measure the concentration of DNA using the Qubit dsDNA HS Kit.

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.

X. qPCR quantitation of template DNA (Day 3)

1. Determine the exact molar concentration of template DNA using an appropriate qPCR assay.

Note that the product obtained in Step IX 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 but not by fluorometry. We use Library Quantification Kits for Illumina (KAPA biosystems) according to the manufacturer's instruction for the quantification, because this kit 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, the amount of obtained template is ~20 fmol, which is sufficient for sequencing in 20 lanes of Illumina HiSeq 2000 or 4 runs of MiSeq.

In addition, the byproducts also make it impossible to directly examine the size of template DNA by electrophoresis. Accordingly, we examine the size of qPCR product. Note that, once PCR reaches to the plateau, the size of PCR-amplified templates cannot be accurately examined under non-denaturing conditions. Thus, we use the standard polyacrylamide/urea/TBE gel electrophoresis. The size is typically 200~500 nt with a peak around 300 nt.

XI. Sequencing (Day 3)

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 the final concentration of 0.5 μM.

Calculation of template volume required for sequencing

1. Calculate the volume of template required in cluster generation with the following equation and parameters.

Platform	HiSeq 2000 or HiSeq 2500 *high output mode*	HiSeq 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 X	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.*

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 N NaOH by diluting 10 N NaOH solution.
3. Denature sequencing templates as follows.

Template DNA solution	x μL
ddH_2O	(19 − x) μL

2N NaOH	1 µL

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.

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 color normalization of fluorescent signals. Alternatively, the phiX control can be added to the sample as described below.

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 N NaOH by diluting 10 N NaOH solution.
3. Denature sequencing templates as follows.

Template DNA solution	x µL
ddH_2O	(11 − x) µL
2N NaOH	0.6 µL

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.
   For the same reason as described above, the phiX control template must be added to the sample. Follow the instruction provided by Illumina for the preparation of denatured phiX control template.
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 N NaOH by diluting 10 N NaOH solution.
3. Denature sequencing templates as follows.

Template DNA solution	x µL
ddH_2O	(76 − x) µL
2N NaOH	4 µL

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.

   For the same reason as described above, the phiX control template must be added to the sample. Follow the instruction provided by Illumina for the preparation of denatured phiX control template.
7. Start run according to the manufacturer's instruction.

References

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