NGS Library Preparation for Whole Genome Bisulfite Sequencing (WGBS) on Illumina® Sequencing Platforms

This protocol describes an optimized procedure for whole genome bisulfite conversion using the QIAGEN® EpiTect® Fast DNA Bisulfite Kit (cat. no. 59824), followed by NGS library preparation using the QIAseq™ Ultralow Input Library Kit (cat. no. 180495).

The workflow is summarized in the following figure:

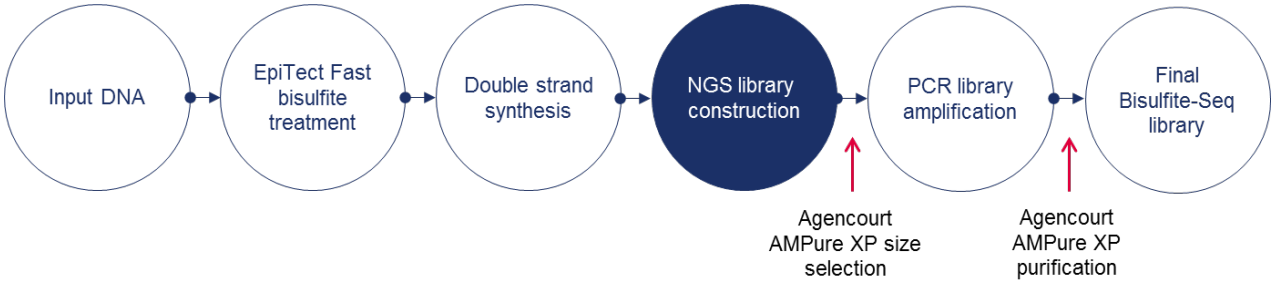

Figure 1. WGBS workflow chart.

IMPORTANT: Please read the handbooks supplied with each required kit for general information on the handling and storage of kit components. Pay careful attention to the required kit for general information on handling and beginning this procedure.

Equipment and reagents

When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

- EpiTect Fast DNA Bisulfite Kit (QIAGEN, cat.no. 59824)
- Klenow Fragment (3'–5' exo-) (5 U/µl) and Klenow buffer (QIAGEN Enzymatics, cat. no. P7010-LC-L)
- Random octamer primer (150 µM) (custom oligos)
- Illumina PCR Primer mix (10 µM) (PCR Primer sequences: 5'-AATGATACGGCGACCACCGA,

5'-CAAGCAGAAGACGGCATACGA) as taken from http://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/experiment-design/illumina-adapter-sequences_1000000002694-01.pdf

- dNTP Mix, PCR Grade (200 µl) (QIAGEN, cat. no. 201900)
- QIAseq Ultralow Input Library Kit (QIAGEN, cat. no. 180495)
- QIAGEN Multiplex PCR Kit (QIAGEN, cat. no. 206143)
- Agencourt® AMPure® XP beads*
- 80% fresh ethanol
- Nuclease-free water
- Buffer EB (QIAGEN, cat. no. 19086)
- Microcentrifuge tubes, PCR tubes or plates
- Sterile pipette tips and pipettes
- DNA LoBind tubes
- Vortexer
- Microcentrifuge
- Thermocycler
- Magnetic stand
- Capillary electrophoresis device (e.g., QIAGEN QIAxcel® Advanced, Agilent® Bioanalyzer, Agilent TapeStation or similar method to assess the quality of DNA library)

For NGS library QC:

- QIAseq Library Quant Array Kit (QIAGEN, cat. no. 33304)

* As an alternative to AMPure XP beads, the GeneRead™ Size Selection Kit (QIAGEN, cat. no. 180514) can also be used for cleanup.

Procedure: Bisulfite conversion of purified DNA

This procedure is for the whole bisulfite conversion of purified DNA prior to NGS library preparation. The following QIAGEN product is required for this procedure: EpiTect Fast DNA Bisulfite Kit (QIAGEN, cat.no. 59824).

Bisulfite conversion of DNA

1. Thaw DNA to be used in the bisulfite reactions. Make sure the Bisulfite Solution is completely dissolved.

Note: If necessary, heat the Bisulfite Solution to 60°C and vortex until all precipitates are dissolved again.

Note: Do not place dissolved Bisulfite Solution on ice.

2. Prepare the bisulfite reactions in 200 µl PCR tubes (not provided) according to Table 1. Add each component in the order listed.

Table 1. Bisulfite conversion of reaction setup

Component	Volume/reaction
DNA (150–1000 ng)	20 µl
Bisulfite Solution	85 µl
DNA Protect Buffer	35 µl
Total reaction volume	**140 µl**

3. Close the PCR tubes and mix the bisulfite reactions thoroughly. Store the tubes at room temperature (15–25°C).

   Note: DNA Protect Buffer should turn from green to blue after addition to the DNA-Bisulfite Solution mixture, indicating sufficient mixing and correct pH for the bisulfite conversion reaction.

4. Perform the bisulfite DNA conversion using a thermal cycler according to the cycling conditions in Table 2. Once complete, the bisulfite conversion should take approximately 40 min.

   Note: If using a thermal cycler that does not allow you to enter the reaction volume (140 µl), set the instrument to the largest volume setting available.

Table 2. Cycling conditions

Step	Incubation time	Temperature
Denaturation	5 min	95°C
Incubation	15 min	60°C
Denaturation	5 min	95°C
Incubation	15 min	60°C
Hold	∞	4°C

Cleanup of bisulfite-converted DNA

5. Once the bisulfite conversion is complete, briefly centrifuge the PCR tubes containing the bisulfite reactions, and then transfer the complete bisulfite reactions to clean 1.5 ml microcentrifuge tubes.

   Transfer of precipitates in the solution will not affect the performance or yield of the reaction.

6. Add 310 µl freshly prepared Buffer BL to each sample. Mix the solutions by vortexing and then centrifuge briefly.
7. Add 250 µl ethanol (96–100%) to each sample. Mix the solutions by pulse vortexing for 15 s, and centrifuge briefly to remove the drops from inside the lid.
8. Place the necessary number of MinElute® DNA spin columns and collection tubes in a suitable rack. Transfer the entire mixture from each tube from step 7 into the corresponding MinElute DNA spin column.
9. Centrifuge the spin columns at maximum speed for 1 min. Discard the flow-through, and place the spin columns back into the collection tubes.
10. Add 500 µl Buffer BW (wash buffer) to each spin column, and centrifuge at maximum speed for 1 min. Discard the flow-through, and place the spin columns back into the collection tubes.
11. Add 500 µl Buffer BD (desulfonation buffer) to each spin column then close the lids of the spin columns, and incubate for 15 min at room temperature (15–25°C).

    If there are precipitates in Buffer BD, avoid transferring them to the spin columns.

    IMPORTANT: The bottle containing Buffer BD should be closed immediately after use to avoid acidification from carbon dioxide in air.

12. Centrifuge the spin columns at maximum speed for 1 min. Discard the flow-through, and place the spin columns back into the collection tubes.
13. Add 500 µl Buffer BW to each spin column and centrifuge at maximum speed for 1 min. Discard the flow-through and place the spin columns back into the collection tubes.
14. Repeat step 13 once.
15. Add 250 µl ethanol (96–100%) to each spin column and centrifuge at maximum speed for 1 min.
16. Place the spin columns into new 2 ml collection tubes, and centrifuge the spin columns at maximum speed for 1 min to remove any residual liquid.

    Optional: Place the spin columns with open lids into a clean 1.5 ml microcentrifuge tube (not provided) and incubate the columns for 5 min at 60°C in a heating block. This step ensures the evaporation of any remaining liquid.

17. Place the spin columns into clean 1.5 ml microcentrifuge tubes (not provided). Add 18 µl RNAse-free water directly onto the center of each spin-column membrane and close the lids gently.
18. Incubate the spin columns at room temperature for 1 min.
19. Centrifuge for 1 min at 15,000 x *g* (12,000 rpm) to elute the DNA.

    Note: We recommend storing purified DNA at 2–8°C for up to 24 h. When storing purified DNA for longer than 24 h, we recommend storage at –20°C.

    Note: During bisulfite treatment, DNA is fragmented and can be used without additional fragmentation for the library preparation.

Procedure: Double strand synthesis

This procedure describes the double strand synthesis of the converted, single-stranded and fragmented DNA after bisulfite treatment. The following products are required: Klenow Fragment (3'–5' exo-) (5 U/µl) and Klenow buffer (QIAGEN Enzymatics, cat. no. P7010-LC-L), Random octamer primer (150 µM) and dNTP Mix, PCR Grade (200µl) (cat. no. 201900).

20. Thaw Klenow buffer at room temperature and dNTP mix, random primer and Klenow Fragment (3'–5' exo-) on ice.
21. Dilute dNTP mix (10 mM) 1:10 in PCR grade water to obtain 1 mM dilution.
22. Pipet components for double strand synthesis in a PCR tube in the order as described in Table 3.

Table 3. Double strand synthesis reaction setup

Component	Volume/reaction
Klenow Buffer (Buffer Blue), 10x	4 µl
dNTP mix 1 mM	4 µl
PCR grade water	12 µl
Random Octamer Primer (150 µM)	2 µl
Klenow fragment exo- (5 units/µl)	3 µl
Total reaction mix volume	**25 µl**

23. Add 15 µl of the bisulfite treated DNA from step 19 and incubate in a thermocycler using the cycling conditions in Table 4.

Table 4. Cycling conditions

Step	Incubation time	Temperature
1	30 min	37°C
2	10 min	68°C
3	Hold	4°C

Procedure: Ultralow input library preparation

This procedure describes the NGS library preparation of the converted, double-stranded and fragmented DNA. The following products are required: QIAseq Ultralow Input Library Kit (QIAGEN, cat. no. 180495), Agencourt AMPure XP beads or GeneRead Size Selection Kit (QIAGEN, cat. no 180514).

24. Program a thermal cycler with the protocols described in Table 5 and Table 6.

Table 5. End-polishing cycling conditions

Step	Incubation time	Temperature
1	30 min	25°C
2	15 min	65°C
3	Hold	4°C

Table 6. Ligation cycling conditions

Step	Incubation time	Temperature
1	10 min	25°C
2	Hold	4°C

End-polishing

25. Set up the End-polishing Reaction Mix on ice according to Table 7.

Table 7. End-polishing reaction setup

Component	Volume/reaction
Input DNA from step 23	40 µl
End-polishing Buffer 10x	5 µl
End-polishing Enzyme Mix	2 µl
RNAse-free H_20	3 µl
Total reaction volume	**50 µl**

26. Mix by gently pipetting. Do not vortex.
27. Load into the thermal cycler and start the End-polishing program. Proceed to the next step.

Adapter ligation

28. During the end-polishing reaction, vortex and spin down the thawed adapter plate.
    Remove the protective adapter plate lid, pierce the foil seal for each well to be used and prepare diluted adapter with TE buffer as directed in the kit handbook.
29. When the thermal cycler reaches the 4°C hold, stop the program.
30. Remove reactions from the thermal cycler and add ligation components on ice according to Table 8. Return any unused 96-plex adapter to –20°C.

Table 8. Adapter ligation reaction setup

Component	Volume/reaction
End-polished DNA from step 27	50 µl
Ultralow Input Ligation Buffer, 4x	25 µl
Ultralow Input Ligase	5 µl
QIAseq Adapter (96-plex plate)	2 µl
RNAse free H_20	18 µl
Total reaction volume	**100 µl**

31. Mix adapter ligation reactions by pipetting 5–6 times.
32. Incubate at 25°C for 10min (see Table 6).
    IMPORTANT: Do not use a thermocycler with a heated lid.
33. When the program is complete, proceed immediately to adapter ligation cleanup.
34. Add 80 µl resuspended Agencourt AMPure XP beads to each sample and mix.
35. Incubate for 5 min at room temperature.
36. Pellet the beads on a magnetic stand and carefully discard the supernatant.

37. Add 200 µl fresh 80% ethanol to each pellet. Pellet the beads on the magnetic stand and carefully discard the supernatant.
38. Repeat step 37 for a total of 2 ethanol washes. Remove excess ethanol.
39. Incubate on the magnetic stand for 5–10 min or until the beads are dry. Over-drying may result in lower DNA recovery. Remove from the magnetic stand.
40. Elute by resuspending in 52.5 µl Buffer EB. Pellet beads on the magnetic stand. Carefully transfer 50 µl supernatant to a new PCR plate.
41. Add 50 µl of resuspended Agencourt AMPure XP beads to each sample.
42. Follow steps 35–39. Elute by resuspending in 23 µl Buffer EB. Pellet the beads on the magnetic stand. Carefully transfer 20 µl of supernatant into a new PCR plate.

Procedure: Amplification of library using the QIAGEN Multiplex PCR Kit

This procedure describes the amplification of an NGS library. The following QIAGEN products are required: QIAGEN Multiplex PCR Kit (QIAGEN, cat. no. 206143), Illumina Library amplification primer mix (from QIAseq Ultralow Input Library Kit, cat. no. 180495), Agencourt AMPure XP beads.

43. Thaw QIAGEN Multiplex PCR Master Mix, RNase-free water and Illumina Primer Mix. Mix thoroughly before use.
44. Prepare the amplification reaction mix according to Table 9.

Table 9. Library amplification reaction setup

Component	Volume/reaction
PCR grade water	4 µl
Multiplex PCR Mastermix 2x	25 µl
Illumina PCR Primer Mix (10 µM)	1 µl
NGS Library	20 µl
Total reaction volume	**50 µl**

Note: For amplification of more libraries, prepare a PCR reaction mix that contains all the components required for multiplex PCR except the NGS library DNA. Add 10% greater volume than the volume required for the total number of reactions to be performed. Then add 20 µl NGS Library DNA to 30 µl of the PCR reaction mix.

45. Program the thermal cycler according to conditions outlined in Table 10.

Table 10. Cycling conditions

Step	Incubation time	Temperature
1	15 min	95°C
2 for 6–8 cycles	30 s	94°C
	30 s	60°C
	1.5 min	72°C
3	10 min	72°C
4	Hold	4°C

46. Place the PCR tubes in the thermal cycler and start the cycling program as outlined in Table 10.
47. After amplification, samples can be stored overnight at 2–8°C, or at –20°C for longer storage.

Purify amplified library

48. Add 50 µl resuspended Agencourt AMPure XP beads to each sample and mix.
49. Incubate for 5 min at room temperature.
50. Pellet the beads on a magnetic stand and carefully discard the supernatant.
51. Add 200 µl fresh 80% ethanol to each pellet. Pellet the beads on the magnetic stand and carefully discard the supernatant.
52. Repeat step 51 for a total of 2 ethanol washes. Remove excess ethanol.
53. Incubate on the magnetic stand for 5–10 min or until the beads are dry. Over-drying may result in lower DNA recovery. Remove from the magnetic stand.
54. Elute by resuspending in 25 µl Buffer EB. Pellet beads on the magnetic stand. Carefully transfer 22 µl supernatant to a new PCR plate.
55. QC library using a capillary electrophoresis device such as the Agilent Bioanalyzer or equivalent methods.

    Note: The library should show a distribution centered around the size of the fragmented DNA plus 120 bp (see Figure 2). The increase in library length reflects the addition of sequencing adapters to the DNA fragments.

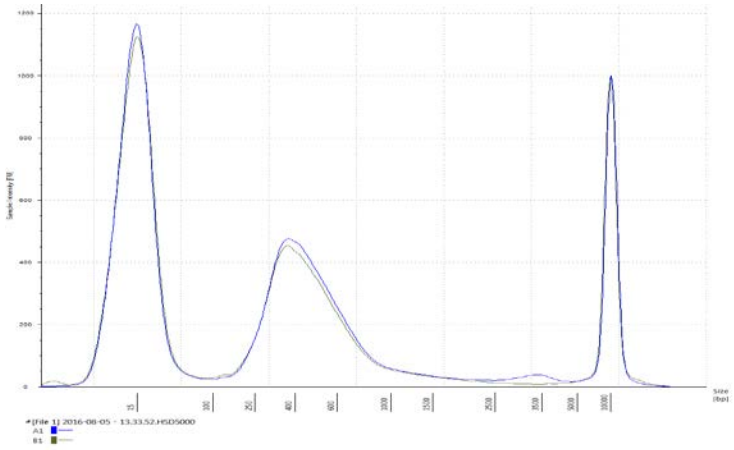

Figure 2. Capillary electrophoresis device trace data.

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Safety data sheets (SDS) for any QIAGEN product can be downloaded from **www.qiagen.com/safety**.

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