

## Supplementary Protocol

# WGBS Libraries Prepared with QIAseq<sup>®</sup> Ultralow Input Library Kit

This protocol describes a streamlined method for whole genome bisulfite conversion using the QIAGEN<sup>®</sup> EpiTect<sup>®</sup> Fast DNA Bisulfite Kit, followed by NGS library preparation using the QIAseq Ultralow Input Library Kit. Refer to Figure 1 for the summarized workflow.

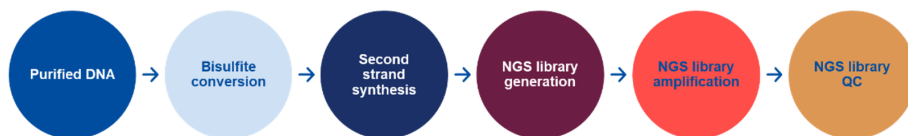


Figure 1. WGBS workflow chart.

Read the *QIAseq Ultralow Input Library Kit Handbook* ([www.qiagen.com/HB-2155](http://www.qiagen.com/HB-2155)) for handling and storage instructions, and carefully review the procedure guidelines before starting.

## 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.

- QIAseq Ultralow Input Library Kits:
  - QIAseq Ultralow Input Library Kit (12) (cat. no. 180492) and QIAseq UDI Y-Adapter Kit (24) (cat. no. 180310); or
  - QIAseq Ultralow Input Lib UDI-A/B/C/D Kit (96), (cat. nos. 180497, 180498, 180499, and 180500)
- EpiTect Fast DNA Bisulfite Kit (50) (cat. no. 59824)
- Klenow Fragment (3'-5' exo-) (5 U/μL) and Klenow buffer (cat. no. P7010-LC-L)
- Random Hexamers (100 μL) (cat. no. 79236)
- dNTP Mix, PCR Grade (200 μL) (cat. no. 201900)
- 96–100% and 80% fresh ethanol
- Buffer EB (cat. no. 19086)
- Microcentrifuge tubes, PCR tubes or plates
- Sterile pipette tips and pipettes
- DNA LoBind tubes
- Vortexer
- Microcentrifuge
- Thermocycler
- Magnetic racks for magnetic bead separation (e.g., DynaMag™-2 /96 Magnet, Thermo Fisher Scientific, cat. no. 12321D)
- QIAxcel® Connect System, Agilent® 2100 Bioanalyzer or similar to evaluate the DNA fragmentation profile, or comparable capillary electrophoresis device, or method to assess the quality of the DNA library.
- Agilent High Sensitivity DNA Kit (Agilent, cat. no. 5067-4626)

- QIAseq Library Quant System (Array Kit [cat. no. 333304]; Assay Kit [cat. no. 333314]) for library quantification

## Procedure: Bisulfite conversion of purified DNA

This procedure is for the whole bisulfite conversion of purified DNA prior to NGS library preparation. This procedure requires the QIAGEN EpiTect Fast DNA Bisulfite Kit.

### Bisulfite conversion of DNA

1. Thaw DNA for 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.

**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 (µL) for fragmented DNA	Volume/reaction (µL) for HMW DNA and FFPE
DNA (150–1000 ng)	20	55
Bisulfite Solution	85	8
DNA Protect Buffer	35	–
<b>Total reaction volume</b>	<b>140</b>	<b>140</b>

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 the 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 following cycling conditions (see Table 2). The complete cycle should take approximately 20 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	Temperature (°C)	Incubation time for fragmented DNA	Incubation time for FFPE DNA	Incubation time for HMW DNA
1. Denaturation	95	5 min	5–8 min	10 min
2. Incubation	60	10–15 min*	15 min	30 min
3. Denaturation	95	5 min	5–8 min	10 min
4. Incubation	60	10–15 min*	15 min	30 min
5. Hold	4	Indefinite	Indefinite	Indefinite

\* cfDNA is strong-fragmented and typically less than 20 ng input, therefore incubation at 10 min is sufficient to completely convert non-methylated Cytosines. Pre-fragmented DNA with the average size of 300 bp will require 15 min incubation.

## Desulfonation and 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.

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

6. Add 310  $\mu\text{L}$  of freshly prepared Buffer BL to each sample. Mix the solutions by vortexing and then centrifuge briefly.
7. Add 250  $\mu\text{L}$  ethanol (96–100%) to each sample. Mix the solutions by pulse vortexing for 15 s, then 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 of 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  $\mu\text{L}$  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  $\mu\text{L}$  Buffer BD (desulfonation buffer) to each spin column. Then, close the lids of the spin columns, and incubate for 15 min at room temperature.

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

**Important:** Close the bottle containing Buffer BD 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  $\mu\text{L}$  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  $\mu\text{L}$  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  $\mu\text{L}$  Buffer RNase-free water directly onto the center of each spin-column membrane, then close the lids gently.
18. Incubate the spin columns at room temperature for 1 min.
19. Centrifuge for 1 min at 15,000  $\times g$  (12,000 rpm) to elute the DNA.

**Note:** We recommend storing purified DNA at  $-20^{\circ}\text{C}$ . Storage of the generated single strands with concentration less than 100 ng/ $\mu\text{L}$  is not recommended for more than 2 weeks since this will lead to material loss.

**Note:** If DNA protect is omitted during bisulfite treatment, DNA fragmentation will occur, and DNA can be used directly for library preparation without further fragmentation. However, heat-induced DNA fragmentation is variable and dependent on the quality of the starting material. Mechanical pre-fragmentation of DNA provides greater standardization but necessitates a higher input amount.

## Protocol: Library Preparation of the bisulfite-converted DNA

### Procedure: Double strand synthesis

This procedure describes the double strand synthesis of the converted, single-stranded, and fragmented DNA after bisulfite treatment and requires Klenow Fragment (3'–5' exo-) (5 U/ $\mu$ L) and Klenow buffer, Random hexamer primer and dNTP Mix, PCR Grade (200  $\mu$ L).

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. Pipette 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 ( $\mu$ L)
Klenow Buffer (Buffer Blue), 10x	4
dNTP mix 1 mM	4
RNase-free water	11
Random Hexamer Primer	3
Klenow fragment exo- (5 units/ $\mu$ L)	3
<b>Total reaction mix volume</b>	<b>25</b>

23. Add 15  $\mu$ L of the bisulfite-treated DNA from step 19 and incubate in a thermocycler using following cycling condition as described in Table 4.

**Table 4. Double-strand synthesis cycling conditions**

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

## Procedure: End-polishing

This procedure describes the NGS library preparation of the converted, double-stranded DNA using QIAseq Ultralow Input Library Kit reagents.

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 (°C)
1	30 min	25
2	15 min	65
3	Hold	4

**Table 6. Adapter ligation cycling conditions**

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

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

**Table 7. End-polishing reaction setup**

<b>Component</b>	<b>Volume/reaction (µL)</b>
Input DNA (from step 23)	40
End-polishing Buffer 10x	5
End-polishing Enzyme Mix	2
RNAse-free water	3
<b>Total reaction volume</b>	<b>50</b>

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 after completing the cycling.

### Procedure: 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 Table 8.

**Table 8. Adapter dilution**

<b>Input DNA Amount</b>	<b>Adapter Dilution Factor</b>
10–99 pg	1:1000
100–999 pg	1:100
1–9 ng	1:10
10–100 ng	No dilution (use undiluted)

29. Remove end-polished DNA from the thermal cycler and prepare ligation reactions as described in Table 9, and mix by pipetting until the suspension appears homogenous.

**Note:** When processing multiple samples, it is possible to prepare a ligation mix consisting of the Ultralow ligation buffer 4x, RNase-free water, and Ultralow Input Ligase. Mix by repeated pipetting up and down. The Ultralow ligation buffer 4x has a high density, ensure the mix is completely homogenous. Then add the 48  $\mu\text{L}$  of this mix to the 50  $\mu\text{L}$  end-polished DNA. Then add 2  $\mu\text{L}$  of the individual adapters to each sample.

**Table 9. Ligation reaction setup**

Component	Volume/reaction ( $\mu\text{L}$ )
End-polished DNA from step 27	50
Ultralow Input Ligation Buffer, 4x	25
Ultralow Input Ligase	5
QIAseq Adapter (96-plex plate)	2
RNase-free water	18
<b>Total reaction volume</b>	<b>100</b>

30. Return any unused 96-plex adapter to  $-20^{\circ}\text{C}$ .
31. Place the ligation mixes in the cycler and run the program outlined in Table 6.

**Important:** Set the thermocycler heated lid off and allow it to cool down before incubating the ligation reactions. If the thermocyclers' heated lid cannot be adjusted, then incubate with the heated lid open.

32. When the program is complete, proceed immediately to adapter ligation cleanup.

### Procedure: Post adapter ligation cleanup using QIAseq Beads

33. Add 80  $\mu$ L resuspended QIAseq beads to each sample and mix.
34. Incubate for 5 min at room temperature.
35. Pellet the beads on a magnetic stand and carefully discard the supernatant.
36. Add 200  $\mu$ L fresh 80% ethanol to each pellet.
37. 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.
40. Remove from the magnetic stand.
41. Elute by resuspending in 52.5  $\mu$ L Buffer EB. Pellet beads on the magnetic stand.
42. Carefully transfer 50  $\mu$ L supernatant to a new PCR plate or tube.
43. Add 40  $\mu$ L resuspended QIAseq beads to each sample and mix.
44. Incubate for 5 min at room temperature.
45. Pellet the beads on a magnetic stand and carefully discard the supernatant.
46. Add 200  $\mu$ L fresh 80% ethanol to each pellet.
47. Pellet the beads on the magnetic stand and carefully discard the supernatant.
48. Repeat step 47 for a total of 2 ethanol washes. Remove excess ethanol.
49. Incubate on the magnetic stand for 5–10 min or until the beads are dry.
50. Remove from the magnetic stand.
51. Elute by resuspending in 23  $\mu$ L Buffer EB. Pellet beads on the magnetic stand.
52. Carefully transfer 20  $\mu$ L supernatant to a new PCR plate or tube.

## Procedure: Amplification of library using the HiFi PCR Master Mix

53. Thaw HiFi PCR Master Mix, 2x, library from step 52, RNase-free water, and Illumina primer mix. Mix thoroughly before use.
54. Prepare a reaction mix according to Table 10.
55. Prepare a volume of reaction mix 10% greater than that required for the total number of reactions to be performed.

**Table 10. Library amplification mix**

Component	Volume/reaction (µL)
PCR grade water	3.5
HiFi PCR Master Mix, 2x	25
Illumina primer Mix (10 µM)	1.5
Library	20
<b>Total reaction volume</b>	<b>50</b>

56. Program the thermal cycler according to the manufacturer's instructions, using the conditions outlined in Table 11.

**Table 11. Library amplification cycling**

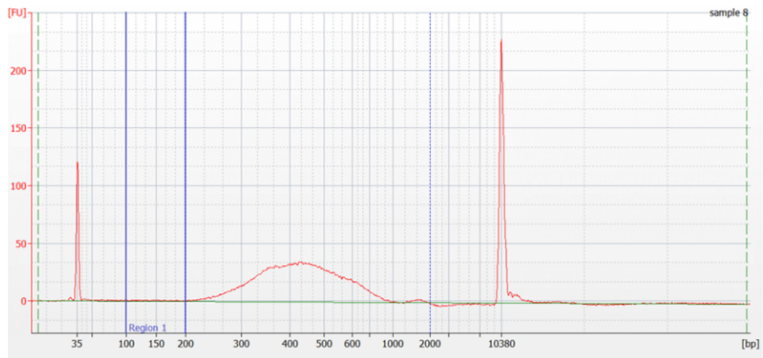
Step	Temperature (°C)	Incubation time
1	95	15 min
	94	30 s
2 for 6–14 cycles*	60	30 s
	72	1.5 min
3	72	10 min
4	4	Hold

\* Use 6 cycles for input >100 ng, 8 cycles for approx. 50 ng, 12 cycles for approx. 10 ng, and 14 cycles for input <10 ng.

57. Place the PCR tubes in the thermal cycler and start the cycling program as outlined in Table 11.
58. After amplification, samples can be stored overnight at 2–8°C, or at –20°C for longer storage.

### Final library purification using QIAseq beads

59. Add 50 µL resuspended QIAseq beads to each sample and mix.
60. Incubate for 5 min at room temperature.
61. Pellet the beads on a magnetic stand and carefully discard the supernatant.
62. Add 200 µL fresh 80% ethanol to each pellet. Pellet the beads on the magnetic stand and carefully discard the supernatant.
63. Repeat step 62 for a total of 2 ethanol washes. Remove excess ethanol.
64. 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.
65. Elute by resuspending in 25 µL RNase-free water. Pellet beads on the magnetic stand. Carefully transfer 22 µL supernatant to a new PCR plate.
66. QC library using electrophoretic devices for the absence of adapter dimer. Figure 2 illustrates a typical size distribution.
67. Quantify using QPCR or Qubit.



**Figure 2. Electrophoretic trace of a WGBS library.** The library was prepared using the QIAseq Ultralow Input Library Kit in accordance with the current protocol. A starting amount of 60 ng of high molecular weight (HMW) DNA was used. For bisulfite treatment, the thermal cycling parameters suitable for HMW DNA were applied. Prior to analysis, the library was diluted 1:3 in RNase-free water and loaded on an Agilent BioAnalyzer HS DNA chip. The final library yield was 6.5 nM.

## Document Revision History

Date	Changes
01/2026	Initial release.

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at [www.qiagen.com](http://www.qiagen.com) or can be requested from QIAGEN Technical Services or your local distributor.

### Limited License Agreement for QIAseq® Ultralow Input Library Kit

Use of this product signifies the agreement of any purchaser or user of the product to the following terms:

1. The product may be used solely in accordance with the protocols provided with the product and this Instructions for Use and for use with components contained in the panel only. QIAGEN grants no license under any of its intellectual property to use or incorporate the enclosed components of this panel with any components not included within this panel except as described in the protocols provided with the product, this Instructions for Use, and additional protocols available at [www.qiagen.com](http://www.qiagen.com). Some of these additional protocols have been provided by QIAGEN users for QIAGEN users. These protocols have not been thoroughly tested or optimized by QIAGEN. QIAGEN neither guarantees them nor warrants that they do not infringe the rights of third-parties.
2. Other than expressly stated licenses, QIAGEN makes no warranty that this panel and/or its use(s) do not infringe the rights of third-parties.
3. This panel and its components are licensed for one-time use and may not be reused, refurbished, or resold.
4. QIAGEN specifically disclaims any other licenses, expressed or implied other than those expressly stated.
5. The purchaser and user of the panel agree not to take or permit anyone else to take any steps that could lead to or facilitate any acts prohibited above. QIAGEN may enforce the prohibitions of this Limited License Agreement in any Court, and shall recover all its investigative and Court costs, including attorney fees, in any action to enforce this Limited License Agreement or any of its intellectual property rights relating to the panel and/or its components.

For updated license terms, see [www.qiagen.com](http://www.qiagen.com).

Trademarks: QIAGEN®, Sample to Insight®, QIAseq® (QIAGEN Group). Registered names, trademarks, etc. used in this document, even when not specifically marked as such, are not to be considered unprotected by law.

01/2026 HB-3770-001 © 2026 QIAGEN, all rights reserved.