November 2017

QIAseq™ Methyl Library Handbook

For DNA library construction for whole genome bisulfite sequencing on Illumina® sequencing platforms

Sample to Insight

QIAGEN

Contents

Handbook Revision History

Document	Changes	Date
HB-2476-001	Initial release	November 2017

Kit Contents

Box 1

QIAseq Methyl Library Kit	**(24)**
Catalog no.	**180502**
No. of preps	**24**
VeraSeq Ultra DNA Polymerase	15 µl
VeraSeq Buffer II, 5X	500 µl
RNase-Free Water	5 x 1.9 ml
Primer Mix Illumina Libr. Amp	2 x 20 µl
dNTP Mix (10 mM/each)	55 µl
BisU DNA repair Enzyme Mix	60 µl
BisU DNA repair Buffer, 5X	500 µl
Ultralow Input Ligase	3 x 65 µl
Ultralow Input Ligation Buffer, 4X	900 µl
Adapter Plate 24-plex Illumina Bar Code	1 plate (24)
Quick-Start Protocol	1

* For adapter sequences, see “Appendix B: Adapter Bar Codes for QIAseq Methyl Libraries”, page 27.

Box 2

QIAseq Methyl Library Kit	**(24)**
Catalog no.	**180502**
No. of preps	**24**
QIAseq Beads	5 ml

Storage

The QIAseq Methyl Library Kit is shipped in two boxes. Box 1 is shipped on dry ice or blue ice and Box 2 is shipped at room temperature. Upon receipt, all components in Box 1 should be stored immediately at –30°C to –15°C in a constant-temperature freezer. All components in Box 2 should be stored immediately at 2–8°C.

Intended Use

The QIAseq Methyl Library Kit is intended for molecular biology applications. This product is not intended for the diagnosis, prevention or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at **www.qiagen.com/safety** where you can find, view and print the SDS for each QIAGEN kit and kit component.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of QIAseq Methyl Library Kit is tested against predetermined specifications to ensure consistent product quality.

Introduction

Epigenetics describes the study of heritable changes in gene function that occur without a change in the nuclear DNA sequence. In addition to RNA-associated silencing and histone modification, a major epigenetic mechanism in higher-order eukaryotes is DNA methylation. Epigenetic changes play a crucial role in the regulation of important cellular processes, such as gene expression and cellular differentiation, and were also identified as key factors in many various diseases.

DNA methylation occurs on cytosine residues, especially in CpG islands, which are GC-rich regions. They are usually clustered around the regulatory region of genes and can affect their transcriptional regulation. Methylation of CpG islands is known to inactivate gene expression and plays an important role in normal and disease development. Cytosine methylation may also occur in non-CpG content, as described for embryonic stem cells.

Whole genome bisulfite sequencing (WGBS), which combines bisulfite-mediated conversion of unmethylated cytosines to uracil and next generation sequencing (NGS), allows genome-wide detection of 5-methylcytosine residues at unprecedented single-base resolution. In turn, this enables the connection of gene activity and the precise localization of a DNA methylation marker.

Clinically relevant specimens for next-generation sequencing can be difficult to obtain or yield limited amounts of nucleic acids. Therefore, researchers wish to use samples as efficiently as possible. Solutions that push the limits of input amounts – without sacrificing sensitivity and performance – are needed to safeguard samples. However, current approaches used to treat samples with bisulfite after NGS library preparation, in order to convert unmethylated cytosines to uracils reveal major challenges, such as significant bisulfite-induced sample loss due to DNA degradation. Traditional library preparation methods for bisulfite sequencing demand very high DNA input amounts, since they require several purification steps or require a large number of PCR cycles during NGS library construction.

The QIAseq Methyl Library procedure delivers a fast and streamlined workflow for NGS library construction starting from bisulfite-converted single-stranded DNA. The protocol consists of a two-step reaction performed in a single tube, with no intermediate purification steps. This approach reduces both DNA loss and the need for higher DNA input. This post-bisulfite library construction protocol does not require extra methylated adapters. Furthermore, optimized enzymes and buffer compositions ensure superior yields of high-quality, NGS-ready libraries – even from low input.

The QIAseq Methyl Library chemistry applies highly efficient priming and double-strand synthesis of the bisulfite-converted single strands, combined with end-polishing of the generated double strand in only one step followed by ultra-efficient adapter ligation. Library amplification uses the VeraSeq Ultra DNA Polymerase which is a high-fidelity enzyme that tolerates the presence of uracil. This is important, since evaluation of errors after bisulfite sequencing is difficult, due to variability that originates from methylation from incomplete conversion and errors introduced during sequencing. Dedicated EpiTect Fast bisulfite conversion protocols deliver complete conversion of unmethylated cytosines and optimal starting material for library generation, without the need for extra fragmentation. After library construction and sequencing on Illumina platforms, bioinformatic analysis is extremely simplified using the Bisulfite Sequencing Plug-in (version 2.0 or later) for the Biomedical Genomics Workbench and CLC Genomics Workbench for non-directional libraries. The entire WGBS workflow is shown in Figure 1.

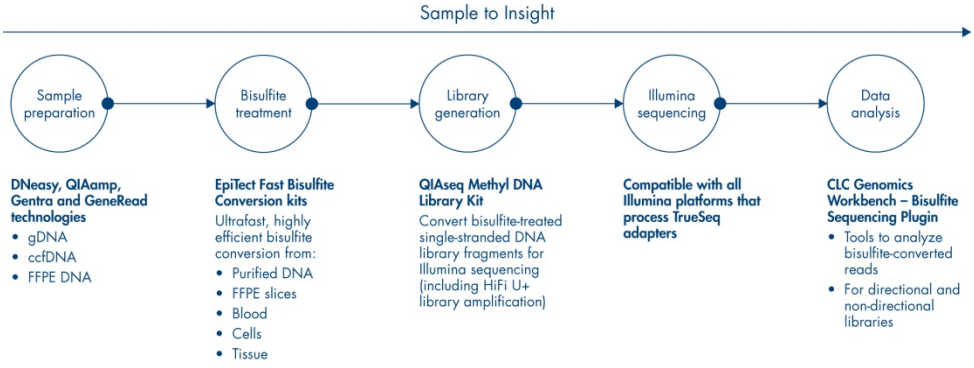

Figure 1. Whole genome bisulfite sequencing (WGBS) workflow.

With the QIAseq Methyl Library Kit, reaction setup is straightforward and handling time is greatly reduced, allowing DNA amplification and library preparation to be completed in less than 5 hours. The kit provides a time-saving, one-tube library preparation protocol that does not require extra DNA fragmentation and sample cleanup between steps, minimizing starting material loss and cross-contamination risk. Co-optimization of bisulfite conversion and library construction processes enables a highly streamlined and efficient protocol and generates a ready-to-use WGBS library in under a day.

The QIAseq Methyl Library Kit introduces priming of optimized, short primers to enormously reduce starting bias and capture all genomic regions with high efficiency. The high conversion efficiency results in high yields of library without the need for high numbers of library amplification cycles, even from low input. The kit allows the construction of highly complex libraries from a wide range of DNA amount and materials with a high percentage of mapped reads, uniform genome coverage for accurate methylation analysis.

Dual-barcoded, plate-format adapters are included with the QIAseq Methyl Library Kit and allow sequencing on Illumina platforms.

Typical library concentrations from a QIAseq Methyl Library Kit reaction are 2–40 nM with an average fragment size of approximately 350 bp, depending on DNA input and cycle number during library amplification.

Principle and procedure

The QIAseq Methyl Library Kit uses an enzyme mix and random, short primers for double-strand synthesis of the single-stranded, bisulfite-treated DNA. In the same reaction setup, enzymes repair and prepare the ends of the fragments for subsequent adapter ligation. Both DNA repair and ligation reactions occur in the same tube, minimizing sample loss. The ligation step is followed by a purification step using QIAseq Beads which are included in the kit. Library amplification is performed using the high-fidelity enzyme VeraSeq Ultra DNA Polymerase (Figure 2). VeraSeq Ultra DNA Polymerase is an engineered, ultra-thermostable, uracil-literate polymerase designed to maximize the speed, accuracy, and length of DNA synthesis during sequencing template preparation. The result is a novel enzyme that can read through uracil, extend a kilobase of sequence in 15 seconds and is 25 times more accurate than *Taq* DNA Polymerase. This enables the amplification of WGBS libraries without sequence bias.

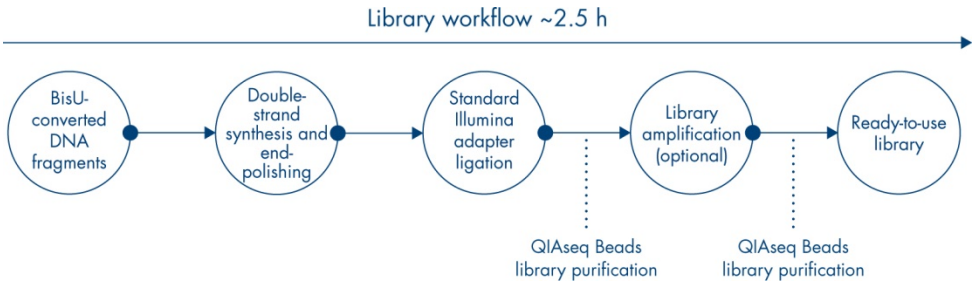

Figure 2. QIAseq Methyl Library workflow.

Dual-barcoded, plate-format adapters are included with the QIAseq Methyl Library Kit. Each adapter well contains a single-use adapter consisting of a unique combination of two 8-nucleotide identification barcodes. By combining one of eight D5 barcodes and one of three D7 barcodes in each ready-to-use adapter, this kit supports up to 24-plexing prior to sequencing (see "Appendix B: Adapter Bar Codes for QIAseq Methyl Libraries", page 27, for barcode IDs). Following library construction, the reaction cleanup and removal of adapter–dimers can be achieved using QIAseq Beads. After library amplification, a final cleanup step is performed with QIAseq Beads.

Description of protocols

This handbook contains protocols for the generation of libraries using bisulfite-converted gDNA, amplification of the libraries and dedicated protocols for bisulfite conversion of gDNA and prefragmented samples (approximately 200 bp) or ccfDNA using EpiTect Fast Bisulfite Conversion kits (cat. nos. 59802, 59824, 59864). The protocol for library construction is described in "Protocol: Library Generation from Bisulfite-Converted DNA", page 16. The subsequent library amplification is described in section "Protocol: Library Amplification", page 21. The dedicated protocols for WGBS using EpiTect Fast DNA Bisulfite Kit are described in "Appendix C: Bisulfite Conversion of Purified gDNA and FFPE DNA Using EpiTect Fast DNA Bisulfite Conversion Kits", page 29 and "Appendix D: Bisulfite Conversion of Prefragmented (~200 bp) and ccfDNA Using EpiTect Fast Bisulfite Conversion Kits", page 33. For additional protocols for direct lysis of cells, blood and FFPE slices, please consult the *EpiTect Fast Bisulfite Conversion Handbook* and use the cycling conditions recommended in "Appendix C: Bisulfite Conversion of Purified gDNA and FFPE DNA Using EpiTect Fast DNA Bisulfite Conversion Kits".

The generated library can be quantified using qRT-PCR and is optimized for use on Illumina sequencing platforms.

Compatible sequencing platforms:

- Illumina HiSeq®
- Illumina MiSeq®
- Illumina NextSeq®

Equipment and Reagents to Be Supplied by User

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 Bisulfite Conversion Kit (QIAGEN, cat. nos. 59802, 59824, 59864)
- PCR tubes or plates
- LoBind® tubes for storage of generated libraries (Eppendorf, cat. no. 0030108094, 0030108116 or 0030108132) or equivalent
- Pipets and pipet tips
- Magnetic racks for magnetic bead separation (e.g., DynaMag™-2 Magnet, Thermo Fisher Scientific, cat. no. 12321D*)
- Thermocycler
- Microcentrifuge
- Vortexer
- NanoDrop® UV-Vis spectrophotometer for DNA and bisulfite-treated DNA quantification
- Ice
- 96–100% ethanol
- 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
- QIAseq Library Quant Assay Kit (QIAGEN, cat. no. 333314) for library quantification

* This is not a complete list of suppliers and does not include many important vendors of biological supplies.

Important Notes

DNA preparation and quality control

High-quality DNA is essential for obtaining good bisulfite conversion, library generation and sequencing results. The most important prerequisite for any DNA sequence analysis experiment is consistent, high-quality DNA from every experimental sample. Therefore, DNA purification procedures, as well as accurate quantification and quality control are critical to the success of the experiment.

General precautions

Use good laboratory practices to minimize cross-contamination of nucleic acid products.

Always use PCR tubes, microcentrifuge tubes and pipet tips that are certified sterile, DNase- and RNase-free.

Before starting, wipe down work area and pipets with an RNase and DNA cleaning product such as RNase Away® (Sigma-Aldrich) or LookOut® DNA Erase (Sigma-Aldrich).

For consistent bisulfite conversion, library construction and amplification, ensure the thermal cycler used in the protocol is in good working order and has been calibrated to within the manufacturer's specifications.

Read the entire protocol before beginning. Take note of stopping points where samples can be frozen at –20°C and plan your workflow accordingly.

Library generation is based on a number of enzymatic reactions and is sensitive to many factors, such as reaction temperature, time and setup conditions and alcohol contamination, as well as the purity and quality of the input DNA.

When working with plates, we recommend mixing by pipetting samples up and down and changing tips to avoid cross-contamination.

Recommended library quantification method

QIAGEN's QIAseq Library Quant Assay Kit (cat. no. 333314), which contains laboratory-verified forward and reverse primers together with a DNA standard, is highly recommended for accurate quantification of the prepared library. Alternatively Qubit® or Agilent Bioanalyzer may be used, but they are not recommended for non-enriched libraries and are less accurate since they measure all fragments (adapter-ligated, non-adapter-ligated and ligated fragment with only one adapter).

Protocol: Library Generation from Bisulfite-Converted DNA

This protocol is for NGS library preparation from bisulfite-converted, single-stranded and fragmented DNA.

Important points before starting

- This protocol is optimized for bisulfite-converted DNA using EpiTect Fast Bisulfite Conversion kits, but can be used with bisulfite conversion methods that generate single-stranded DNA with fragment mean size between 200–1500 bp.
- Samples of 10 pg – 500 ng gDNA (optimal: 1–100 ng) as input in bisulfite conversion can be used for library generation using the QIAseq Methyl Library Kit.
- If starting with a low input level, note that quantification of single-stranded DNA after bisulfite conversion will not be accurate.
- Avoid DNA contamination of reagents by using separate laboratory equipment (e.g., pipets, filter pipet tips, reaction vials, etc.).
- Enzymes and enzyme mixes should be thawed on ice. All other components can be thawed at room temperature (15–25°C) but placed on ice immediately after thawing.
- If using less than 500 pg input, the libraries will show lower mapping efficiencies.
- Set up all reactions on ice.

Things to do before starting

- Thaw BisU DNA Repair Buffer, 5x and Ultralow Input Ligation Buffer, 4X and place on ice.
- Prepare fresh 80% ethanol.
- All buffers and reagents should be vortexed before use to ensure thorough mixing.
- All enzyme mixes should be placed on ice until use.

- Program the thermal cycler. For increased speed and convenience, all incubation steps of the protocol can be preprogrammed and saved on a thermal cycler in advance.

Procedure

1. Before starting with the setup of the reactions and reagent mixes program a thermal cycler with the protocols described in Table 1 and Table 2.

Table 1. Thermal cycling for BisU DNA repair reactions

Step	Temperature	Incubation time
1	30°C	30 min
2	68°C	15 min
3	4°C	Hold

Note: Use thermocycler with a heated lid set at 75°C.

Table 2. Thermal cycling for ligation of adapters to end-polished fragments

Step	Temperature	Incubation time
1	20°C	15 min
2	4°C	Hold

Note: Use thermocycler with lid off.

BisU DNA repair reaction

2. Prepare bisulfite converted DNA dilution 10 pg – 500 ng (optimal: 1–100 ng) in RNase-free Water. If no quantification occurs between EpiTect Fast protocol and library preparation, use total volume of the EpiTect Fast Eluate (approximately 16 µl).

   Note: During bisulfite conversion, desulfonation and column purification of bisulfite-converted DNA, approximately 30–50% of DNA will be lost. Please calculate DNA input accordingly to begin with optimal input for library preparation.

3. Set up the BisU DNA repair reaction mix on ice according to Table 3 and mix by gently vortexing. Keep on ice until further processing.

   Note: If working with more samples, dilute BisU- converted DNA in a standard volume (e.g., 20 µl) and set up a BisU DNA repair reaction master mix by pipetting BisU DNA Repair Buffer, 5x RNase-free Water and BisU DNA Repair Enzyme Mix. Scale up for the number of required reactions and then add 10%.

Table 3. BisU DNA repair reaction setup

Component	Volume/reaction
BisU DNA repair Buffer, 5x	10 µl
RNase-free Water	Variable
BisU DNA repair Enzyme Mix	2 µl
BisU-converted DNA	Variable
Total reaction volume	**50 µl**

4. Add diluted BisU DNA to each BisU DNA Repair Reaction Mix on ice to a total volume reaction of 50 µl and gently vortex to mix.
5. Briefly spin down the tubes, immediately transfer to the thermocycler and start the programmed BisU DNA repair cycling program (Table 1) to allow double-strand synthesis and end-polishing of the fragments.
6. Place samples on ice, after completion of thermal cycling.

Adapter ligation

7. During BisU DNA repair cycling, vortex and spin down the Adapter Plate. Pierce the plate foil seal and dilute adapters in RNase-free Water (Table 4).
8. For adapter ligation, use the dilutions in Table 4, depending on the DNA input in the complete workflow, including EpiTect Fast bisulfite conversion.

Table 4. Adapter dilution

DNA input in complete workflow	Adapter dilution in RNase-free Water
<500 pg	1:50
500 pg – 50ng	1:10
>50 ng	1:2

9. Transfer 4 µl of one adapter to each sample. Track the bar codes used and freeze remaining adapters.
10. Prepare the ligation master mix on ice according to Table 5. Mix by gently vortexing and spin down shortly to collect liquids on the bottom of the tube.

Table 5. Ligation master mix setup

Component	Volume/reaction
Ultralow Input Ligation Buffer, 4X	25 µl
Ultralow Input Ligase	5 µl
RNase-free Water	16 µl
Total reaction volume	**46 µl**

Note: Scale up for the number of required reactions, then add 10%.

11. Add 46 µl ligation master mix to each sample to generate a total volume of 100 µl. Vortex to mix, then spin down and place in the thermocycler.
12. Run the programmed ligation cycling program (Table 2). After completion of ligation step proceed immediately with purification of the generated library.

Library purification using QIAseq Beads

13. Add 50 µl (0.5x) mixed QIAseq Beads to each sample and vortex.

    Note: If working with plates, to avoid contamination during opening the sealing tapes we recommend mixing by pipetting. Always change pipet tips after each mixing step.

14. Incubate for 5 min at room temperature to bind library fragments on the beads.

15. Pellet the beads on a magnetic stand until supernatant is clear (approximately 5 min) and carefully discard the supernatant.
16. Add 200 µl fresh 80% ethanol to each pellet to wash the beads immobilized on the magnet.
17. Discard the supernatant and repeat the washing step 16. After repeating the washing step, discard the supernatant. Carefully remove all remaining ethanol droplets from the tube inner walls, since remaining ethanol contamination may inhibit subsequent reactions.
18. Incubate on the magnetic stand for 5–10 min or until the beads are dry (visual control). Avoid over-drying since this may result in lower DNA recovery. Remove from the magnetic stand.
19. Elute by resuspending the beads in 55 µl RNase-free Water. Pellet beads on the magnetic stand until the supernatant is clear (approximately 5 min).
20. Carefully transfer 50 µl supernatant to a new tube and proceed with the second purification, below
21. Add 60 µl (1.2x) QIAseq Beads to each sample, mix and follow steps 14–18.
22. Elute by resuspending beads in 25 µl RNase-free Water. Pellet the beads on the magnetic stand until supernatant is clear (approximately 5 min) and transfer 20 µl supernatant into a new LoBind tube. The purified library can be stored at –20°C.

Protocol: Library Amplification

This procedure describes the NGS library amplification using the VeraSeq Ultra DNA Polymerase.

1. Program the thermal cycler according to the manufacturer's instructions, using the conditions given in Table 6.

   Note: Cycle number of library amplification will vary depending on the staring material and should be estimated for each individual experiment. Table 7 provides some recommendations for the number of cycles that should be used for different input amounts in the complete workflow, starting with EpiTect Fast bisulfite conversion of DNA.

Table 6. Library amplification cycling conditions

Step	Temperature	Incubation time
1	98°C	30 s
2 (6–18 cycles)	98°C	5 s
	55°C	10 s
	72°C	15 s
3	72°C	5 min
4	4°C	Hold

Note: Use a thermocycler with heated lid on.

Table 7. Recommended PCR cycle numbers for library amplification

DNA input in complete workflow	Number of PCR cycles
<500 pg	16–18
500 pg – 1ng	14
1 ng – 50 ng	10
>50 ng	6

2. Thaw library DNA from step 22 of "Protocol: Library Generation from Bisulfite-Converted DNA", 5X VeraSeq Buffer II, RNase-free Water, dNTP Mix and Illumina primer mix and place on ice after thawing. Place VeraSeq Ultra DNA Polymerase directly onto ice. Mix thoroughly before use. Prepare reaction mix on ice.
3. Prepare the amplification reaction mix according to Table 8. Vortex to mix and spin down to collect liquid in the bottom of the tube.

Table 8. Library amplification reaction mix

Component	Volume/reaction
RNase-free Water	17.25 µl
5X VeraSeq Buffer II	10 µl
dNTP Mix	1.25 µl
Illumina primer mix	1 µl
VeraSeq Ultra DNA Polymerase	0.5 µl
Total	**30 µl**

Note: Scale up for the number of required reactions, then add 10%.

4. Add 30 µl amplification mix to 20 µl library (from step 22 of "Protocol: Library Generation from Bisulfite-Converted DNA"), vortex to mix, then spin down and place on ice.
5. Place the PCR tubes in the thermal cycler and start the programmed library amplification cycling program (Table 7).
6. After amplification, samples can be stored overnight at 2–8°C, or at –20°C for longer storage.

Final library purification using QIAseq Beads

7. Add 55 µl (1.1x volume) QIAseq Beads to 50 µl amplified library and mix well by pipetting up and down.
8. Incubate for 5 min at room temperature to bind library fragments on the beads.

9. Pellet the beads on a magnetic stand until supernatant is clear (approximately 5 min) and carefully discard the supernatant.
10. Add 200 µl fresh 80% ethanol to each pellet to wash the beads immobilized on magnet.
11. Discard the supernatant and repeat the washing step 10.
12. After repeating the washing step, discard the supernatant. Carefully remove all remaining ethanol droplets from the tube inner walls, since remaining ethanol contamination may inhibit subsequent reactions.
13. Incubate on the magnetic stand for 5–10 min or until the beads are dry. Avoid over-drying since this may result in lower DNA recovery. Remove from the magnetic stand.
14. Elute by resuspending and incubating beads for 5 min at room temperature in 25 µl RNase-free Water.
15. Pellet the beads on the magnetic stand for 5 min and transfer 20 µl supernatant into a new LoBind tube. Store at –20°C until sequencing.
16. Assess the quality of the library using a capillary electrophoresis device or comparable method. Check for the correct size distribution (Figure 3) of library fragments and for the absence of adapters or adapter–dimers.

    Note: The median size of the DNA inserts is approx. 250 bp and should be shifted by the size of the adapters 120 bp that were ligated to the library fragments.

    Note: The median fragment size can be used for subsequent qPCR-based quantification methods.

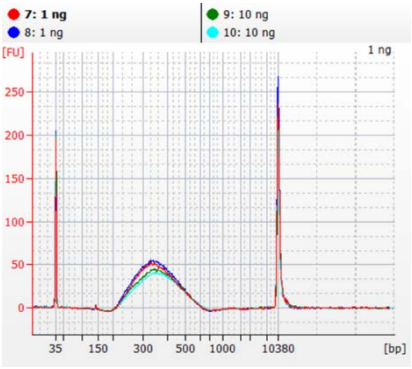

Figure 3. Capillary electrophoresis device trace of generated libraries. Capillary electrophoresis device trace data showing the correct size distribution of library fragment and the absence of adapters or adapter–dimers. Shown are replicate libraries starting from 1 ng or 10 ng bisulfite-converted DNA. 1 ng library was amplified for 14 cycles and 10 ng library was amplified using 10 cycles of PCR. Libraries were diluted 1:10 and loaded on an Agilent High Sensitivity DNA chip.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: **www.qiagen.com/FAQ/FAQList.aspx**. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and/or protocols in this handbook or sample and assay technologies (for contact information, visit **www.qiagen.com**).

	Comments and suggestions
Low library yields from library preparation protocol	
a) Bisulfite-converted DNA yields were lower as expected	Quantify the yield of bisulfite-converted DNA using NanoDrop or Agilent RNA chips. Please note that very low amounts will not be quantified accurately. EpiTect Fast bisulfite conversion, column desulfonation and purification lead to 30–50% DNA loss depending on the input amount and quality of starting material. Take that into consideration while designing the experiment and adjust input for optimal result.
b) Suboptimal reaction conditions due to low DNA quality	DNA fragmentation was too high due to low-integrity DNA input. Adjust denaturation time during the EpiTect Fast protocol.
c) Suboptimal reaction conditions due to ethanol contamination	Ensure that ethanol residue has been fully removed during EpiTect washing steps and residual liquid removal, since ethanol contamination inhibits the subsequent enzymatic reactions.
d) Suboptimal reaction conditions during double-strand synthesis and end-polishing	Ensure that cycling conditions and incubation temperatures are programmed correctly.
e) Inefficient library amplification	Inefficient adapter–dimer removal using QIAseq Beads. Mix beads thoroughly and use the recommended amount for purification. Beads are overdried. Make visual control during drying of beads, humidity of environment may accelerate or slow the drying procedure. Ethanol residues inhibit the reaction. Ensure that all liquids have been removed prior to beads drying. If uncertain, perform a short spin to collect the liquid droplets and remove carefully before drying the beads.

		Comments and suggestions
Unexpected signal peaks in capillary electrophoresis device traces		
a)	Presence of shorter peaks between 60 and 120 bp	These peaks represent library adapters and adapter–dimers that occur when there is no, or insufficient, adapter depletion after library preparation. As adapter–dimers can form clusters on the flow cell and will be sequenced, this will reduce the capacity of the flow cell for the library fragments, even though a low ratio of adapter—dimers versus library will not be a problem. QIAseq Beads efficiently remove adapter–dimers, as well as free adapter molecules.
b)	Presence of larger library fragments after library enrichment	When performing library enrichment , if the fragment population shifts higher than expected after adapter ligation and PCR enrichment (e.g., more than the expected 120 bp shift), this may be a PCR artifact due to over-amplification of the DNA library. Reduce the number of amplification cycles.
c)	Incorrect library fragment size after adapter ligation	During library preparation, adapters of approximately 60 bp are ligated to both ends of the DNA library fragments. This should be reflected on a capillary electrophoresis device by a shift in size of all library fragments of 120 bp. If using library adapters from other suppliers, please refer to the size information given in the respective documentation. The absence of a clear size shift may indicate no, or only low, adapter ligation efficiency. Be sure to use the parameters and incubation times described in the handbook for end-repair, A-addition and ligation, as well as the correct starting amount of DNA.

Appendix A: Dilution Recommendation for Optimal Clustering on Illumina Platforms

Table 9 lists recommendations for optimal library concentration for optimal clustering using MiSeq chemistries Version V2 or V3 with libraries generated from gDNA, ccfDNA or prefragmented DNA.

Table 9. Optimal library concentration

DNA	Molarity	Raw density (K/mm^2)
Bisulfite-converted gDNA	15–17.5 pM	1000–1200
Bisulfite-converted FFPE DNA	15–17.5 pM	1000–1200
ccfDNA	10 pM	1000–1200
Prefragmented DNA (approx. 200 bp)	10 pM	1000–1200

Appendix B: Adapter Bar Codes for QIAseq Methyl Libraries

The bar code sequences used in the QIAseq Methyl Library Kit 96-plex adapter and 24-plex adapter plate are listed in Table 10. Indices 501–508 and 701–703 correspond to the respective Illumina adapter bar codes. The layout of the 24-plex single-use adapter plate is shown in Figure 4.

Table 10. Adapter bar codes used in the QIAseq FX DNA Library Kit 96-plex Adapter

D50X bar code name	i5 bases for entry on the sample sheet	D70X bar code name	I7 bases for entry on the sample sheet
D501	TATAGCCT	D701	ATTACTCG
D502	ATAGAGGC	D702	TCCGGAGA
D503	CCTATCCT	D703	CGCTCATT
D504	GGCTCTGA	–	–
D505	AGGCGAAG	–	–
D506	TAATCTTA	–	–
D507	CAGGACGT	–	–
D508	GTACTGAC	–	–

	1	2	3	4	5	6	7	8	9	10	11	12
A	501/701	501/702	501/703									
B	502/701	502/702	502/703									
C	503/701	503/702	503/703									
D	504/701	504/702	504/703									
E	505/701	505/702	505/703									
F	506/701	506/702	506/703									
G	507/701	507/702	507/703									
H	508/701	508/702	508/703									

Figure 4. QIAseq Methyl 24-plex Adapter Plate layout.

Appendix C: Bisulfite Conversion of Purified gDNA and FFPE DNA Using EpiTect Fast DNA Bisulfite Conversion Kits

Bisulfite conversion of DNA

Thaw DNA to be used in the bisulfite reactions. Make sure the Bisulfite Solution is completely dissolved and equilibrated to room temperature.

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.

IMPORTANT: Do not use carrier RNA.

2. Prepare the bisulfite reactions in 200 µl PCR tubes (not supplied) according to Table 11. Add each component in the order listed.

Table 11. Preparation of bisulfite reactions

Component	Volume/reaction
DNA (100 pg – 500 ng)*	Variable (maximum 55 µl)
RNase free Water	Variable
Bisulfite Solution	85 µl
Total reaction volume	**140 µl**

*Optimal: 1ng – 100 ng.

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 Table 12. The complete cycle should take approximately 80 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.

   IMPORTANT: If using strongly digested DNA, for example DNA purified from FFPE material, we recommend reducing denaturation time to 5 min and bisulfite conversion incubation time to 15 min per cycle.

Table 12. Bisulfite conversion cycling conditions

Step	Temperature	Incubation time gDNA	Incubation time FFPE DNA
Denaturation	95°C	10 min	8 min
Incubation	60°C	30 min	30 min
Denaturation	95°C	10 min	8 min
Incubation	60°C	30 min	30 min
Hold	20°C	Indefinite	Indefinite

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. Avoid storing single-stranded, bisulfite-converted DNA for longer than 2–3 weeks.

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

Appendix D: Bisulfite Conversion of Prefragmented (~200 bp) and ccfDNA Using EpiTect Fast Bisulfite Conversion Kits

This protocol can be used with purified ccfDNA for example using the QIAamp® MinElute ccfDNA Kit or QIAamp MinElute ccfDNA Midi Kit.

Bisulfite conversion of DNA

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

   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.

   IMPORTANT: Do not use carrier RNA.

2. Prepare the bisulfite reactions in 200 µl PCR tubes (not supplied) according to Table 13. Add each component in the order listed.

Table 13. Preparation of bisulfite reactions

Component	Volume/reaction
DNA (100 pg – 500 ng)*	Variable (maximum 40 µl)
RNase-free Water	Variable
Bisulfite Solution	85 µl
DNA Protect Buffer	15 µl
Total reaction volume	**140 µl**

*Optimal: 1 ng – 100 ng.

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 following cycling conditions (Table 14). The complete cycle should take approximately 30 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 14. Bisulfite conversion cycling conditions

Step	Temperature	Incubation time
Denaturation	95°C	5 min
Incubation	60°C	10 min
Denaturation	95°C	5 min
Incubation	60°C	10 min
Hold	20°C	Indefinite

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.

Ordering Information

Product	Contents	Cat. no.
QIAseq Methyl Library Kit (24)	Enzymes, Buffers, Reagents for 24 double-strand synthesis and end-polishing of bisulfite-converted DNA, ligation of Illumina adapters, library purification and amplification. Generated libraries are for use with Illumina Instruments	180502
Related products		
QIAseq Library Quant Assay Kit	Laboratory-verified forward and reverse primers for 500 x 25 µl reactions (500 µl); DNA Standard (100 µl); Dilution Buffer (30 ml); (1.35 ml x 5) GeneRead™ qPCR SYBR® Green Mastermix	333314
EpiTect Fast Bisulfite Kit (10)	Trial kit for 10 preps: Deparaffinization Solution, Lysis Buffer, Proteinase K, Bisulfite Solution, DNA Protect Buffer, MinElute DNA Spin Columns, Carrier RNA, and Buffers	59802
EpiTect Fast DNA Bisulfite Kit (50)	For 50 preps: Bisulfite Solution, DNA Protect Buffer, MinElute DNA Spin Columns, Carrier RNA, and Buffers	59824
EpiTect Fast LyseAll Bisulfite Kit (50)	For 50 preps: Lysis Buffer, Proteinase K, Bisulfite Solution, DNA Protect Buffer, MinElute DNA Spin Columns, Carrier RNA, and Buffers	59864

QIAamp MinElute ccfDNA Mini Kit (50)	For 50 preps (1 or 2 ml sample input volume each): QIAamp UCP MinElute Columns, QIAGEN Proteinase K, Magnetic Bead Suspension, Buffers, Bead Elution Tubes, Collection Tubes (1.5 ml and 2 ml)	55204
QIAamp MinElute ccfDNA Midi Kit (50)	For 50 preps (4 or 5 ml sample input volume each): QIAamp UCP MinElute Columns, QIAGEN Proteinase K, Magnetic Bead Suspension, Buffers, Bead Elution Tubes, and Collection Tubes (1.5 ml and 2 ml)	55284

* Other kit sizes/formats available; see www.qiagen.com.

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