# Whole Genome Bisulfite Sequencing Protocol

This protocol combines NEBNext oligos, the KAPA Hyper Prep kit, and a bisulfite conversion kit to generate WGBS libraries. Please look at the manufacturer's instructions, product information, and referenced papers for more details.

**The following kits and reagents are required:**

i) QIAquick PCR Purification Kit (QIAGEN, cat# 28104)
ii) NEBNext® Multiplex Oligos for Illumina® (Methylated Adaptor, Index Primers Set 1) (NEB cat# E7535S)
iii) KAPA Hyper Prep Kit (ROCHE, cat# KK8500, KK8502, KK8504)
iv) Bisulfite conversion kit (ex. Zymo Gold)
   - ❖ There is no specific conversion kit recommended due to differing conversion rates seen between experiments. Optimization is recommended.

v) PfuTurbo Cx Hotstart DNA Polymerase (Aligent, cat# 600410)
vi) KAPA Library Quantification Kit (ROCHE, cat# KK4854)
vii) AMPure XP beads (Beckman, cat# A63881)
viii) Fragment Analyzer kit DNF-474-0500
ix) Qubit™ dsDNA HS (ThermoFisher, cat# Q32854)

## I. Preparation genomic DNA

Use a gDNA extraction protocol comparable for bisulfite conversion like Bradley's or a commercial kit like QIAGEN.

## II. Fragmentation

Optimum size of the fragmented gDNA is around 250bp (peak size). Use a Covaris S220/E220 sonicator to shear DNA into small fragments.

1) Prepare 5 µg of gDNA.
2) Mix the gDNA samples with 0.5% W/W (25 ng) lambda DNA and top up to a final volume of 130 µl in TE buffer or 10mM Tris-Cl (pH8.0).
3) Fragment the DNA using the following settings:

| Peak Incident Power | 175W |
|---|---|
| Duty Factor | 10% |
| Cycle per burst | 200 |
| Time | 3mins, 30 seconds on 30 seconds off |
| Water level | 12.5 |
| Temperature | 4°C – 6°C |
| Volume | 130 µl |

4) Verify the size of the fragmented DNA on a 1.2% gel or on a Fragment Analyzer using the DNF-474 kit.
5) Purify the DNA by using QIAquick PCR purification Kit and elute in 52µl of EB or 10mM Tris-Cl (pH8.0).

## III. End Repair and A-Tailing

The following uses the KAPA Hyperprep kit (Cat# KK8502).

6) Prepare the following reaction mix on ice:

| **End Repair & A-Tailing Mix** | |
|---|---|
| **Reagent** | **Volume (µL)** |
| Fragmented DNA Sample (1ug) | 50 |
| End Repair & A-Tailing Buffer | 7 |
| End Repair & A-Tailing Enzyme Mix | 3 |
| **Total Volume** | **60** |

7) Vortex gently and spin down briefly.
8) Incubate the reaction in a thermocycler for 30 minutes at 60 °C, lid at 85 °C, store at 4 °C.
9) Proceed to Adapter ligation.

## IV. Methylated Adapter Ligation

The following reaction volume works for 100 ng – 1 µg of DNA.
Methylated adapter MUST be used to avoid conversion of "C" in the adapter to "T" during bisulfite conversion in the following step. CT conversions occurring within the adapter will lead to a change in the adapter sequence resulting in a failure of the final library sequencing.

- ❖ Bring AMPure XP beads to room temperature before using.

10) Prepare the following reaction mix on ice:

| Ligation Mix | |
|---|---|
| **Reagent** | **Volume (µL)** |
| DNA Sample | 60 |
| Ligation buffer | 30 |
| Methylated Adapter from NEB | 2.5 |
| DNA Ligase | 10 |
| PCR-grade Water | 7.5 |
| **Total Volume** | **110** |

11) Incubate the reaction for 15 minutes at 20ºC.
12) Add 3 µl of USER enzyme to the ligation mixture and mix well.
13) Incubate the reaction for 15 minutes at 37 °C.
14) Purify the DNA with 1x AMPure XP beads (113 µl).

## V. Bisulfite Conversion of DNA

15) Follow the procedure of corresponding Bisulfite conversion kit (such as Zymo Gold).
16) Elute the converted DNA in 10 µl of elution buffer.
    - ❖ SAFE STOPPING POINT – Store DNA in -80ºC.

## VI. Amplification of BS converted library

17) Prepare the following reaction:

| PCR Mix | |
|---|---|
| **Reagent** | **Volume (µL)** |
| BS converted DNA | 10µl |
| 10x Pfu CX Buffer | 5µl |
| 10uM NEBNext Universal PCR Primer | 2.5µl |
| 10uM NEBNext Index PCR Primer | 2.5µl |
| 10mM dNTP | 1.5µl |
| Milli-Q / ddH2O | 27.5µl |
| Pfu Turbo CX Polymerase | 1µl |
| **Total Volume** | **50µl** |

18) Set up the following cycling condition on the thermal cycler:

| Thermocycler Conditions | | | |
|---|---|---|---|
| **Stage 1** | 95°C | 2 minutes | |
| | 98°C | 30 seconds | |
| **Stage 2** | 98°C | 15 seconds | Repeat 4-8 cycles |
| | 60°C | 30 seconds | |
| | 72°C | 4 minutes | |
| **Stage 3** | 72°C | 10 minutes | |
| | 4°C | Hold | |

- 4-8 cycles should use for the amplification. If the DNA concentration use for bisulfite conversion is low, more cycles should be use.

19) Double size select DNA with 0.7x AMPure XP beads (keeping the eluent), followed by 0.2x purification.
20) Elute in 20 µL EB
21) Quantify the DNA using both Qubit and KAPA Library Quantification Kit.
22) Run 5ng of DNA on the Fragment Analyzer using DNF-474 analysis to check the average size of library prepared.
23) If necessary, pool libraries together for sequencing.

- A considerable amount of ssDNA might be present in the final library due to the DNA damage during bisulfite conversion. Quantification of the library should be performed cautiously, taking into account the existence of ssDNA.