September 2025

QIAseq® Targeted Methyl Handbook

Targeted next-generation sequencing (NGS) of methylated DNA

Sample to Insight

Table of Contents

Kit Contents

QIAseq Targeted Methyl Panel (including catalog and custom panels)	(8)	(96)	Custom (96)	Custom (384)
Catalog no.	**335501**	**335511**	**335602**	**333603**
No. of samples	**8**	**96**	**96**	**384**
Targeted Methyl Panel Mix	90 µL	1000 µL	1000 µL	4 x 1000 µL
TM-repair Buffer 10x	400 µL	400 µL	400 µL	4 x 400 µL
T4 Polynucleotide Kinase	12 µL	125 µL	125 µL	4 x 125 µL
Ligase Buffer 2x	1.6 mL	3 x 1.6 mL	3 x 1.6 mL	12 x 1.6 mL
DNA Ligase	75 µL	600 µL	600 µL	4 x 600 µL
GeneRead®DNAseq 5x PCR Buffer	230 µL	1.8 mL	1.8 mL	2 x 1.8 mL
UPCR Buffer 5x	60 µL	500 µL	500 µL	4 x 500 µL
dNTP Mix (10 mM each)	55 µL	120 µL	120 µL	4 x 120 µL
HotStarTaq®DNA Polymerase 6 U/µL	60 µL	480 µL	480 µL	4 x 480 µL
Nuclease-free Water	4 x 1.5 mL	50 mL	50 mL	4 x 50 mL
TM Stop Solution	250 µL	250 µL	250 µL	4 x 250 µL
QIAseq Beads	10 mL	2 x 38.4 mL	2 x 38.4 mL	6 x 38.4 µL

QIAseq Methyl DNA Panel

Catalog no.	Product name	Total number of primers	Approx. panel size (kb)
MHS-001Z	Human Breast Cancer Panel	1400	280
MHS-002Z	Human Colorectal Cancer Panel	749	149.6
MHS-201Z	Immuno-Oncology Panel	1204	240.8
MHS-202Z	Human T-cell Infiltration Panel	1415	283

QIAseq Methyl DNA 8-Index Kit	(8)
Catalog no.	335580
No. of samples	8
IL-Me-N7## adapter* contains 8 single-use tubes of molecularly indexed adapters with each tube corresponding to one sample index	10 µL
Il-S502 Index Primer*	40 µL
IL-Forward Primer	45 µL
IL-Universal Primer	45 µL
QIAseq A Read 1 Primer I (100 µM)	30 µL

* For index sequences, see “Appendix B: Adapter Bar Codes for QIAseq Targeted Methyl Panel DNA Libraries”.

QIAseq Methyl DNA 96-Index A, B, C, or D set	(96–384)
Catalog nos.	335591 335592 335593 335594
No. of samples	96–384
IL-Me-N701-N712 (A or C) or IL-Me-N716-N729 (B or D) adapter plate; each plate contains 12 molecularly indexed single use adapters with each well corresponding to one sample index	8 µL each
IL-S502-S511 (A or B set) or IL-S513-S522 (C or D set) Primer Plate with 4 index primer arrays; each array well contains one single use index primer and IL-Universal PCR primer pair for PCR amplification and sample indexing; kit can process up to 384 samples	Lyophilized
IL-Forward Primer	310 µL
QIAseq A Read 1 Primer I (100 µM)	24 µL
12-cap strips	16

Shipping and Storage

The QIAseq Methyl DNA Library Kits and QIAseq Methyl DNA 96-Index A, B, C, or D sets, with exception of the QIAseq Beads and Ligase Buffer, are shipped on dry ice. QIASeq Beads and Ligase Buffer are shipped on cooling packs. The kits, including all reagents and buffers, should be stored immediately upon receipt at –30°C to –15°C in a constant-temperature freezer. QIAseq Beads should be stored at 4–8°C upon delivery and not frozen. Ligase buffer should be stored at –30°C to –15°C in a constant-temperature freezer upon delivery. If stored under these conditions, the kits are stable until the date indicated on the kit label.

Intended Use

The QIAseq Targeted Methyl Panels and QIAseq Methyl DNA Index Kits are intended for molecular biology applications. These products are 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 Targeted Methyl Panels and QIAseq Methyl DNA Index Kits are 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 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.

QIAseq Targeted Methyl Panels enable Sample to Insight®, targeted next-generation sequencing (NGS) to interrogate DNA methylation degree. This highly optimized solution facilitates sensitive DNA methylation detection using integrated unique molecular indexes (UMIs) from cells, tissues, and biofluids. The required amount of template for a single QIAseq Targeted Methyl sequencing reaction ranges from 1 ng to 200 ng for fresh gDNA, from 10 ng to 200 ng for FFPE DNA, or from 10 ng to 200 ng for cfDNA.

QIAseq targeted methylation sequencing, which combines bisulfite-mediated conversion of unmethylated cytosines to uracil and NGS, allows detection of 5-methylcytosine residues at unprecedented single-base resolution. The introduced target enrichment technology enhances DNA methylation NGS by enabling users to sequence specific regions of interest instead of the entire genome, which effectively increases sequencing depth and sample throughput while minimizing costs. The use of UMI allows accurate methylation calling.

Clinically relevant specimens for NGS can be difficult to obtain or can yield limited amounts of nucleic acids. Therefore, it is preferred to use samples as efficiently as possible. Solutions that push the limits of input amounts in the sub-nanogram range – without sacrificing sensitivity and performance – are needed to safeguard samples. Traditional hybrid capture DNA methylation analysis methods demand higher DNA input amounts.

The QIAseq Targeted Methyl Panel Library procedure delivers a 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 step, which reduces DNA loss. After ligation of the first index adaptor with UMI, a high efficient target enrichment reaction and library amplification completes the library preparation workflow, which takes 6 hours with only 2 hours hands-on time. The total workflow, including bisulfite conversion using the EpiTect® Fast chemistry, can be completed in only one day.

The adapted EpiTect Fast Bisulfite Conversion chemistry, including the DNA protect reagent, delivers an optimal starting material for target enrichment and library generation because it allows complete conversion of unmethylated cytosines while avoiding fragmentation of the DNA during bisulfite treatment.

The QIAseq Targeted Methyl Panels are also compatible with enzymatic conversion. For guidance on DNA input and fragmentation prior to treatment, please refer to the recommendations provided by the respective vendor.

The easy-to-use online primer design tool allows primer design and validation for the specified regions of interest. A summary of the predicted quality and covered regions is provided. After library construction and sequencing on Illumina platforms, bioinformatic analysis is extremely simplified using the CLC Genomics Workbench for methylation for the CLC Genomics Workbench 12.0.2 or the online tool on GeneGlobe® (**geneglobe.qiagen.com/analyze**). Collectively, QIAseq Targeted Methyl Panels are a Sample to Insight solution for methylation analysis of targeted genomic regions using NGS (Figure 1).

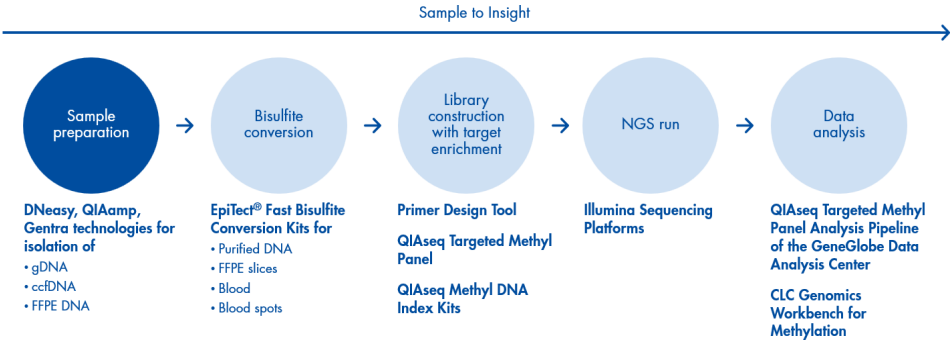


Figure 1. Targeted Methylation Sequencing Workflow.

Typical library concentrations from a QIAseq Targeted Methyl Panel Library Kit reaction are 2–25 nM depending on DNA input and cycle number during library amplification, with an average fragment size of approximately 450 bp for gDNA and FFPE samples and 350 bp for cfDNA.

Principle and procedure

QIAseq Targeted Methyl Panels are provided as single-tube primer mixes with up to 5000 primers per panel. QIAseq Targeted Methyl DNA Panels are designed to enrich selected genes and regions using 1–100 ng high-molecular–weight DNA, 10–200 ng FFPE DNA, or 10–200 ng cfDNA. Lower input amounts are possible. However, this will lead to fewer sequenced UMI and reduced unique mapping. The QIAseq Targeted Methyl Panel workflow (Figure 2) starts with bisulfite-converted DNA, which is generated with the EpiTect Fast chemistry.

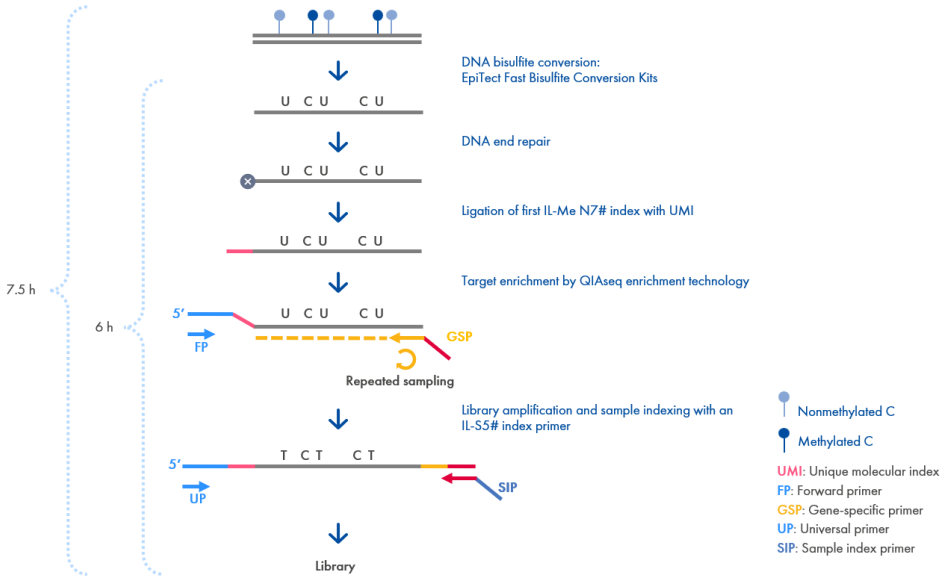


Figure 2. QIAseq Targeted Methyl Panel workflow.

The first step of the library construction uses an enzyme mix with an optimized buffer to repair and prepare the ends of the single-stranded fragments for subsequent adapter ligation. Both DNA end repair and ligation reactions occur in the same tube, minimizing sample loss. With the adapter ligation, each original DNA molecule is assigned a unique sequence or index, referred to as a UMI with a 12-base design of alternate random and cytosine bases, which reduces the risk of primer mispriming on the UMI sequences. Statistically, this process provides 4^8 possible indexes per adapter, and each DNA molecule in the sample receives a unique UMI sequence. In addition, this ligated adapter also contains the first sample index. Nonligated adapters will be removed in a purification step using QIAseq Beads, which are included in the kit. Target enrichment is performed post-UMI assignment to ensure that DNA molecules containing UMIs are sufficiently enriched in the sequenced library. For enrichment, ligated DNA molecules are subject to several cycles of targeted PCR using one region-specific primer and one universal primer complementary to the ligated adapter. A universal PCR is

ultimately carried out to amplify the library and add platform-specific adapter sequences and additional sample indexes.

Next-generation sequencing

QIAseq Targeted Methyl Panels are platform agnostic and are compatible with most medium- and high-throughput sequencers, including Illumina NGS systems (MiniSeq®, MiSeq®, NextSeq® 500/550, NextSeq 1000, NextSeq 2000, NovaSeq 6000, Novaseq X Plus) and the AVITI™ system using the Cloudbreak Freestyle™ sequencing chemistry (Element Biosciences, Inc.). QIAseq Targeted Methyl Panel libraries require a custom sequencing primer for Read 1 (QIAseq A Read1 Primer I) and 151 or 251 bp paired-end (PE) reads if starting with high-molecular–weight gDNA, and 151 bp PE reads if starting with cfDNA and FFPE-DNA.

Data analysis

The QIAseq Targeted Methyl Panel Analysis pipeline is available at **geneglobe.qiagen.com/analyze**. To enter the QIAseq Targeted Methyl analysis portal, select Next-Generation Sequencing, DNA, and QIAseq Targeted Methyl Panels. The pipeline automatically performs all steps necessary to generate a report of DNA methylation status for each targeted cytosine.

The CLC Genomics Workbench for Methylation for the CLC Genomics Workbench is also available to run the same type of analysis on CLC platforms.

Description of protocols

This handbook contains protocols for bisulfite conversion of purified gDNA, FFPE-DNA, and cfDNA using EpiTect Fast Bisulfite Conversion Kit (cat. nos. 59802, 59824, and 59864), as well as generation and amplification of libraries using the freshly bisulfite-converted DNA. The generated library can be quantified using qRT-PCR and is optimized for use on Illumina sequencing platforms.

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 DNA Bisulfite Conversion Kits (QIAGEN, cat. nos. 59802, 59824, 59720, or 59826, 59720)
- PCR tubes or plates
- LoBind® tubes for storage of generated libraries (Eppendorf, cat. nos. 0030108094, 0030108116, or 0030108132)
- pipettes and pipette tips
- Magnetic stand for magnetic bead separation (e.g., DynaMag™-2 /96 Magnet, Thermo Fisher Scientific, cat. no. 12321D)
- Thermal cycler
- Microcentrifuge
- Vortexer
- NanoDrop™ UV-Vis spectrophotometer for DNA- and bisulfite-treated DNA quantification
- Ice
- 96–100% ethanol
- QIAxpert®

- 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 QIAGEN, cat. no. 333304; Assay Kit QIAGEN, cat. no. 333314) for library quantification

When validating a new panel, it is recommended to perform experiments using fully methylated and non-methylated control DNA such as Human HCT116 DKO Methylated DNA and Human HCT116 DKO Non-Methylated DNA (Zymo Research, cat. no. D5014-2 and D5014-1, respectively).

* 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. Residual traces of proteins, salts, or other contaminants may either degrade the DNA or decrease the efficiency of, if not block completely, the enzymatic activity necessary for optimal targeted enrichment. Therefore, DNA purification procedures as well as accurate quantification and quality control are critical to the success of the experiment. Sample purity can be checked with the QIAxpert.

The QIAGEN kits listed in Table 1 are recommended for the preparation of genomic DNA samples from cells, tissues, FFPE tissues, and serum/plasma samples. For best results, all DNA samples should be resuspended in DNase-free Water and RNase-free Water or, alternatively, in DNase-free 10 mM Tris buffer pH 8.0.

DNA isolation can be automated using several QIAGEN instruments. The EZ2 Connect (cat. no. 9003210, 9003220, or 9003230) can be used to isolate gDNA, cfDNA, or FFPE DNA.

Important: Do not use DEPC-treated water.

Important: Ensure that samples are RNA-free. RNA contamination will cause inaccuracies in DNA concentration measurements. Do not omit the recommended RNase treatment step in DNA extraction kits.

Important: If genomic DNA samples must be harvested from biological samples for which kits are not available, please contact QIAGEN Technical Support representatives for suggestions.

Table 1. Recommended kits for purification of DNA and bisulfite conversion of DNA

Kit	Staring material	Cat. no.
QIAamp® DNA Mini Kit	Small amounts of cells and tissue	51304
MagAttract HMW DNA Kit	Blood, tissue, and cells	67563
QIAamp DNA FFPE Tissue Kit	Formalin-fixed, paraffin-embedded tissues	56404
QIAamp DNA FFPE Advanced Kit	QIAamp DNA FFPE Advanced Kits	56604
QIAamp Circulating Nucleic Acid Kit	Animal and human plasma and serum	55114
EZ1&2 DNA Tissue Kit (48)	Blood and tissue	953034
EZ1&2 cfDNA Kit	Plasma and serum	954854
EZ1&2 DNA FFPE Kit	Formalin-fixed, paraffin-embedded tissues	954404
EZ1&2 DNA FFPE UNG Kit (48)	Formalin-fixed, paraffin-embedded tissues + Uracil-N-glycosylase treatment	954414
EpiTect Fast DNA Bisulfite Kit	Purified DNA	59824
EpiTect LyseAll Bisulfite Kit	3 mm punched out circles from dried blood spot on QIAcard FTA, QIAcard Bloodstain, or QIAcard FTA Elute	59104

DNA quantification

The concentration and purity should be determined by measuring the absorbance in a spectrophotometer, such as a QIAxpert. As the spectral properties of nucleic acids are highly dependent on pH, we recommend preparing dilutions and measure absorbance in 10 mM Tris·Cl, pH 8.0. The A_{260}/A_{280} ratio should be >1.8.

DNA integrity

DNA integrity can be checked using the QIAxcel Connect or Agilent TapeStation® instruments. Although bisulfite conversion includes DNA Protect, a buffer to protect DNA from degradation, DNA breakage to a certain degree will occur during this treatment and before target enrichment PCR. High-molecular–weight DNA generally yields better results than fragmented DNA. Intact DNA usually has better coverage uniformity, because it will have less damage over bisulfite conversion. In this way, more UMIs will be captured and longer reads will be obtained, which will increase mapping efficiency, the coverage of the targeted regions, and methylation calling will therefore be more sensitive. While working with FFPE samples, which are strongly fragmented with a DIN number lower than 6, it will be required to adjust input in order to increase coverage.

Important: During bisulfite conversion, it is expected to lose approximately 50% of the DNA input. This should be considered during experimental planning.

DNA input amount

The number of UMIs captured from the original DNA sample correlates with the DNA input amount and sequencing depth. Adequate sequencing of captured UMIs requires relatively deep sequencing coverage. Table 2 provides general information about the estimated average number of captured UMIs at each CpG position with 40 ng and 100 ng fresh DNA at the given sequencing depth. The sensitivity in detection of methylation degree differences is strongly dependent on the level of methylation itself. Methylation degree differences in higher methylation levels can be performed accurately with less captured molecules, while lower methylation degree needs deeper sequencing for accurate detection of smaller differences. For accurate methylation calls of unknown methylation status, a coverage of at least 100 UMI reads per CpG is recommended.

Table 2. Expected UMI and read number, based on fresh DNA of DIN 7–8 input in the complete workflow, including bisulfite conversion

	Fresh gDNA – 3 read pairs/UMI			
	Mean UMI per primer	**Mean read per primer**	**Mean UMI per CpG**	**Mean read per CpG**
40 ng	185	607	107	152
100 ng	343	864	169	210

Note: Approximately 50% of DNA may be lost during bisulfite conversion.

Sequencing capacity and sample multiplex level

Sample multiplexing level is determined by the size of the panel, required depth of coverage, and sequencing platform read capacity. For the Illumina platforms, sample indexes are available to multiplex up to 384 samples per run. In Table 3, general guidelines are provided for the number of samples that can be multiplexed in different sequencing platforms, based on panel size and read depth. Fine-tuning the read depth is possible after the first run. QIAseq Targeted Methyl Panel libraries require a custom sequencing primer for Read 1 (QIAseq A Read1 Primer I) and 151 bp paired-end reads. If the sample integrity allows the generation of longer fragments, 250 bp paired-end reads will increase the mapping efficiency.

Table 3. Number of multiplexed samples based on panel size with 500x mean coverage*

			Number of primers in the panel				
Instrument	**Version**	**Capacity paired-end reads (millions)**	**100**	**1000**	**2000**	**3000**	**5000**
MiniSeq	Mid output	16	80	8	4	3	2
MiniSeq	High output	50	250	25	13	9	5
MiSeq	V2	30	150	15	8	6	3
NextSeq 500/550	Mid output	260	1300	131	70	40	25
NextSeq 500/550	High output	800	4000	400	200	130	77
NextSeq 1000/2000	P1 flow cell	200	1000	100	55	35	23
NextSeq 1000/2000	P2 flow cells	800	4000	400	230	150	90
NextSeq 1000/2000	P3 flow cells	2400	12000	1200	600	400	250
NovaSeq 6000	SP (2 lanes per flow cell)	1600	8000	800	400	250	150
NovaSeq 6000	S1 (2 lanes per flow cell)	3200	16000	1600	800	530	320
AVITI	AVITI 2x150 Sequencing Kit Cloudbreak FS Low Output	250	1300	131	70	40	25
AVITI	AVITI 2x150 Sequencing Kit Cloudbreak FS Medium Output	500	2600	260	140	80	50
AVITI	AVITI 2x150 Sequencing Kit Cloudbreak FS High Output	1000	6000	520	280	160	100

* Based on 2 x 151 bp paired-end read on Illumina platform.

General precautions

- Use good laboratory practices to minimize cross-contamination of nucleic acid products.
- Always use PCR tubes, microcentrifuge tubes, and pipette tips that are certified sterile and free of DNase and RNase.
- Before starting, wipe down the work area and pipettes with an RNase and DNA cleaning product such as RNase Away® (Sigma-Aldrich) or LookOut® DNA Erase (Sigma-Aldrich).
- For consistent bisulfite conversion and library construction and amplification, ensure that the thermal cycler used in the protocol is in good working order and has been calibrated to the manufacturer's specifications.
- Read the entire protocol before beginning. Take note of the stopping points where samples can be frozen at –30°C to –15°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.
- **Important**: When working with plates, we recommend mixing by pipetting samples up and down and changing tips to avoid cross-contamination. Increase the number of pipetting rounds when mixing magnetic beads to ensure complete resuspension. In the purification steps, including mixing of QIAseq Beads with the samples, ensure that the beads are resuspended homogeneously without any visual clumps.
- **Important**: Equilibrate QIAseq Beads at room temperature prior to use.

Recommended library quantification method

QIAGEN's QIAseq Library Quant System (Array Kit, cat. no. 333304, and 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, QIAxcel Connect System or Agilent Bioanalyzer may be also used for quantification and qualification of the amplified libraries. Well-defined libraries without adapter dimers or signs of overamplification can also be quantified using Qubit, with concentrations typically aligning with electropherogram-based estimates.

Protocol: Bisulfite Conversion of Unmethylated Cytosines in DNA using EpiTect Fast DNA Kits (Spin Column)

This protocol enables bisulfite conversion of DNA in a volume of up to 20 µL (high concentration protocol).

The QIAseq Targeted Methyl Panel Library was optimized for 1–200 ng gDNA,10–200 ng FFPE DNA, and 10–200 ng cfDNA as starting material in the complete workflow including bisulfite conversion. If starting with this input DNA total eluates from EpiTect Fast bisulfite conversion can be directly used in the subsequent library preparation without further quality control steps.

Important points before starting

- Read carefully the *EpiTect Fast Bisulfite Conversion Handbook* for general recommendations in handling of reagents and background information on the conversion chemistry.
- 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.
- Perform all centrifugation steps at room temperature.
- During bisulfite treatment, it is estimated that approximately 30–60% DNA will be degraded and lost. DNA input in bisulfite conversion higher than 40 ng will increase mapping rate and region coverage. Use RNA carrier in the EpiTect Fast procedure if using gDNA less than 10 ng and FFPE-DNA less than 40 ng.

Things to do before starting

- Prepare the kit reagents as described in "Preparation of reagents", page 19 of the *EpiTect Fast Bisulfite Conversion Handbook (06/2019)*.
    - BW and BD buffer must be complemented with ethanol (96–100%).
    - Depending on the kit size, add 30 mL ethanol to 13 mL BW concentrate or 120 mL ethanol to 52 mL BW concentrate.
    - Add 27 mL ethanol (96–100%) to Buffer BD and store at 2–8°C. Invert the bottle several times before starting the procedure and make sure to close the bottle immediately after use. White precipitates may form in the Buffer BD–ethanol mix after some storage time. These precipitates will not affect the performance of Buffer BD. However, avoid transferring precipitates to the MinElute® DNA spin column.
- If required, prepare Carrier RNA as outlined in "Preparation of reagents", page 19 of the *EpiTect Fast Bisulfite Conversion Handbook (06/2019)*, by resuspending the lyophilized Carrier RNA in 310 µL RNase-free water. This amount will be sufficient for 50 samples and can be added to BL buffer with ratio 1:100 v/v. For example, when processing 10 samples, mix 35 µL of reconstituted Carrier RNA with 3.5 mL of Buffer BL. This yields a final Carrier RNA concentration of 10 µg/mL in Buffer BL and already includes a 10% surplus.
- Equilibrate samples and buffers to room temperature.

  Optional: Set a thermomixer, heating block, or heated orbital incubator to 60°C to dissolve the Bisulfite Solution.

Procedure

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 completely 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 4. Add each component in the order listed.

   Note: If the sample is very low concentrated and the volume is not limited, 2 bisulfite reactions per sample can be set up and combined into one column during desulfonation and cleanup step (step 10 on page 27).

Table 4. Bisulfite reaction components

Component	Volume (µL) per reaction
Nuclease-free Water	Variable*
Bisulfite Solution	85
DNA Protect Buffer	35
Total reaction volume	**140**

* The combined volume of DNA and Nuclease-free Water must total 20 µL to allow maximum DNA Protect Buffer in the reaction.

3. Close the PCR tubes, mix the bisulfite reactions thoroughly by pulse vortexing, and spin down. Store the tubes at room temperature.

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. Program the thermal cycler according to Table 5 below. 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 5. Bisulfite conversion thermal cycling conditions

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

* For samples that consistently show incomplete conversion, extending the duration of 60°C incubation step to up to 20 min may be required to ensure complete bisulfite conversion of DNA.

5. Place the PCR tubes containing the bisulfite reactions into the thermal cycler. Start the thermal cycling incubation.

   Important: Because the bisulfite reaction is not overlaid with mineral oil, only thermal cyclers with heated lids are suitable for this procedure. It is important to use PCR tubes that close tightly.

Cleanup of bisulfite-converted DNA

6. 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.

7. Add 310 µL freshly prepared Buffer BL containing 10 µg/mL Carrier RNA (see "Preparation of reagents", page 16 in the *EpiTect Fast Bisulfite Conversion Handbook, 06/2019*) to each sample. Mix the solutions by vortexing and then centrifuge briefly.

   Note: Carrier RNA is not necessary when using >10 ng of gDNA. If using strongly fragmented DNA, FFPE-DNA, or cfDNA, adding Carrier RNA to experiments with input less than 40 ng will decrease DNA loss and increase coverage.

8. 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.

9. 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 8 into the corresponding MinElute DNA spin column.

10. Depending on the sample starting concentration, proceed to step 10a or 10b and 10c.

    a. Centrifuge the spin columns at maximum speed for 1 min. Discard the flow-through and place the spin columns back into the collection tubes. Proceed with step 11.

    b. When working with low concentrated samples and 2 bisulfite reactions per sample have been set up, load the second reaction into the MinElute DNA spin column used for the first bisulfite reaction from the identical sample.

       Note: Make sure that the columns are tracked with sample numbers to avoid any cross contamination of samples during reloading of the column.

    c. Centrifuge the spin columns 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 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.

12. Add 500 µL Buffer BD (desulfonation buffer) to each spin column and incubate for 15 min at room temperature.

    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.

    Note: It is important to close the lids of the spin columns before incubation.

13. Centrifuge the spin columns at maximum speed for 1 min. Discard the flow-through and place the spin columns back into the collection tubes.

14. 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.

15. Repeat step 14 once.

16. Add 250 µL ethanol (96–100%) to each spin column and centrifuge at maximum speed for 1 min.

17. 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.

18. 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.

    Note: If heating block is not available, repeat the spin columns step using a fresh collection tube to avoid any residual ethanol carryover.

19. Place the spin columns into clean 1.5 mL microcentrifuge tubes (not provided) and add 22 µL Nuclease-free Water directly onto the center of each spin-column membrane and close the lids gently.

20. Incubate the spin columns at room temperature for 1 min.
21. Centrifuge for 1 min at 15,000 × *g* (12,000 rpm) to elute the DNA.

    Note: Bisulfite-treated DNA can be stored at –15°C to –35°C. Do not exceed storage of bisulfite-treated DNA for 2 weeks.

Protocol: Library Generation from Bisulfite-Converted gDNA and FFPE DNA

This procedure describes the NGS library preparation starting from bisulfite-converted, single-stranded DNA. It includes end repair of the bisulfite-converted DNA, adapter ligation and introduction of UMIs, target enrichment with the QIAseq Methyl Panel, library amplification with introduction of the second index, and library cleanup steps using magnetic beads.

Important points before starting

- This protocol is optimized for bisulfite-converted DNA using Spin Column-based EpiTect Fast Bisulfite Conversion Kits but can be used with bisulfite conversion methods that generate single-stranded DNA with fragment mean size between 200 and 2000 bp.
- If starting with a low input level, note that the quantification of single-stranded DNA using photometric methods after bisulfite conversion will not be accurate.
- Storage of low concentrated bisulfite-treated DNA for longer than 2 weeks is not recommended prior to targeted methylation enrichment.
- Avoid DNA contamination of reagents by using separate laboratory equipment (e.g., pipettes, filter pipette tips, reaction vials, etc.).
- Set up all reactions on ice.

Things to do before starting

- Prepare fresh 70% ethanol.
- Enzymes and enzyme mixes should be thawed on ice and placed on ice until use. Avoid extended vortexing of the enzymes and enzyme mixes.

- All other components can be thawed at room temperature (15–25°C) but should be placed on ice immediately after thawing. Equilibrate Ligase Buffer 2x at room temperature.
- Equilibrate QIAseq Beads at room temperature and mix well to resuspend before use.
- All buffers and reagents should be vortexed before use to ensure thorough mixing and spun down to collect all liquids on the bottom of the tubes.
- Program the thermal cycler. To avoid prolonged incubation of reaction mixes on ice and for increased speed and convenience, all incubation steps of the protocol can be preprogrammed and saved on a thermal cycler in advance.

Procedure

End repair of bisulfite-converted DNA

1. Thaw bisulfite-converted DNA form previous step (21) and use the total volume of 20 µL for the End repair reaction.
2. Setup the bisulfite-converted DNA repair reaction mix on ice according to Table 6. Mix by pulse vortexing (3–4 times) and spin down. Keep the reaction on ice.

Table 6. Bisulfite-converted DNA repair reaction mix setup

Component	Volume (µL) per reaction
TM-repair buffer, 10x	3
Nuclease-free Water	6
T4 Polynucleotide Kinase	1
Bisulfite-converted DNA	20
Total reaction volume	**30**

3. Program a thermal cycler with the protocols described in Table 7.

Important: Use a thermocycler with heated lid at 75°C. If not possible, run incubation at 37°C with open lid and close the heated lid for incubation at 65°C.

Table 7. Bisulfite-converted DNA repair cycling conditions

Step	Temperature (°C)	Incubation time (min)
1	37	30
2	65	20
3	4	Hold

4. Transfer the reaction mix from step 2 to the thermocycler and start the bisulfite-converted DNA repair cycling program (Table 7). Place the samples on ice after cycling completion.

Adapter ligation

5. During bisulfite-converted DNA repair cycling, prepare the ligation mix according to Table 8. Mix thoroughly by pulse vortexing and spin down.

   Note: Ligase buffer 2x should be equilibrated at room temperature. The ligase buffer has a high density. Avoid carryover of big droplets with the pipette tip and pipette carefully into the tube ensuring the accurate amount of buffer.

Table 8. Ligation mix

Component	Volume (µL) per reaction
Ligase Buffer, 2x	45
Nuclease-free Water	6
DNA Ligase	4
Total reaction volume	**55**

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

6. Add 55 µL ligation master mix to each 30 µL end-repaired DNA sample from the previous step and mix by pulse vortexing and spin down.

7. Add 5 µL of IL-Me-N7## adapter to the ligation mixes from the previous step and track the used adapters.

   Important: Only one single-indexed adapter should be used per ligation reaction. Open one adapter tube at a time if using 8-index adapters and avoid cross-contamination.

   For 96-index adapters supplied in a plate (layout shown in Figure 3), use a multichannel pipette to add the appropriate amount of adapters and change the pipette tips after each pipetting step to avoid cross-contamination. Spin down the plate to collect liquid on the bottoms of wells before pipetting.

   Note: The foil covering the 96-index adapters is pierceable with a pipette tip.

IL-Me-N701-N715 Adapter Plate in QIAseq 96-Index I Set A or Set C

	1	2	3	4	5	6	7	8	9	10	11	12
A	N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715
B	N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715
C	N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715
D	N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715
E	N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715
F	N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715
G	N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715
H	N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715

IL-Me-N701-N715 Adapter Plate in QIAseq 96-Index I Set B or Set D

	1	2	3	4	5	6	7	8	9	10	11	12
A	N716	N718	N719	N720	N721	N722	N723	N724	N726	N727	N728	N729
B	N716	N718	N719	N720	N721	N722	N723	N724	N726	N727	N728	N729
C	N716	N718	N719	N720	N721	N722	N723	N724	N726	N727	N728	N729
D	N716	N718	N719	N720	N721	N722	N723	N724	N726	N727	N728	N729
E	N716	N718	N719	N720	N721	N722	N723	N724	N726	N727	N728	N729
F	N716	N718	N719	N720	N721	N722	N723	N724	N726	N727	N728	N729
G	N716	N718	N719	N720	N721	N722	N723	N724	N726	N727	N728	N729
H	N716	N718	N719	N720	N721	N722	N723	N724	N726	N727	N728	N729

Figure 3. Layout of sample adapters in QIAseq 96-Index I Set A, B, C, or D. A through H of each plate have adapters. Each well in each row contains one single-use adapter.

8. After adding the adapters, mix by short vortexing, spin down, and place the samples on ice.

9. Program a thermal cycler with the protocol described in Table 9.

   Important: Use a thermal cycler with the heated lid off. Ensure that lid is cooled down to room temperature.

Table 9. Ligation cycling conditions

Step	Temperature (°C)	Incubation time
1	25	15 min
2	4	Hold

10. Place ligation mixes from step 8 in the thermal cycler and run the ligation cycling program (Table 9).
11. After cycling is complete, proceed directly with cleanup of the ligated fragments.

Cleanup of ligated fragments

12. For sample purification, mix 90 µL (1x) QIAseq Beads with each sample by pulse vortexing. Ensure that the beads are equilibrated at room temperature and resuspended homogeneously without any visual clumps.
13. Incubate for 5 min at room temperature. Pulse spin the tube to collect all liquid on the bottom, immobilize beads on a magnet for approximately 5 min, and discard the clear supernatant.

    Note: Time for immobilization depends on the type of magnet and on the tubes that are being used. Ensure that the supernatant is clear by visual inspection.
14. Add 200 µL fresh 70% ethanol to each bead pellet immobilized on the magnet. Do not disturb the pellet.
15. Discard the supernatant. Carefully remove all remaining ethanol droplets from the tube inner walls.

16. Incubate on the magnetic stand for 5–10 min until the beads are dry. Over-drying may result in lower DNA recovery. Remove from the magnetic stand.

17. Elute by carefully resuspending in 55 µL Nuclease-free Water. Incubate for 5 min at room temperature. Immobilize beads and transfer 52 µL supernatant to a new tube.

18. Mix 52 µL (1x) QIAseq Beads with each sample by pulse-vortexing and repeat steps 13–16.

    Note: Visually inspect that the pellet is completely dry to avoid ethanol carry-over and inhibition in the next reaction.

19. Elute by resuspending beads in 20 µL Nuclease-free Water. Incubate for 5 min at room temperature. Immobilize the beads and transfer 17 µL of supernatant into a new LoBind tube. Avoid any magnetic bead carry over with the ligated DNA fragments. Ligated DNA may be stored overnight at –15°C to –30°C if needed.

Target enrichment

20. Thaw DNA from step 19 if stored at –15°C to –30°C and amplification reagents on ice. Mix all reagents gently, spin down, and place on ice.

21. Prepare a reaction mix according to Table 10. Add each component in the order listed in this table.

Table 10. Target enrichment reaction mix

Component	Volume (µL) per reaction
Nuclease-free Water	1.6
GeneRead DNAseq Panel 5x PCR Buffer	8
dNTP Mix (10 mM)	0.8
QIAseq Targeted Methyl Panel	8
IL Forward Primer	1.6
HotStarTaq DNA Polymerase	3
Total	**23**

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

22. Carefully mix 17 µL of ligated and purified DNA from step 19 with 23 µL target enrichment reaction mix, spin down, and place on ice.

23. Program a thermal cycler with the protocols described in Table 11.

    Important: Set the thermal cycler heated lid on.

Table 11. Target enrichment cycling conditions

		Incubation time	
Step	**Temperature (°C)**	**<2000 primer/tube**	**>2000 primer/tube**
1	95	13 min	13 min
2	98	2 min	2 min
3 (8 cycles)	98	15 s	15 s
	65	10 min	15 min
4	72	5 min	5 min
5	4	Hold	Hold

24. Place the PCR tubes in the thermal cycler and start the preprogrammed target enrichment cycling with the conditions outlined in Table 11.
25. After cycling is complete, **quickly** transfer samples on ice.
26. Add 2 µL of ice-cold TM Stop Solution to the 40 µL sample mix and immediately place samples back on ice.

    Note: After addition of TM Stop Solution, DNA can be stored overnight at –15°C to –30°C, if needed.

Cleanup of the target enrichment reaction

27. For sample purification, carefully mix 42 µL (1x) QIAseq Beads with each sample by pulse-vortexing. Ensure that the beads are equilibrated at room temperature and resuspended heterogeneously without any visual clumps.
28. Incubate for 5 min at room temperature. Shortly spin down and collect all liquid on the tube bottom and immobilize beads on a magnet for approximately 5 min and discard the clear supernatant.

    Note: Time for immobilization depends on the type of magnet. Ensure that supernatant is clear by visual control.
29. Add 200 µL fresh 70% ethanol to each bead pellet immobilized on the magnet.
30. Discard the supernatant. Carefully remove all remaining ethanol droplets from the tube inner walls.
31. Incubate on the magnetic stand for 3–7 min until the beads are dry. Over-drying may result in lower DNA recovery, so visual control is strongly recommended. Remove from the magnetic stand.
32. Elute by resuspending in 55 µL Nuclease-free Water. Incubate for 5 min at room temperature. Immobilize the beads and transfer 52 µL supernatant to a new tube.

33. Mix 52 µL (1x) QIAseq Beads with each sample by pulse-vortexing and repeat steps 28–31.

    Note: The beads need to be completely dried to avoid ethanol carryover and inhibition in the next reaction. Remove any ethanol droplets with a pipette.

34. Elute by resuspending beads in 20 µL Nuclease-free Water. Incubate for 5 min at room temperature. Immobilize the beads and transfer 17 µL of supernatant into a new tube. Avoid any magnetic bead carry-over. Store at –15°C to –30°C.

Library amplification

This step will amplify the library and add the second index.

For library amplification use the number of cycles indicated in Table 12.

Table 12. Typical required cycles for library amplification

	50–150 primer per pool		151–2000 primer per pool		2001–5000 primer per pool	
Input (ng)	**gDNA**	**FFPE-DNA**	**gDNA**	**FFPE-DNA**	**gDNA**	**FFPE-DNA**
1	26	28	25	27	24	26
10	23	26	22	25	21	24
50	21	24	20	23	19	22
100	19	21	18	20	17	19

Note: For strongly fragmented FPPE samples (DIN < 6) 2 cycles can be added to the number of cycles recommended in Table 12 to increase yields.

35. Thaw the DNA (from step 34 above) and amplification reagents on ice.

36. Prepare the Universal PCR (UPCR) reaction mix:

    a. If working with QIAseq Methyl DNA 8-Index Kit, prepare the UPCR reaction mix by adding the components in the order according to Table 13.

b. If using the QIAseq Methyl DNA 96-Index I Set A, B, C, or D, prepare the mix as described in Table 14. Track the number of the used indexes.

Table 13. Universal PCR mix if using QIAseq Methyl DNA 8-Index I

Component	Volume (µL) per reaction
DNA of step 35	13.4
QIAseq UPCR Buffer 5x	4
IL Universal primer	0.8
IL-S502 Index Primer	0.8
HotStarTaq DNA Polymerase	1
Total	**20**

Table 14. Universal PCR mix if using QIAseq Methyl DNA 96-Index I Set A, B, C, or D*

Component	Volume (µL) per reaction
Nuclease-free Water	1.6
QIAseq UPCR Buffer 5x	4
HotStarTaq DNA Polymerase	1
Total	6.6

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

* Applies to QIAseq IL-S5 Index Primer Plate in Set A, B, C, or D. The final library dual sample index is determined by the combination of the IL-N7 Adapter Plate and the QIAseq IL-S5 Index Primer Plate. Total sample index level can be up to 384-plex if using QIAseq 96-index Set A, B, C, and D together.

37. Mix the UPCR reactions as follows.

a. Mix the reaction from step 36a by pulse vortexing, spin down, and place on ice.

Note: Total volume of reaction is 20 µL.

b. If working with QIAseq Methyl DNA 96-index I Set A, B, C, or D, add 13.4 µL of the DNA from step 35 to one well of the QIAseq IL-S5 Index Primer Plate in Set A, B, C, or D, as illustrated in Figure 4.

c. Add 6.6 µL of the universal PCR mix prepared according to Table 14 to each well of the adapter plate already including the DNA. Seal the plate, mix, spin down and place on ice.

Note: Total volume of reaction is 20 µL.

IL-S502-S511 Index Primer Plate in QIAseq 96-Index I Set A or Set C

	1	2	3	4	5	6	7	8	9	10	11	12
A	S502	S502	S502	S502	S502	S502	S502	S502	S502	S502	S502	S502
B	S503	S503	S503	S503	S503	S503	S503	S503	S503	S503	S503	S503
C	S505	S505	S505	S505	S505	S505	S505	S505	S505	S505	S505	S505
D	S506	S506	S506	S506	S506	S506	S506	S506	S506	S506	S506	S506
E	S507	S507	S507	S507	S507	S507	S507	S507	S507	S507	S507	S507
F	S508	S508	S508	S508	S508	S508	S508	S508	S508	S508	S508	S508
G	S510	S510	S510	S510	S510	S510	S510	S510	S510	S510	S510	S510
H	S511	S511	S511	S511	S511	S511	S511	S511	S511	S511	S511	S511

IL-S513-S522 Index Primer Plate in QIAseq 96-Index I Set B or Set D

	1	2	3	4	5	6	7	8	9	10	11	12
A	S513	S513	S513	S513	S513	S513	S513	S513	S513	S513	S513	S513
B	S515	S515	S515	S515	S515	S515	S515	S515	S515	S515	S515	S515
C	S516	S516	S516	S516	S516	S516	S516	S516	S516	S516	S516	S516
D	S517	S517	S517	S517	S517	S517	S517	S517	S517	S517	S517	S517
E	S518	S518	S518	S518	S518	S518	S518	S518	S518	S518	S518	S518
F	S520	S520	S520	S520	S520	S520	S520	S520	S520	S520	S520	S520
G	S521	S521	S521	S521	S521	S521	S521	S521	S521	S521	S521	S521
H	S522	S522	S522	S522	S522	S522	S522	S522	S522	S522	S522	S522

Figure 4. Layout of IL-S5 Index Primer Plate in QIAseq 96-Index I Set A, B, C, or D. Each well contains one predispensed dried sample index primer and universal primer pair for a single reaction. In Universal PCR step, IL-Me-N7 Adapter Plate in Set A, B, C, or D used in ligation must be paired with IL-S5 Index Primer Plate in Set A, B, C, or D, respectively.

38. Place the tubes or plates with the reaction mixes from step 37a or 37c in the cycler and start the cycling program as outlined in Table 15.

Table 15. Library amplification cycling conditions (UPCR)

Step	Temperature (°C)	Incubation time
1	95	13 min
2	98	2 min
3 (Y-cycles*)	98 62	15 s 2 min
4	72	5 min
5	4	Hold

* Y-cycles: cycle number extracted from Table 12.

39. After cycling completion, proceed with library purification. Alternatively, the amplified library can be stored at –30°C to –15°C.

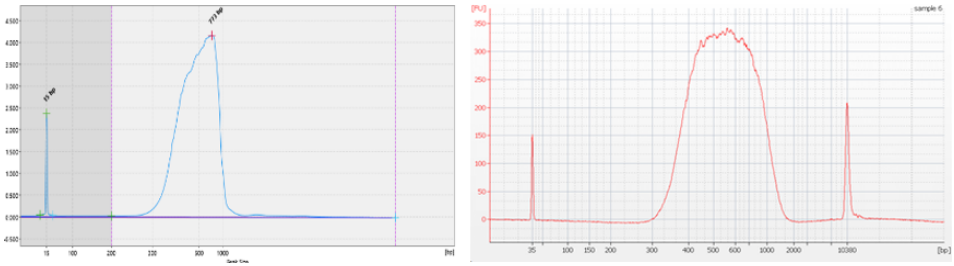
Cleanup of amplified library

40. Add 80 µL of ice-cold Nuclease-free Water to the 20 µL sample from step 39 and mix.
41. Add 100 µL (1x) QIAseq Beads to each sample and mix thoroughly by pulse vortexing. Ensure that the beads are equilibrated at room temperature and resuspended homogeneously without any visual clumps.
42. Incubate for 5 min at room temperature. Immobilize the beads on a magnet and discard the clear supernatant.
43. Add 200 µL fresh 70% ethanol to each bead pellet immobilized on the magnet.
44. Discard the supernatant. Carefully remove all remaining ethanol droplets from the tube inner walls.
45. Incubate on the magnetic stand for 5–10 min until the beads are dry. Over-drying may result in lower DNA recovery. Remove from the magnetic stand.

46. Elute by carefully resuspending in 25 µL Nuclease-free Water. Incubate for 5 min at room temperature. Immobilize the beads on a magnet and transfer 20 µL supernatant to a new LoBind tube.

    The libraries can be stored at –30°C to –15°C prior to quantification using the QIAseq Library Quant System.

    Assess the quality of the library using a capillary electrophoresis device or comparable method. Check for the correct size distribution (Figure 5) of library fragments. The majority of the library fragments are between 300 and 1000 bp. The median fragment size can be used for subsequent qPCR-based quantification methods.



A **B**

Figure 5. Capillary electrophoresis device trace of generated libraries. Capillary electrophoresis device trace data showing the correct size distribution of library fragments. **[A]** Shown is a library starting from 40 ng bisulfite-converted DNA using the QIAseq Targeted Methyl T Cell Infiltration Panel (MHS-202Z). The library was diluted 1:10 and loaded on a QIAxcel High Sensitivity Cartridge (with DMSO). **[B]** Shown is the same library as in **A**. The library was diluted 1:5 and loaded on an Agilent High Sensitivity DNA chip.

Protocol: Library Generation from Bisulfite-Converted cfDNA

This procedure describes the NGS library preparation starting from bisulfite-converted, single-stranded cfDNA, and includes end repair of the bisulfite converted DNA fragments, adapter ligation and introduction of UMIs, target enrichment with the QIAseq Methyl Panel, library amplification with introduction of the second index, and library cleanup steps using magnetic beads.

Important points before starting

- This protocol is optimized for bisulfite-converted cfDNA using spin column-based EpiTect Fast Bisulfite Conversion Kits, but can be used with bisulfite conversion methods or enzymatic conversion methods that generate single-stranded DNA with fragment mean size of approximately 170 to 300 bp.
- The fragment size of cfDNA isolated from plasma will vary depending on sample stabilization and purification method. Ensure to use cfDNA free from gDNA contamination because this will alter the evaluated methylation profile.
- cfDNA isolated from urine samples will show a higher variable fragment size profile than cfDNA isolated from plasma. Be sure to understand the origin of the nucleic acid fragments and standardize the purification method to evaluate samples with similar profile excluding variable gDNA content.
- Samples of 10–200 ng cfDNA can be used in the targeted methylation protocol as input in complete workflow starting with EpiTect Fast bisulfite conversion. The total eluate of 20 µL can be used in the library generation protocol.

  Note: During bisulfite treatment, it is estimated that approximately 30–60% DNA will be degraded and lost. DNA input in bisulfite conversion higher than 40 ng will increase

region coverage. Use RNA carrier in the EpiTect Fast procedure if using less than 40 ng cfDNA.

- Avoid DNA contamination of reagents by using separate laboratory equipment (e.g., pipettes, filter pipette tips, reaction vials, etc.).
- Set up all reactions on ice.

Things to do before starting

- Prepare fresh 70% ethanol.
- Enzymes and enzyme mixes should be thawed on ice and placed on ice until use.
- All other components can be thawed at room temperature but should be placed on ice immediately after thawing. Equilibrate Ligase Buffer 2x at room temperature.
- Equilibrate QIAseq beads at room temperature and mix well to resuspend.
- All buffers and reagents should be vortexed before use to ensure thorough mixing and should be spun down to collect all liquids on the bottom of the tubes.
- All enzyme mixes should be placed on ice until use. Avoid extended vortexing of the enzymes and enzyme mixes.
- 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

End repair of bisulfite-converted cfDNA

1. Thaw the bisulfite-converted cfDNA from step 20 on page 29 ("Protocol: Bisulfite Conversion of Unmethylated Cytosines in DNA using EpiTect Fast DNA Kits") and use the

total volume of 20 µL for the end repair reaction.

2. Set up the bisulfite-converted cfDNA repair reaction mix on ice according to Table 16. Mix by pulse vortexing (3–4 times) and spin down. Keep reaction on ice.

Table 16. Bisulfite-converted cfDNA repair reaction mix setup

Component	Volume (µL) per reaction
TM-repair buffer, 10x	3
Nuclease-free Water	6
T4 Polynucleotide Kinase	1
Bisulfite-converted cfDNA	20
Total reaction volume	**30**

3. Program a thermal cycler with the protocol described in Table 17.

   Important: Use a thermocycler with heated lid at 75°C. If not possible, run incubation at 37°C with open lid, and then close the heated lid for incubation at 65°C.

Table 17. Bisulfite-converted cfDNA repair cycling conditions

Step	Temperature (°C)	Incubation time (min)
1	37	30
2	65	20
3	4	Hold

4. Transfer the reaction mix from step 2 to the thermal cycler and start the bisulfite-converted DNA repair cycling program (Table 17). Place samples on ice after cycling completion.

Adapter ligation

5. During bisulfite-converted cfDNA repair cycling, prepare the ligation mix according to Table 18. Mix thoroughly by pulse vortexing and spin down.

   Note: Ligase buffer 2x should be equilibrated at room temperature. The ligase buffer has a high density. Mix well by pulse vortexing before pipetting. Avoid carryover of big droplets with the pipette tip and pipette carefully into the tube ensuring the accurate amount of buffer.

Table 18. Ligation mix

Component	Volume (µL) per reaction
Ligase Buffer, 2x	45
Nuclease-free Water	6
DNA Ligase	4
Total reaction volume	**55**

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

6. Add 55 µL ligation master mix to each 30 µL end-repaired cfDNA sample from the previous step and mix by pulse vortexing and then spin down.

7. Add 5 µL of IL-Me-N7## adapter to the ligation mixes from the previous step and track the used adapters.

   Important: Only one single-indexed adapter should be used per ligation reaction. Open one adapter tube at a time if using 8-index adapters and avoid cross-contamination.

   For 96-index adapters supplied in a plate (layout described in Figure 3), use a multichannel pipette to transfer the appropriate amount of adapters. Spin down the plate to collect liquid on the bottom of the wells before pipetting.

   Note: The foil covering the 96-index adapters is pierceable with a pipette tip.

8. After adding the adapters, mix by short vortexing, spin down, and place samples on ice.
9. Program a thermal cycler with the protocol described in Table 19.

   Important: Use a thermal cycler with the heated lid off. Ensure that the lid is cooled down to room temperature.

Table 19. Ligation cycling conditions

Step	Temperature (°C)	Incubation time
1	25	15 min
2	4	Hold

10. Place the ligation mixes from step 8 in the thermal cycler and run the ligation cycling program (Table 19).
11. After cycling is complete, proceed directly with cleanup of the ligated fragments.

Cleanup of ligated fragments

12. For sample purification, mix 108 µL (1.2x) QIAseq Beads with each sample by pulse vortexing. Ensure that the beads are equilibrated at room temperature and are completely resuspended without any visual clumps.
13. Incubate for 5 min at room temperature. Shortly spin down and collect all liquid on the tube bottom and immobilize the beads on a magnet for approximately 5 min and discard the clear supernatant.

    Note: The time for immobilization depends on the type of magnet. Ensure that supernatant is clear by visual inspection.
14. Add 200 µL fresh 70% ethanol to each bead pellet immobilized on the magnet.
15. Discard the supernatant. Carefully remove all remaining ethanol droplets from the tube inner walls. Do not disturb the immobilized magnetic bead pellet.

16. Incubate on the magnetic stand for 5–10 min until the beads are dry. Over-drying may result in lower DNA recovery. Remove from the magnetic stand.
17. Elute by carefully resuspending in 55 µL Nuclease-free Water. Incubate for 5 min at room temperature. Immobilize the beads and transfer 52 µL supernatant to a new tube.
18. Mix 62.4 µL (1.2x) QIAseq Beads with each sample by pulse vortexing and repeat steps 13–16.

    Note: Visually inspect that the pellet is completely dry to avoid ethanol carryover and inhibition in the next reaction.
19. Elute by resuspending beads in 20 µL Nuclease-free Water. Incubate for 5 min at room temperature. Immobilize the beads and transfer 17 µL of supernatant into a new LoBind tube. Avoid any magnetic beads carry-over with the ligated DNA fragments. Ligated DNA may be stored overnight at –15°C to –30°C if needed.

Target enrichment

20. Thaw the DNA from step 19 if stored at –15°C to –30°C and the amplification reagents on ice. Mix all reagents gently, spin down, and place on ice.
21. Prepare a reaction mix according to Table 20. Add each component in the order listed in this table.

Table 20. Target enrichment reaction mix

Component	Volume (µL) per reaction
Nuclease-free Water	1.6
GeneRead DNAseq Panel 5x PCR Buffer	8
dNTP Mix (10 mM)	0.8
QIAseq Targeted Methyl Panel	8
IL Forward Primer	1.6
HotStarTaq DNA Polymerase	3
Total	**23**

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

22. Carefully mix 17 µL of ligated and purified DNA from step 21 on the previous page with 23 µL target enrichment reaction mix, spin down, and place on ice.

23. Program a thermal cycler with the protocols described in Table 21. Set the cycler heated lid on.

Table 21. Target enrichment cycling conditions

		Incubation time	
Step	**Temperature (°C)**	**<2000 primer/tube**	**>2000 primer/tube**
1	95	13 min	13 min
2	98	2 min	2 min
3 (8 cycles)	98	15 s	15 s
	65	10 min	15 min
4	72	5 min	5 min
5	4	Hold	Hold

24. Place the PCR tubes in the thermal cycler and start the pre-programmed target enrichment cycling with the conditions outlined in Table 21.
25. After cycling is complete, quickly transfer samples on ice.
26. Add 2 µL of ice-cold TM Stop Solution to the 40 µL sample mix and immediately place the samples back on ice.

    Note: After addition of TM Stop Solution, the DNA can be stored overnight at –15°C to –30°C, if needed.

Cleanup of the target enrichment reaction

27. For sample purification, carefully mix 50.4 µL (1.2x) QIAseq Beads with each sample by pulse vortexing. Ensure that the beads are equilibrated at room temperature and are completely mixed, and that no clumps are visible.
28. Incubate for 5 min at room temperature. Shortly spin down and collect all liquid on the tube bottom and immobilize beads on a magnet for approximately 5 min and discard the clear supernatant.

    Note: The time for immobilization depends on the type of magnet. Ensure that supernatant is clear by visual inspection.
29. Add 200 µL fresh 70% ethanol to each bead pellet immobilized on the magnet.
30. Discard the supernatant. Carefully remove all remaining ethanol droplets from the tube inner walls. Do not disturb the immobilized magnetic bead pellet.
31. Incubate on the magnetic stand for 5–10 min until the beads are dry. Over-drying may result in lower DNA recovery; visual inspection is strongly recommended. Remove from the magnetic stand.
32. Elute by carefully resuspending in 55 µL Nuclease-free Water. Incubate for 5 min at room temperature. Immobilize beads and transfer 52 µL supernatant to a new tube.

33. Mix 62.4 µL (1.2x) QIAseq Beads with each sample by pulse vortexing and repeat steps 28–31.

    Note: Beads need to be completely dried to avoid ethanol carryover and inhibition in the next reaction. Remove any ethanol droplets with a pipette.

34. Elute by resuspending the beads in 20 µL Nuclease-free Water. Incubate for 5 min at room temperature. Immobilize the beads and transfer 17 µL of supernatant into a new tube. Avoid any magnetic beads carry-over. Store at –15°C to –30°C.

Library amplification

This step will amplify the library and add the second index.

For library amplification, use the number of cycles as indicated in Table 22.

Table 22. Typical required cycles for library amplification

Input cfDNA (ng)	50–150 primers per pool	151–2000 primers per pool	2001–5000 primers per pool
1	24	23	22
10	22	21	20
50	21	20	18
100	20	19	17

35. Thaw DNA from step 34 and amplification reagents on ice.

36. Prepare the Universal PCR (UPCR) reaction mix:

    a. If working with QIAseq Methyl DNA 8-Index Kit prepare the UPCR reaction mix by adding the components in the order according to Table 23.

    b. If using the QIAseq Methyl DNA 96-Index I Set A, B, C, or D, prepare the mix as described in Table 24. Track the number of the used indexes.

Table 23. Universal PCR mix if using QIAseq Methyl DNA 8-Index I

Component	Volume (µL) per reaction
DNA of step 34	13.4
QIAseq UPCR Buffer 5x	4
IL Universal primer	0.8
IL-S502 Index Primer	0.8
HotStarTaq DNA Polymerase	1
Total	**20**

Table 24. Universal PCR mix if using QIAseq Methyl DNA 96-Index I Set A, B, C, or D*

Component	Volume (µL) per reaction
Nuclease-free Water	1.6
QIAseq UPCR Buffer 5x	4
HotStarTaq DNA Polymerase	1
Total	**6.6**

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

* Applies to QIAseq IL-S5 Index Primer Plate in Set A, B, C, or D. The final library dual sample index is determined by the combination of the IL-N7 Adapter Plate and the QIAseq IL-S5 Index Primer Plate. Total sample index level can be up to 384-plex if using QIAseq 96-index Set A, B, C, and D together.

37. Mix the UPCR reactions as described below.

    a. Mix the reaction from step 36a (on the previous page) by pulse vortexing, spin down, and place on ice.

       Note: Total volume of reaction is 20 µL.

    b. If working with QIAseq Methyl DNA 96-Index I Set A, B, C, or D, add 13.4 µL of the DNA from step 33 to one well of the QIAseq IL-S5 Index Primer Plate in Set A, B, C, or

D, as illustrated in Figure 4.

c. Add 6.6 µL of the universal PCR mix prepared according to Table 24 to each well of the adapter plate already including the DNA. Seal the plate, mix, spin down, and place on ice.

Note: Total volume of reaction is 20 µL.

38. Place the tubes or plates with the reaction mixes from step 37a or 37c in the cycler and start the cycling program as outlined in Table 25.

Table 25. Library amplification cycling conditions (UPCR)

Step	Temperature (°C)	Incubation time
1	95	13 min
2	98	2 min
3 (Y-cycles*)	98	15 s
	62	2 min
4	72	5 min
5	4	Hold

* Y-cycles: cycle number extracted from Table 22 on page 53.

39. After cycling completion, proceed with library purification. Alternatively, the amplified library can be stored at –30°C to –15°C.

Cleanup of amplified library

40. Add 80 µL of ice-cold water to the 20 µL sample from step 39 and mix by pulse vortexing.

41. Add 120 µL (1.2x) QIAseq Beads to each sample and mix thoroughly by pulse vortexing. Ensure that the beads are resuspended homogeneously without any visual clumps.

42. Incubate for 5 min at room temperature. Briefly spin down, immobilize the beads on a magnet until supernatant is clear, and discard the clear supernatant.

43. Add 200 µL fresh 70% ethanol to each bead pellet immobilized on the magnet.

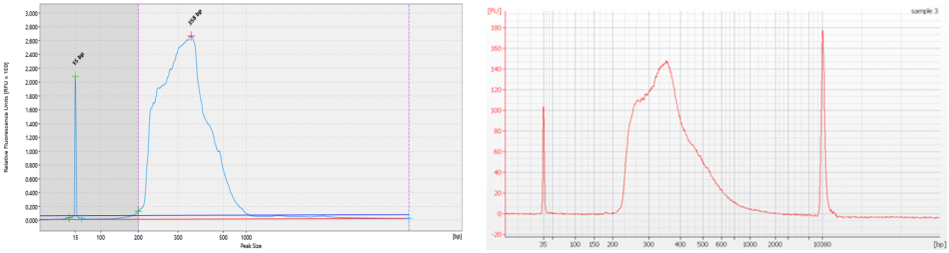
44. Discard the supernatant. Carefully remove all remaining ethanol droplets from the tube inner walls. Do not disturb the immobilized bead pellet.

45. Incubate on the magnetic stand for 5–10 min until the beads are dry. Over-drying may result in lower DNA recovery. Remove from the magnetic stand.

46. Elute by carefully resuspending in 25 µL Nuclease-free Water. Incubate for 5 min at room temperature. Immobilize beads on a magnet and transfer 20 µL supernatant to a new LoBind tube.

    The libraries can be stored at –30°C to –15°C prior to quantification using the QIAseq Library Quant System.

    Assess the quality of the library using a capillary electrophoresis device or comparable method. Check for the correct size distribution (Figure 6) of library fragments. The majority of the library fragments are between 200 and 600 bp. The median fragment size can be used for subsequent qPCR-based quantification methods.



A B

Figure 6. Capillary electrophoresis device trace of generated libraries. Capillary electrophoresis device trace data showing the correct size distribution of library fragments. **[A]** Shown is a library starting from 10 ng bisulfite-converted cfDNA isolated from PAX-stabilized plasma using the QIAseq Targeted Methyl T Cell Infiltration Panel (MHS-202Z). The library was diluted 1:5 and loaded on a QIAxcel High Sensitivity Cartridge (with DMSO). **[B]** Shown is the same library as in **A**. The library was diluted 1:5 and loaded on an Agilent High Sensitivity DNA chip. The mean library fragment size of 350 bp represent exactly the size of cfDNA of approximately 170 bp plus the adapter length.

Protocol: Normalization of Libraries with QIAseq Universal Normalizer Kits (Optional)

QIAseq Universal Normalizer Kit (cat. no. 180615) features a bead-based normalization method that enables normalizing most library types for Illumina to approximately 4 nmol/L without the need for quantification using dPCR, qPCR, or electrophoresis. The QIAseq Library Normalizer workflow allows adjusting the library concentration in as little as 60 minutes and enables balanced library representation at optimal flow cell utilization.

This protocol describes the modification of any Illumina library that has intact P5/P7 ends. After modification libraries can be normalized using the QIAseq Normalizer workflow.

Things to do before starting

- Read carefully the *QIAseq Normalizer Kit Handbook* for further recommendations in handling of reagents, background information, and troubleshooting guide.
- Be sure your libraries and preparation workflow meet the requirements for use of the QIAseq Normalizer Kit.
- If libraries are frozen, thaw them on ice. Once libraries are thawed, mix them thoroughly by quickly vortexing to avoid any localized concentrations. Briefly spin down libraries.
- Thaw QIAseq HiFi PCR Master Mix and the Normalizer Primer Mix on ice. Once the reagents are thawed, mix them thoroughly by quickly vortexing to avoid any localized concentrations. Briefly spin down the vortexed reagents before use.
- Prepare fresh 80% ethanol.
- Equilibrate the QIAseq Beads to room temperature (15–25°C). Directly before use, vortex the beads thoroughly to homogenize.

Important points before starting

- The input material used for library preparation is of good quality and/or is accurately quantified.
- The laboratory staff is experienced with the library preparation method in use.
- The conditions for the library preparation method are well established in your laboratory (e.g., input amount, adapter concentrations per dilution, PCR settings, cleanup procedures, and other workflow parameters) so your library yields are consistent with concentrations well above 15–20 nmol/L in a minimal volume of 15 µL and free of adapter dimer peaks.
- The planned number of libraries in the sequencing pool is 6 or greater.

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.

- LoBind tubes for storage of generated libraries (Eppendorf, cat. no. 0030108094, 0030108116, or 0030108132) or equivalent
- 96-well PCR plates or PCR tubes/strips
- Foil seal for 96-well plates, heat resistant
- 100% ethanol (ACS grade)
- Nuclease-free Water
- pipette tips and pipettes
- Vortexer
- Microcentrifuge and/or plate centrifuge for 96-well plates

- Thermal cycler
- Magnetic stand (e.g., DynaMag™,2 Magnet or DynaMag™, 96 Side Skirted Magnet, Thermo Fisher, cat. no. 12027)
- QIAGEN QIAxcel, Agilent Bioanalyzer, or similar method to assess the quality of DNA library (optional)

Procedure

Library modification for use with QIAseq Universal Normalizer Kit

1. Set up a thermal cycler with a heated lid according to Table 26.

   Note: If your libraries are likely to have very low concentrations (<10 nmol/L) or only small volumes of library are available (<15 µL), increase the PCR cycles to 5 cycles.

Table 26. Thermal cycling parameters

Time	Temperature (°C)	Number of cycles
2 min	98	1
20 s	98	4–5*
30 s	60	
30 s	72	
2 min	72	1
∞	4	Hold

* 5 cycles work equally efficient with libraries having a concentration of 10–30 nM.

2. Prepare the library modification reaction mix on ice according to Table 27. Mix the components in a PCR tube/strip or 96-well plate.

Table 27. Reaction mix for Normalizer modification PCR

Component	Volume (µL) per reaction
HiFi PCR Master Mix, 2x	25
Normalizer Primer Mix	1.5
Finalized Illumina library*	≤23.5
RNase-free Water	variable
Total reaction volume	**50**

* If available, always use 23.5 µL of finalized Illumina library. Otherwise use all available volume and fill with RNase-free Water.

3. Transfer the PCR tube/strip or plate to the thermal cycler and start the program.
4. Once PCR is complete, transfer the whole reaction to a new 1.5 mL Eppendorf tube and add 50 µL of homogenized QIAseq Beads to each reaction.
5. Mix by thoroughly pipetting up and down or by vortexing. Incubate the mixture for 5 min at room temperature.
6. If required, pulse spin the tubes to collect all liquid at the bottom.
7. Pellet the beads on a magnetic stand for 5 min or until the solution is clear.
8. Carefully discard the supernatant without disturbing the pellet.
9. Wash the beads by adding 200 µL fresh 80% ethanol to each pellet. Pellet the beads on the magnetic stand and carefully discard the supernatant.
10. Repeat step 9 for a total of 2 ethanol washes.
11. Remove as much excess ethanol as possible.
12. Incubate on the magnetic stand for 5–10 min or until the beads are dry. Over-drying of beads may result in lower DNA recovery. Remove from the magnetic stand.

13. Elute modified libraries by resuspending in 32.5 µL of RNase-free Water or 10 mM Tris·Cl, pH 8.0. Pellet the beads on the magnetic stand and carefully transfer 30 µL of the supernatant into a new tube.

    Note: If required, the elution volume can be reduced to 20 µL to increase the library concentration. The QIAseq Normalizer procedure will require 15 µL of modified library.

14. The collected supernatant contains the modified library and can be used for normalization. Proceed to protocol "QIAseq Library Normalization for use with all QIAseq Normalizer Kits (Tube format)", next section. Alternatively, modified libraries can be stored for later use at –15°C to –35°C.

QIAseq Library Normalization for use with all QIAseq Normalizer Kit (tube format)

This protocol describes the normalization of previously modified QIAseq Targeted Methyl Libraries. Only use libraries that have been modified/amplified using the Normalizer Primer Mix. Using nonmodified libraries will lead to normalization failure. QIAseq Normalization will yield double-stranded libraries at a concentration of approximately 4 nmol/L. Normalized libraries can be pooled for sequencing at equal volumes without further quantification.

15. Preheat a heating block for 1.5 mL tubes to 55°C.

16. Homogenize the bottle(s) of Normalizer Wash Buffer by vortexing and preheat the whole reagent bottle(s) to 55°C in a water bath. Alternatively, use a heating block for 1.5/2.0 mL tubes or Falcon tubes. Fill the homogenized wash buffer into compatible receptacles to preheat. Prepare 450 µL wash buffer (400 µL is required) per normalization reaction. Leave the wash buffer at 55°C until use.

17. Thoroughly mix the Normalizer Reagent by vortexing for 60 s. Make sure that the pellet of beads is completely dissolved and the solution is well homogenized. It is not required to equilibrate the Normalizer Reagent to room temperature before use.

18. Directly before use, pulse spin the Normalizer Reagent at low force to collect all liquid at the bottom. Then use a 200 µL pipette to homogenize the reagent. Be sure to disperse a sediment of beads that may have formed during pulse spin.
19. To normalize each library, pipette 5 µL of homogenized Normalization Reagent into a 1.5 mL tube. Leave the tubes at room temperature.
20. Add 15 µL of the modified library to be normalized to the 1.5 mL tube containing Normalization Reagent. Mix well by pipetting or vortexing.
21. Incubate for 10 min at room temperature.
22. Add 200 µL pre-warmed Normalizer Wash Buffer (55°C) to each tube.
23. Pellet the beads on a magnetic stand for 2 min and wait until the solution is clear.

    Important: Make sure all beads have pelleted. If you are uncertain, leave the tubes on the magnet for 5 min.
24. Carefully discard the supernatant without disturbing the pellet.
25. With the tube on the magnet add 200 µL pre-warmed Normalizer Wash Buffer (55°C) to each pellet.
26. Switch the tube position on the magnet to wash the beads. Wait until all beads have pelleted. Alternatively, turn the tube by 180° to force the beads to opposite side of the tube.
27. Carefully discard the supernatant without disturbing the pellet. Remove as much remaining liquid as possible, then proceed to the next step immediately.

    Note: Drying the beads is not required.
28. Add 26 µL Normalizer Elution Buffer to each pellet and mix well by vortexing. Make sure the pellet is completely dissolved. It is not required to equilibrate the Normalizer Elution Buffer to room temperature before use.

29. Tap the tube on the benchtop to collect the liquid at the bottom. If there is remaining liquid in the lid, pulse spin the tube at low force. Do not spin to form a compact bead pellet.
30. Incubate for 5 min at 55°C in a heating block.
31. Pulse spin to collect all liquid at the bottom, then pellet the beads on a magnetic stand for 2 min and wait until the solution is clear.
32. Carefully transfer 25 µL of the supernatant to a new tube.

    Important: Do not discard the supernatant. The supernatant contains the library ready for sequencing.
33. Proceed to "Guidelines: Pooling and sequencing of normalized libraries for use with all QIAseq Normalizer Kit". Alternatively, the normalized libraries can be stored at –15°C to –35°C for up to 3 months.

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 than expected	Quantify the yield of bisulfite-converted DNA using NanoDrop, QIAxpert, 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. If using FFPE DNA, increase input depending on degree of degradation.
b) Sub-optimal reaction conditions due to ethanol contamination	Ensure that ethanol residue has been fully removed during EpiTect washing steps and bead clean-up during the library construction. Remove any residual liquid from columns and tubes, since ethanol contamination inhibits the subsequent enzymatic reactions.
c) Sub-optimal reaction conditions during enzymatic reactions	Ensure that cycling conditions and incubation temperatures are programmed correctly.
d) Inefficient library amplification	Inefficient adapter–dimer removal using QIAseq Beads. Mix the beads thoroughly and use the recommended amount for purification. The beads are overdried. Make visual control during drying of beads, as 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 carefully remove before drying the beads.
Unexpected signal peaks in capillary electrophoresis device traces	

	Comments and suggestions
a) Presence of shorter peaks between 90 bp and 180 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. In the case of DNA, the DNA was very strongly fragmented and short fragments were overamplified. Perform a second round of QIAseq Beads clean-up with 0.8x QIAseq Beads for gDNA and FFPE preparations and 1x QIAseq Beads for cfDNA libraries before sequencing.
b) Presence of larger library fragments after library enrichment	When performing library enrichment, if the fragment population shifts higher than expected range or has a second wide peak running into the marker peak, this may be a PCR artifact due to over-amplification of the DNA library. Reduce the number of amplification cycles. If quantification of the library will be performed over Q-PCR this higher peak will not affect sequencing. Alternatively, if quantification needs to be performed over Agilent, run a second library amplification for 2 cycles and repeat bead clean-up. When the difference between BioAnalyzer and QPCR is high, it is recommended to confirm quantity of the library by a third method (e.g., Qubit) as this may come from dilution steps inaccuracies.
Methylation degree of Cytosines in CHH and CHG content is higher than 2%	
a) Coverage is too low	Verify if coverage of Cytosines on CHH and CHG context is higher than 300x and if there are SNV found on this position.
b) Conversion of non-methylated cytosines is incomplete	Follow exactly the workflow steps as described in the section *Protocol: Bisulfite Conversion of Unmethylated Cytosines in DNA using EpiTect Fast DNA Kits*. Ensure that the cycler maintain the programmed cycling condition. Avoid prolonged exposition of BD buffer or column with BD buffer to air. In case of repeated incomplete conversion especially when using input of 1–2 µg, for example when performing bulk bisulfite treatment for running multiple library replicates, you can increase the conversion time at 60°C from 10 min up to 20 min.

Appendix A: Sequencing Setup on Illumina MiSeq

Important points before starting

- Recommendations for library dilution concentrations and library loading concentrations are based on QIAseq Library Quant System.
- QIAseq A Read1 Primer I (Custom Read 1 Sequencing Primer) **must** be used when performing sequencing on Illumina platform.
- Paired-end sequencing **should** be used for QIAseq Targeted Methyl Panel on Illumina platform.
- For complete instructions on how to denature and dilute sequencing libraries, PhiX Libraries, to prepare custom index primers, and set up a sequencing run, please refer to the system-specific Illumina documents.

  Denature and Dilute Libraries for the MiSeq system (**support-docs.illumina.com/IN/MiSeq_DnD/Content/MiSeq/DnD-MiSeq.htm?protocol=standard**)

Sequencing preparations for MiSeq without addition of PhiX Library

- Create the Run using Local Run Manager (Figure 7a). Sample index of QIAseq Targeted Methyl Panel is compatible with Illumina Nextera® XT v2 adapter sample index system.
- Set the Run Settings as follows:
  - Library Prep Kit: Nextera XT
  - Index Kit: Select **Nextera XT v2 Index Kit Set A/ B/ C or D**

    - Index Reads: Select **2**
    - Read Type: Select **Paired End**
- Read Lengths: Set “Read1: 151, Index 1: 8, Index 2: 8, Read 2: 151”
- **Important**: Check the box “Custom Primers” for “Read 1”(Figure 7b)
- **Important**: Set Adapter Trimming “On”
- Save the run or export the sample sheet.

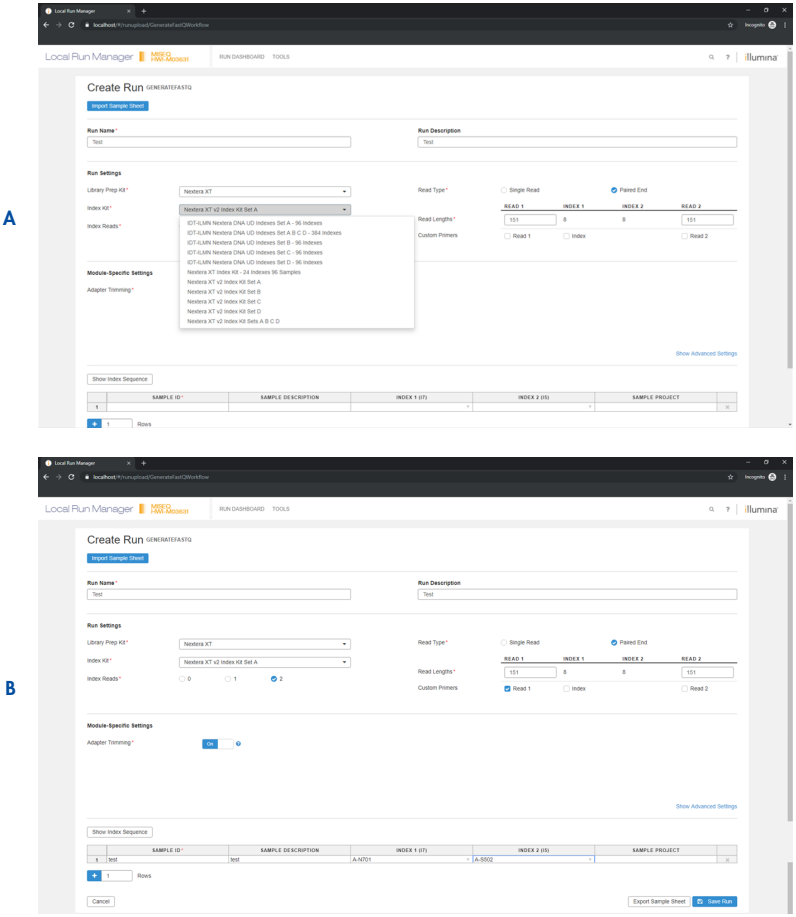


Figure 7. Run Setup using Local Run Manager.

Proceed with library dilution and denaturation as described in the next steps.

1. Dilute libraries to 1.8–2 nM for MiSeq. Combine libraries with different sample indexes in equimolar amounts if a similar sequencing depth is needed for each library.

   Note: Recommendations for library dilution concentrations are based on QIAseq Library Quant System.

   Note: If combining libraries with the same number of primers, pool equal volumes of the individual libraries at 1.8–2 nM together. If the libraries have different primer numbers, then combine the libraries at volume ratios according to their number of primers. For example, Library A has 3000 primers at 1.8 nM and Library B has 600 primers at 1.8 nM. Combining 30 µL of Library A with 6 µL of Library B will result in similar coverage depth for both Libraries A and B in the same sequencing run.

2. Prepare and load the library onto a MiSeq according to the MiSeq System Denature and Dilute Libraries Guide. The final library concentration is 9–10 pM on the MiSeq.

3. Use 597 µL HT1 (Hybridization Buffer) to dilute 3 µL QIAseq A Read 1 Custom Primer I (provided) to obtain a final concentration of 0.5 µM. Load 600 µL of the diluted QIAseq A Read 1 Primer I to position 18 of the MiSeq reagent cartridge (Figure 8).

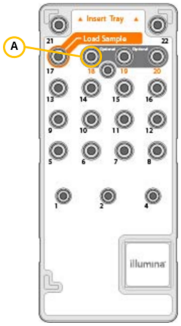


Figure 8. Loading the QIAseq A Read 1 Primer I into position 18, shown by A. For more details, refer to the Illumina protocol in *MiSeq System Custom Primers Guide* (**support.illumina.com/content/dam/illumina-support/documents/documentation/system_documentation/miseq/miseq-system-custom-primers-guide-15041638-01.pdf**).

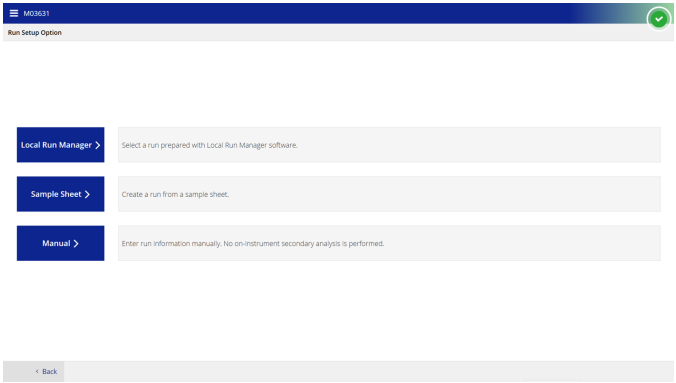
4. Start the run by selecting one of the options Local Run Manager or Sample Sheet (Figure 9a).
    - If you choose the **Local Run Manager** option, select from the drop list the saved run that will be started and follow the instructions of the Software.
    - If you select **Sample Sheet**, browse the sample sheet saved for this run and follow the instructions of the software for the next steps.
    - If you start the run by selecting **Manual**, enter the information in "Run Setup" as follows:

      Read Type: Select **Paired End**.

      Read Length: Set "Read1: 151, Index 1: 8, Index 2: 8, Read 2: 151"

      Important: Check the box Custom Primers "Read 1" and follow the next steps as required by the MiSeq Control Software (Figure 9b).

A



B

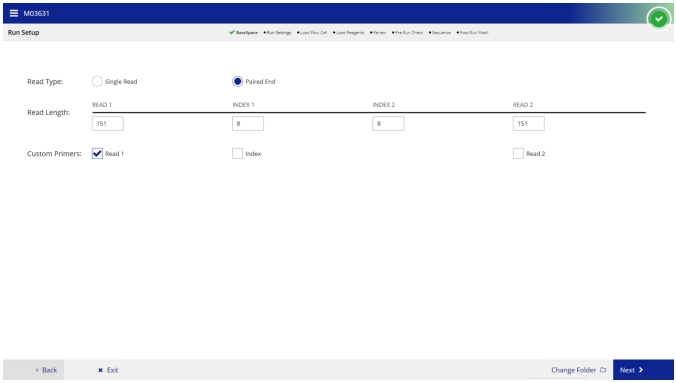


Figure 9. Starting a Sequencing Run on MiSeq.

5. Upon completion of the sequencing run, proceed to "Appendix C: Data Analysis Using QIAGEN's QIAseq Targeted Sequencing Data Analysis Portal or the Biomedical Genomics Workbench" on page 81.

Sequencing preparations for MiSeq with addition of PhiX Library

Addition of PhiX is recommended for Methylation sequencing because the libraries are of lower complexity due to base composition after the conversion of non-methylated C to T and will increase the Q30 up to 93–95%. For PhiX spike-in recommendation depending on instrument and chemistry, please consult Appendix C.

1. Create the Run using Local Run Manager (Figure 10 on the next page). The sample index of QIAseq Targeted Methyl Panel is compatible with Illumina Nextera® XT v2 adapter sample index system.
2. Set the Run Settings as follows:
    - Library Prep Kit: Nextera XT
    - Index Kit: Select **Nextera XT v2 Index Kit Set A / B/ C or D**
    - Index Reads: Select **2**
    - Read Type: Select **Paired End**
    - Read Lengths: Set "Read1: 151, Index 1: 8, Index 2: 8, Read 2: 151"
    - **Important**: Do not check Custom Primer for "Read 1"(Figure 10)
    - **Important**: Set Adapter Trimming "On"
3. Save the run or export the sample sheet.

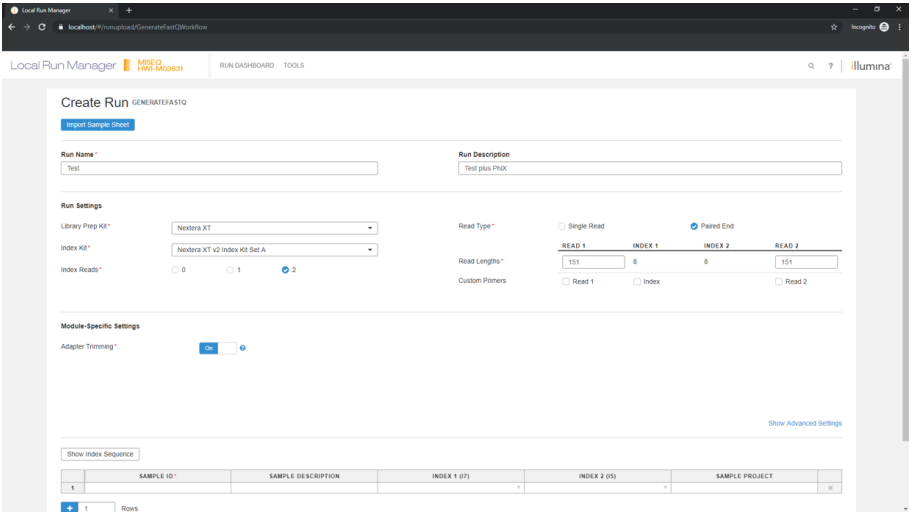


Figure 10. Run Setup using Local Run Manager when sequencing with PhiX.

4. Proceed with library dilution as described in the next steps:

   a. Dilute libraries to 1.8–2 nM for MiSeq. Combine libraries with different sample indexes in equimolar amounts if a similar sequencing depth is needed for each library.

      Note: Recommendations for library dilution concentrations are based on QIAseq Library Quant System.

      Note: If combining libraries with the same number of primers, pool equal volumes of the individual libraries at 1.8–2 nM together. If the libraries have different primer numbers, then combine the libraries at volume ratios according to their number of primers. For example, Library A has 3000 primers at 1.8 nM and Library B has 600 primers at 1.8 nM, combining 30 µL of Library A with 6 µL of Library B will result in similar coverage depth for both Libraries A and B in the same sequencing run.

b. Prepare and load the library onto a MiSeq according to the MiSeq System Denature and Dilute Libraries Guide (Denature and Dilute Libraries for the MiSeq system, **support- docs.illumina.com/IN/MiSeq_ DnD/Content/MiSeq/DnD-MiSeq.htm?protocol=standard**). The final library concentration is 9–10 pM on the MiSeq.

c. Denature and Dilute PhiX Control to a final concentration of 12.5 pM according to Illumina recommendations (*Denature and Dilute Libraries for the MiSeq system*) and add 60 µL denatured and diluted PhiX library to 540 µL denatured and diluted library pool. That is 10% PhiX.

d. Use slimmer tips (e.g., CLS4853 Corning® gel-loading pipette tips or similar) to pipette 3.4 µL of QIAseq A Read 1 Primer (100 µM) I into position 12 of the MiSeq reagent cartridge (Figure 11). This position contains 680 µL of the Illumina Read1 (HP10) primer, adding 3.4 µL of QIAseq A Read 1 Primer (100 µM) will give a final concentration of 0.5 µM. Mix the primers by pipetting up and down.

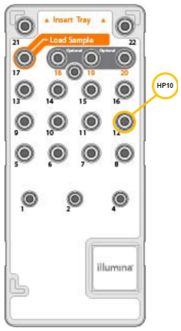


Figure 11. Loading the QIAseq A Read 1 Primer I into position 12 shown by HP10 in the figure.

5. Start the run using the MiSeq Control Software (Figure 12a) by selecting one of the following options.

- If you choose the **Local Run Manager** option, select from the drop list the saved run that will be started and follow the instructions of the software.
- If you select **Sample Sheet**, browse the sample sheet saved for this run and follow the instructions of the software for the next steps.
- If you start the run by selecting **Manual**, enter the information in "Run Setup" as follows.

  Read Type: Select **Paired End**

  Read Length: Set "Read1: 151, Index 1: 8, Index 2: 8, Read 2: 151"

Important: Do **not** check Custom Primer for Read 1 and follow the next steps as required by the MiSeq Control Software (Figure 12b).

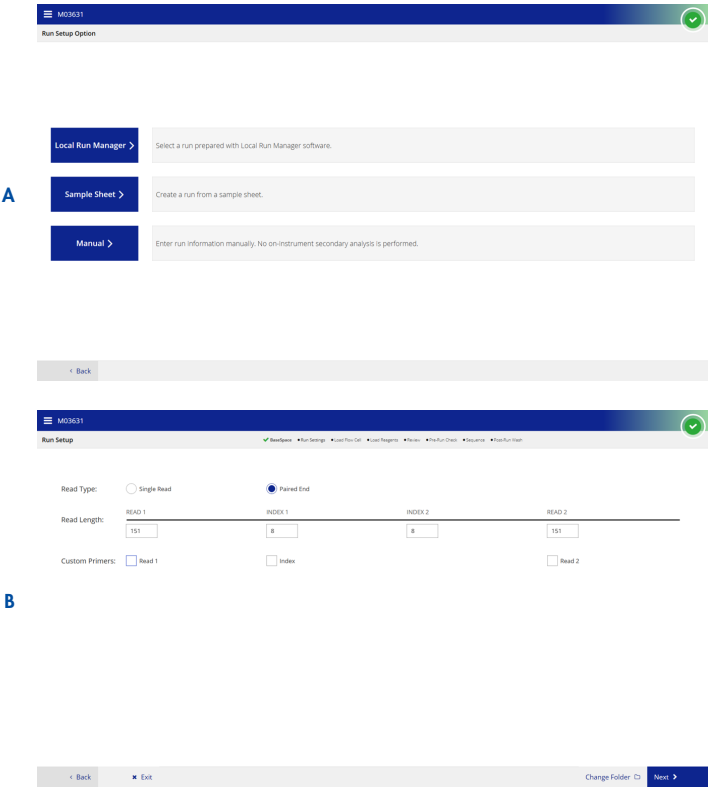


Figure 12. Run Setup using MiSeq Control Software when using PhiX.

6. Upon completion of the sequencing run, proceed to "Appendix C: Data Analysis Using QIAGEN's QIAseq Targeted Sequencing Data Analysis Portal or the Biomedical Genomics Workbench" on page 81.

Important: When sequencing library pools with low complexity (e.g., fewer than 8 libraries or libraries from heavily fragmented DNA or FFPE) PhiX library spike-in will improve the sequencing quality. In general, the addition of PhiX, even in concentration lower than 10%,

will improve signal complexity and can be used to easily perform root cause analysis in case of low quality runs.

Follow Illumina's recommendations on how to spike in custom primers into the Illumina sequencing primer when conducting sequencing runs with PhiX (**knowledge.illumina.com/library- preparation/general/library- preparation- general-reference_material-list/000001542**).

Appendix B: Sequencing Setup for Different Sequencers

The following table provides recommendations for loading of the flow cells with Library and custom sequencing read 1 primer. Note that optimal loading concentration may vary from pool to pool depending on the quality and library average fragment size. Optimization of loading may be helpful for instruments such as NextSeq or NovaSeq. It may be of great value to sequence the pool using MiSeq Nano reagent to evaluate the quality and clustering efficiency before loading the NextSeq or NovaSeq.

Sequencer	Library loading concentration (pM)	PhiX (%)	Custom sequencing primer loading concentration (µM)	Custom sequencing primer loading position	Select custom primer in Run setup
MiSeq	9–10	–	0.5	18	yes
MiSeq	9–10	10	0.5	12	no
NextSeq 500/550 MidOutput	1.2	15	0.3	20	no
NextSeq 1000/2000 XLEAP-SBS chemistry	600–650	20	0.3 with Illumina primer*	VP21	yes
NextSeq 1000/2000 Standard SBS	600–650	20	0.3 with Illumina primer†	BP14	yes
NovaSeq X+	140	20	0.3	28	no
Cloudbreak Freestyle§	14–15	20	1	R1	n/a

* NextSeq 1000/2000 XLEAP-SBS Read Primer Kit, cat. no. 20112859

† NextSeq 1000/2000 Standard SBS Read Primer Kit, cat. no. 20046117

§ For exact recommendations for Run setup and reagent loading, refer to *AVITI™ System User Guide* (Element Biosciences) (**go.elementbiosciences.com/aviti-system-user-guide-ma-00008**)

For setup updates for all Illumina sequencers, refer to **knowledge.illumina.com/library-preparation/general/library-preparation-general-reference_material-list/000001542**

Appendix C: Adapter Bar Codes for QIAseq Targeted Methyl Panel DNA Libraries

IL-MeN7 index sequences		IL-S5 index sequences	
Name	**Sequence**	**Name**	**Sequence**
IL-N701	TCGCCTTA	IL-S502	CTCTCTAT
IL-N702	CTAGTACG	IL-S503	TATCCTCT
IL-N703	TTCTGCCT	IL-S505	GTAAGGAG
IL-N704	GCTCAGGA	IL-S506	ACTGCATA
IL-N705	AGGAGTCC	IL-S507	AAGGAGTA
IL-N706	CATGCCTA	IL-S508	CTAAGCCT
IL-N707	GTAGAGAG	IL-S510	CGTCTAAT
IL-N710	CAGCCTCG	IL-S511	TCTCTCCG
IL-N711	TGCCTCTT	IL-S513	TCGACTAG
IL-N712	TCCTCTAC	IL-S515	TTCTAGCT
IL-N714	TCATGAGC	IL-S516	CCTAGAGT
IL-N715	CCTGAGAT	IL-S517	GCGTAAGA
IL-N716	TAGCGAGT	IL-S518	CTATTAAG
IL-N718	GTAGCTCC	IL-S520	AAGGCTAT
IL-N719	TACTACGC	IL-S521	GAGCCTTA
IL-N720	AGGCTCCG	IL-S522	TTATGCGA

IL-MeN7 index sequences		IL-S5 index sequences	
Name	**Sequence**	**Name**	**Sequence**
IL-N721	GCAGCGTA		
IL-N722	CTGCGCAT		
IL-N723	GAGCGCTA		
IL-N724	CGCTCAGT		
IL-N726	GTCTTAGG		
IL-N727	ACTGATCG		
IL-N728	TAGCTGCA		
IL-N729	GACGTCGA		

Appendix D: Data Analysis Using QIAGEN's QIAseq Targeted Sequencing Data Analysis Portal or the Biomedical Genomics Workbench

After sequencing, the results can be analyzed using QIAGEN's QIAseq targeted methylation sequencing data analysis portal. Our data analysis pipeline will perform read trimming (removing primer sequences), mapping to the reference genome, UMI counting, and methylation calling.

Alternatively, data from QIAseq Targeted Methyl Panels can be analyzed using the Genomics Workbench 12.0.2 and higher, which allows you to optimize analysis parameters to your specific panel. The parameters can then be locked for routine use. Contact your account manager for further details.

1. Use Chrome or Firefox, as the portal is not compatible with Internet Explorer (IE). Log in or create a QIAGEN account at **www.qiagen.com**
2. Log in to the GeneGlobe Data Analysis Center at **geneglobe.qiagen.com/analyze/**
3. Under **Analyze your data**, select **NGS**.
4. Select **QIAseq Targeted Methyl Panels**.
5. Select one option from the drop-down menu and select **Start Analysis**.
6. Upload or manage your data in the tab **Read Files**. Upload FASTQ files by selecting **Upload New Files**.

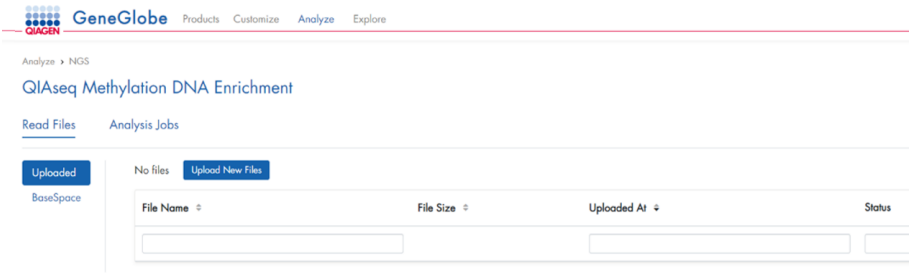


Figure 13. File Upload tab of the QIAseq Targeted Sequencing Data Analysis Portal.

7. Once upload of FASTQ files is completed, choose the **Analysis Jobs** tab and click on **Create New Jobs**.

8. Type in a Job Title in the respective box, select a panel catalog number under **Catalog**, and then select **Analyze** to start the job. Once analysis is completed, download the zipped folder.

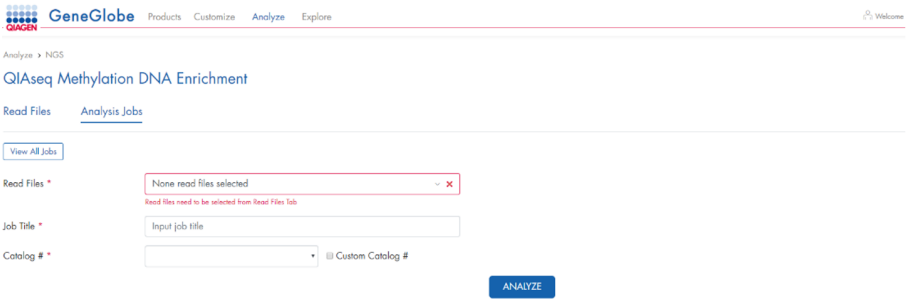


Figure 14. Analysis job creation tab of the QIAseq Targeted Sequencing Data Analysis Portal.

Appendix E: Bisulfite Conversion of Unmethylated Cytosines in DNA using EpiTect Fast 96 Bisulfite Kit

Protocol: Bisulfite conversion using EpiTect Fast 96 plates

This protocol enables bisulfite conversion of DNA amounts of 50–500 ng gDNA, 100–500 ng FFPE, and 50–500 ng cfDNA in a volume of 20 µL (high concentration protocol).

If starting with this input DNA, total eluates from EpiTect Fast bisulfite conversion can be directly used in the subsequent library preparation without further quality control steps.

Important points before starting

- Read carefully the *EpiTect Fast Bisulfite Conversion Handbook* for general recommendations on handling of reagents and background information on the conversion chemistry.
- 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.
- Perform all centrifugation steps at room temperature.
- For DNA input < 50 ng, the use of the EpiTect plates (96) is not recommended.
- During bisulfite treatment, it is estimated that approximately 30–60% DNA will be degraded and lost. DNA input in bisulfite conversion higher than 40 ng will increase mapping rate and region coverage. Use Carrier RNA in the EpiTect Fast procedure if using DNA less than 100 ng. For cfDNA, use Carrier RNA for any input.

Things to do before starting

- Prepare the kit reagents as described in "Preparation of reagents", page 19 of the *EpiTect Fast Bisulfite Conversion Handbook* (06/2019).
    - BW and BD buffer must be complemented with ethanol (96–100%).
    - Add 120 mL ethanol to BW concentrate, invert the bottle several times, and store at room temperature.
    - Add 27 mL ethanol (96–100%) to Buffer BD and store at 2–8°C. Invert the bottle several times before starting the procedure and make sure to close the bottle immediately after use. White precipitates may form in the Buffer BD–ethanol mix after some storage time. These precipitates will not affect the performance of Buffer BD. However, avoid transferring precipitates to the MinElute DNA spin column.
- If required, prepare Carrier RNA as out lined in "Preparation of reagents", page 16 of the *EpiTect Fast Bisulfite Conversion Handbook (06/2019)*, by resuspending the lyophilized Carrier RNA in 1350 µL RNase-free water. Split the dissolved Carrier RNA into conveniently sized aliquots (e.g., 675 µL aliquots for processing 96 reactions) and store at –30°C to –15°C. Aliquots can be stored for up to 1 year. Add 300 µL of the dissolved Carrier RNA to one bottle of Buffer BL to obtain a final concentration of 10 µg/mL. Carrier RNA is not necessary if >100 ng DNA is used. If Buffer BL contains precipitates, dissolve by heating (maximum 70°C) with gentle agitation.
- Equilibrate samples and buffers to room temperature.

  Optional: Set a thermomixer, heating block, or heated orbital incubator to 60°C to dissolve the Bisulfite Solution.

Procedure

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 completely 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 28 below. Add each component in the order listed.

   Note: The combined volume of DNA and RNase-free Water must total 20 µL for high concentration samples.

   Note: If using a multichannel pipette to dispense DNA Protect Buffer, use the provided EpiTect 96 DNA Protect Buffer Reservoir. Commonly used polystyrene reservoirs are sensitive to the solvent in this buffer.

Table 28. Bisulfite reaction components

Component	Volume (µL) per reaction
DNA	Variable (maximum 20 µL)
Nuclease-free Water	Variable*
Bisulfite Solution	85
DNA Protect Buffer	35
Total reaction volume	**140**

* The combined volume of DNA and Nuclease-free Water must total 20 µL.

3. Seal the EpiTect 96 Conversion Plate using EpiTect 96 Cover Foil (provided) and mix the bisulfite reactions by vortexing thoroughly. Centrifuge the plate briefly at 650 × *g* (approximately 2000 rpm) to collect the reactions in the bottom of the wells. Store the plate at room temperature.

   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 DNA bisulfite conversion reaction.

   Note: If the lid of the thermal cycler is not compatible with the provided EpiTect 96 Cover Foils, cap strips can be used (see "Equipment and Reagents to Be Supplied by User" on page 14).

4. Perform the bisulfite DNA conversion using a thermal cycler. Program the thermal cycler according to Table 29 below. 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 29. BisU conversion thermal cycling conditions

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

* For samples that consistently show incomplete conversion, extending the 60°C incubation step to up to 20 min may be required to ensure complete bisulfite conversion of DNA.

5. Place the PCR plates containing the bisulfite reactions into the thermal cycler. Start the thermal cycling incubation.

Important: Because the bisulfite reaction is not overlaid with mineral oil, only thermal cyclers with heated lids are suitable for this procedure. Seal the plates and ensure that the seal is tight.

Cleanup of bisulfite-converted DNA using a centrifuge

1. Once the bisulfite conversion is complete, briefly centrifuge the EpiTect 96 Conversion Plate containing the bisulfite reactions at 650 × *g*.
2. Place an EpiTect 96 Plate on top of an S-Block. Mark the plate for later identification.
3. When working with DNA less than 100 ng, dispense 310 µL freshly prepared Buffer BL containing 10 µg/mL Carrier RNA (see "Preparation of reagents") into the required EpiTect 96 Plate wells.

   Note: Proceed with the cleanup protocol within 5 min of applying Buffer BL.
4. Transfer the complete bisulfite reactions from step 1 to the EpiTect 96 Plate and mix with Buffer BL by pipetting up and down 4 times. Precipitates in the bisulfite reactions will not affect performance or yield.
5. Add 250 µL ethanol (molecular biology grade, 96–100%) to each sample and mix by pipetting up and down 4 times.
6. Seal the EpiTect 96 Plate with an AirPore Tape Sheet. Load the S-Block and EpiTect 96 Plate into the centrifuge plate holder, then place the holder into the rotor bucket. Centrifuge at 5800 × *g* for 1 min.

   Optional: AirPore Tape Sheets help prevent cross-contamination.
7. Remove the AirPore Tape Sheet. Add 500 µL Buffer BW to each sample and seal the EpiTect 96 Plate with a new AirPore Tape Sheet. Centrifuge at 5800 × *g* for 1 min. Empty the S-Block.

8. Remove the AirPore Tape Sheet. Add 250 µL Buffer BD to each sample and seal the EpiTect 96 Plate with a Tape Pad (provided). Incubate for 15 min at room temperature. If there are precipitates in Buffer BD, avoid transferring them to the plate.

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

9. Centrifuge at 5800 × *g* for 1 min.

10. Remove the Tape Pad. Add 500 µL Buffer BW to each sample and seal the EpiTect 96 Plate with a new AirPore Tape Sheet. Centrifuge at 5800 × *g* for 1 min. Empty the S-Block.

11. Remove the AirPore Tape Sheet. Add 500 µL Buffer BW to each sample and seal the EpiTect 96 Plate with a new AirPore Tape Sheet. Centrifuge at 5800 × *g* for 1 min.

12. Remove the AirPore Tape Sheet. Add 250 µL ethanol (96–100%) to each sample and seal the EpiTect 96 Plate with a new AirPore Tape Sheet. Centrifuge at 5800 × *g* for 1 min.

13. Dispose of the S-Block appropriately. Remove the AirPore Tape Sheet and place the EpiTect 96 Plate on top of an EpiTect Elution Plate. Centrifuge at 5800 × *g* for 15 min.

    Important: If using a centrifuge with adjustable temperature, set the temperature to 60°C. The heat generated during centrifugation ensures evaporation of residual ethanol in the sample. If no centrifuge with adjustable temperature can be used, the EpiTect 96 Plate can be dried after centrifugation in an clean incubator for 30 min at 60°C.

14. Place the EpiTect 96 Plate on top of a new EpiTect Elution Plate.

15. To elute DNA, add 70 µL Buffer EB to each sample using a multichannel pipette.

    Important: For complete elution of bound DNA, ensure that the elution buffer is dispensed directly onto the center of each membrane on the EpiTect 96 Plate.

16. Centrifuge at 5800 × *g* for 1 min. Seal the elution plate for storage using Tape Pads. The average eluate volume is approximately 40 µL from 70 µL elution buffer.

17. The purified DNA can be stored at –30°C to –15°C for up to 2 weeks.

Protocol: Library generation from bisulfite-converted gDNA and FFPE DNA in 96-well format

This procedure describes the NGS library preparation starting from bisulfite-converted, single-stranded DNA. It includes end repair of the bisulfite-converted DNA, adapter ligation and introduction of UMIs, target enrichment with the QIAseq Methyl Panel, library amplification with introduction of the second index, and library cleanup steps using magnetic beads.

Important points before starting

- This protocol is optimized for bisulfite-converted DNA using EpiTect Fast 96 Bisulfite Kit (cat. no. 59720).
- Avoid DNA contamination of reagents by using separate laboratory equipment (e.g., pipettes, filter pipette tips, reaction vials, etc.).
- Set up all reactions on ice.

Things to do before starting

- Prepare fresh 70% ethanol.
- Enzymes and enzyme mixes should be thawed on ice and placed on ice until use.
- All other components can be thawed at room temperature (15–25°C) but placed on ice immediately after thawing. Equilibrate Ligase Buffer 2x at room temperature.
- All buffers and reagents should be vortexed before use to ensure thorough mixing and spin down to collect all liquids on the bottom of the tubes.
- All enzyme mixes should be placed on ice until use. Avoid extended vortexing of the enzymes and enzyme mixes.

- Program the thermal cycler. To avoid prolonged incubation of reaction mixes on ice and for increased speed and convenience, all incubation steps of the protocol can be preprogrammed and saved on a thermal cycler in advance.

Procedure

End repair of bisulfite-converted DNA

1. Thaw bisulfite-converted DNA form previous step (16) and use the total volume of 20 µL for the End repair reaction.
2. Setup the bisulfite-converted DNA repair reaction mix on ice according to Table 30. Mix by pulse vortexing (8–10 times) and spin down. Keep the reaction on ice.

Table 30. Bisulfite-converted DNA repair reaction mix for 96 reactions

Component	Volume (µL) per 96 reactions plus 10%
TM-repair buffer, 10x	316.8
Nuclease-free Water	633.6
T4 Polynucleotide Kinase	105.6

3. Use a microdispensing pipette to transfer 10 µL of the master mix of Table 3 in each well of a 96 0.2 mL-well PCR plate.
4. Add the 20 µL bisulfite-converted DNA from previous step (16) to the master mix plate. Total volume is 30 µL.
5. Seal the plate with an adhesive foil or cap-strips and mix by repeatedly vortexing 8–10 times. Make sure that all wells of the plates are mixed, and then spin down.
6. Program a thermal cycler with the protocols described in Table 31.

   Note: Use a thermal cycler with heated lid at 75°C. If not possible, run incubation at 37°C with open lid and close the heated lid for incubation at 65°C.

Table 31. Bisulfite-converted DNA repair cycling conditions

Step	Temperature (°C)	Incubation time (min)
1	37	30
2	65	20
3	4	Hold

7. Place the plate from step 5 into the thermal cycler and start the bisulfite-converted DNA repair cycling program, Table 31 above. Place samples on ice after cycling completion.

Adapter ligation

8. During bisulfite-converted DNA repair cycling, prepare the ligation mix according to Table 32 below. Mix thoroughly by pulse vortexing and spin down.

   Note: Ligase buffer 2x should be equilibrated at room temperature. The ligase buffer has a high density. Avoid carryover of big droplets with the pipette tip and pipette carefully into the tube ensuring the accurate amount of buffer.

Table 32. Ligation mix

Component	Volume (µL) per 96 reactions plus 10%
Ligase Buffer, 2x	4752
Nuclease-free Water	633.6
DNA Ligase	422.4
Total master mix volume	**5808**

9. Add 55 µL ligation master mix to each 30 µL end-repaired DNA sample from the previous step and mix by pulse vortexing and spin down.
10. Add 5 µL of IL-Me-N7## adapter to the ligation mixes from the previous step and track the used adapters.

Important: Only one single-indexed adapter should be used per ligation reaction. Open one adapter tube at a time if using 8-index adapters and avoid cross-contamination.

For 96-index adapters supplied in a plate (layout described in Figure 3 on page 34), use a multichannel pipette to add the appropriate amount of adapters and change pipette tips after each pipetting step to avoid cross-contamination. Spin down the plate to collect liquid on the bottom of the wells before pipetting.

Note: The foil covering the 96-index adapters is pierceable with a pipette tip.

11. After adding the adapters, mix by pulse vortexing (10 times), spin down. Repeat mixing and spin down once and place samples on ice.
12. Program a thermal cycler with the protocol described in Table 33 below.

    Important: Use a thermal cycler with heated lid off. Ensure that lid is cooled down to room temperature.

Table 33. Ligation cycling conditions

Step	Temperature (°C)	Incubation time
1	25	15 min
2	4	Hold

13. Place ligation mixes from step 10 in the thermal cycler and run the ligation cycling program (Table 33 above).
14. After cycling is complete, proceed directly with cleanup of the ligated fragments.

Cleanup of ligated fragments

When working with plates use 8-channel pipettes to transfer and mix the samples. To avoid cross contamination, change pipette tips after each completion of mixing step. Before pipetting, verify that all pipetting tips are fitted well on the pipette. Visually inspect if all pipette tips have the same volume. Try to avoid bubble formation during pipetting.

15. Fill 15 mL volume of beads in a reagent reservoir. Ensure that the beads are resuspended homogeneously without any visual clumps

16. For sample purification, mix 90 µL (1x) QIAseq Beads with each sample by pulse vortexing. Ensure that the beads are equilibrated at room temperature and resuspended homogeneously without any visual clumps.

17. Seal the plate and incubate for 5 min at room temperature. Pulse spin the plate to collect all liquid on the bottom, immobilize beads on a 96-magnetic stand for approximately 5 min, and discard the clear supernatant using the multichannel pipette.

    Note: Time required for immobilization depends on the type of magnet and on tubes that are being used. Ensure that the supernatant is clear by visual inspection.

18. Fill 25 mL of freshly prepared 70% ethanol in a reagent reservoir and pipette 180 µL of 70% ethanol into each bead pellet immobilized on the magnet.

19. Discard the supernatant. Carefully remove all remaining ethanol droplets from the tube inner walls.

20. Incubate on the magnetic stand for 7–10 min until the beads are dry. Over-drying may result in lower DNA recovery. Remove from the magnetic stand.

21. Elute by carefully resuspending in 55 µL Nuclease-free Water. To pipette Nuclease-free Water, use a repeated pipettor or microdispensing pipette. Incubate for 5 min at room temperature. Immobilize beads and transfer 52 µL supernatant to a new plate.

22. Mix 52 µL (1x) QIAseq Beads with each sample by pulse-vortexing and repeat steps 17–20.

    Note: Visually inspect that the pellet is completely dry to avoid ethanol carryover and inhibition in the next reaction.

23. Elute by resuspending beads in 20 µL Nuclease-free Water. Incubate for 5 min at room temperature. Immobilize the beads and transfer 17 µL of supernatant into a new PCR

plate. Avoid any magnetic bead carryover with the ligated DNA fragments. Ligated DNA may be stored overnight at –15°C to –30°C if needed.

Target enrichment

24. Thaw DNA from step 23 if stored at –15°C to –30°C and amplification reagents on ice. Mix all reagents gently, spin down, and place on ice.

25. Prepare a reaction mix according to the table below. Add each component in the order listed in this table.

Table 34. Target enrichment reaction mix

Component	Volume (µL) per 96 reactions plus 10%
Nuclease-free Water	169
GeneRead DNAseq Panel 5x PCR Buffer	844.8
dNTP Mix (10 mM)	84.5
QIAseq Targeted Methyl Panel	844.8
IL Forward Primer	169
HotStarTaq DNA Polymerase	316.8
Total	**2428.9**

26. pipette with a microdispensing pipette 23 µL of the target enrichment reaction mix into the 17 µL of ligated and purified DNA from step 23. Mix by pipetting up and down 10 times, spin down, and place on ice.

27. Program a thermal cycler with the protocols described in Table 35 on the facing page. Set the thermal cycler heated lid on.

Table 35. Target enrichment cycling conditions

		Incubation time	
Step	**Temperature (°C)**	**<2000 primer/tube**	**>2000 primer/tube**
1	95	13 min	13 min
2	98	2 min	2 min
3 (8 cycles)	98	15 s	15 s
	65	10 min	15 min
4	72	5 min	5 min
5	4	Hold	Hold

28. Place the PCR tubes in the thermal cycler and start the preprogrammed target enrichment cycling with the conditions outlined in Table 35 above.
29. After cycling is complete, **quickly** transfer samples on ice.
30. Add 2 µL of ice cold TM Stop Solution to the 40 µL sample mix and immediately place samples back on ice.

    Note: After the addition of TM Stop Solution, DNA can be stored overnight at –15°C to –30°C, if needed.

Cleanup of the target enrichment reaction

31. Add 10 mL of well mixed beads into a reagent reservoir.
32. For sample purification, mix carefully 42 µL (1x) QIAseq Beads with each sample by pipetting up and down 10x using a multichannel pipette. Ensure that the beads are equilibrated at room temperature and resuspended homogeneously without any visual clumps.
33. Seal the plate and incubate for 5 min at room temperature. Briefly spin down and collect all liquid on the plate bottom and immobilize beads on a 96-magnetic stand for

approximately 5 min and discard the clear supernatant.

Note: Time for immobilization depends on the type of magnet. Ensure that the supernatant is clear by visual inspection.

34. Fill 25 mL of freshly prepared 70% ethanol in a reagent reservoir and pipette 180 µL of 70% ethanol to each bead pellet immobilized on the magnet.
35. Discard the supernatant. Carefully remove all remaining ethanol droplets from the tube inner walls.
36. Incubate on the magnetic stand for 5–10 min until the beads are dry. Over-drying may result in lower DNA recovery, so visual inspection is strongly recommended. Remove from the magnetic stand.
37. Elute by resuspending in 55 µL Nuclease-free Water. Incubate for 5 min at room temperature. Immobilize beads and transfer 52 µL supernatant to a new tube.
38. Mix 52 µL (1x) QIAseq Beads with each sample by pipetting up and down 10x and repeat steps 34–36.

    Note: Beads need to be completely dried to avoid ethanol carryover and inhibition in the next reaction. Remove any ethanol droplets with a pipette.
39. Elute by resuspending beads in 20 µL Nuclease-free Water. Incubate for 5 min at room temperature. Immobilize the beads and transfer 17 µL of supernatant into a new tube. Avoid any magnetic bead carry over. Store at –15°C to –30°C.

Library amplification

This step will amplify the library and add the second index.

For library amplification, use the number of cycles indicated in Table 36 on the facing page.

Table 36. Typical required cycles for library amplification

	50–150 primer per pool		151–2000 primer per pool		2001–5000 primer per pool	
Input (ng)	**gDNA**	**FFPE-DNA**	**gDNA**	**FFPE-DNA**	**gDNA**	**FFPE-DNA**
50	29	–	28	–	27	–
100	27	29	26	28	25	27
200	25	27	24	26	23	25

40. Thaw DNA (from step 39 on the previous page) and amplification reagents on ice.
41. Prepare a reaction mix by adding the components in the order according to Table 37 below.
42. Mix by pulse vortexing and spin down and place on ice.

    Note: Total volume of the amplification reaction is 20 µL.

Table 37. Universal PCR mix if using QIAseq Methyl DNA 96-index I Set A, B, C, or D*

Component	Volume (µL) per 96 reactions plus 10%
Nuclease-free Water	169
QIAseq UPCR Buffer 5x	422.4
HotStarTaq DNA Polymerase	105.6
Total	697

Note: Applies to QIAseq IL-S5 Index Primer Plate in Set A, B, C, or D. The final library dual sample index is determined by the combination of the IL-N7 Adapter Plate and the QIAseq IL-S5 Index Primer Plate. Total sample index level can be up to 384-plex if using QIAseq 96-index Set A, B, C, and D together.

43. Add 13.4 µL of each DNA from step 34 to one well of the QIAseq IL-S5 Index Primer Plate in Set A, B, C, or D, as illustrated in Figure 4 on page 42.
44. Add 6.6 µL of the universal PCR mix prepared according to Table 14 to each well of the adapter plate already including the DNA. Seal the plate, mix, spin down, and place on

ice.

Note: Total volume of reaction is 20 µL.

45. Place the tubes or plates with the reaction mixes from step 44 in the cycler and start the cycling program as outlined in Table 38 below.

Table 38. Library amplification cycling conditions (UPCR)

Step	Temperature (°C)	Incubation time
1	95	13 min
2	98	2 min
3 (Y-cycles*)	98	15 s
	62	2 min
4	72	5 min
5	4	Hold

* Y-cycles: cycle number extracted from Table 12.

46. After cycling completion, proceed with library purification. Alternatively, the amplified library can be stored at –30°C to –15°C.

Cleanup of amplified library

47. Add 11 mL of well mixed beads into a reagent reservoir.

48. Add 80 µL of ice cold nuclease-free water using a microdispensing pipette to the 20 µL sample from step 46 and mix.

49. Add 100 µL (1x) QIAseq Beads to each sample and mix thoroughly by pipetting up and down 10x.

50. Incubate for 5 min at room temperature. Immobilize beads on a magnet and discard the clear supernatant.

51. Fill 25 mL of freshly prepared 70% ethanol in a reagent reservoir and pipette 180 µL of 70% ethanol to each bead pellet immobilized on the magnet.

52. Discard the supernatant. Carefully remove all remaining ethanol droplets from the tube inner walls.

53. Incubate on the magnetic rack for 5–10 min until the beads are dry. Over-drying may result in lower DNA recovery. Remove from the magnetic stand.

54. Elute by carefully resuspending in 25 µL Nuclease-free Water. Incubate for 5 min at room temperature. Immobilize beads on a magnet and transfer 20 µL supernatant to a new LoBind tube.

The libraries can be stored at –30°C to –15°C prior to quantification using the QIAseq Library Quant System.

Assess the quality of the library using a capillary electrophoresis device or comparable method. Check for the correct size distribution of library fragments. Most of the library fragments are between 300–1000 bp. The median fragment size can be used for subsequent qPCR-based quantification methods.

Protocol: Library generation from bisulfite-converted cfDNA in 96-well format

This procedure describes the NGS library preparation starting from bisulfite-converted, single stranded DNA. It includes end repair of the bisulfite-converted DNA, adapter ligation, and introduction of UMIs, target enrichment with the QIAseq Methyl Panel, library amplification with introduction of the second index, and library cleanup steps using magnetic beads.

Important points before starting

- This protocol is optimized for bisulfite-converted cfDNA using EpiTect Fast 96 Bisulfite Kit (cat. no. 59720).
- This procedure is also applicable for cfDNA converted using enzymatic methods.
- Avoid DNA contamination of reagents by using separate laboratory equipment (pipettes, filter pipette tips, reaction vials, etc.).
- Set up all reactions on ice.

Things to do before starting

- Prepare fresh 70% ethanol.
- Enzymes and enzyme mixes should be thawed on ice and placed on ice until use.
- All other components can be thawed at room temperature but placed on ice immediately after thawing. Equilibrate Ligase Buffer 2x at room temperature.
- All buffers and reagents should be vortexed before use to ensure thorough mixing and spin down to collect all liquids on the bottom of the tubes.
- All enzyme mixes should be placed on ice until use. Avoid extended vortexing of the enzymes and enzyme mixes.

- 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

End repair of bisulfite-converted cfDNA:

1. Thaw bisulfite-converted DNA from step 16 ("Protocol: Library generation from bisulfite-converted gDNA and FFPE DNA in 96-well format") and use the total volume of 20 µL for the end repair reaction.
2. Set up the bisulfite-converted DNA repair reaction mix on ice according to Table 39. Mix by pulse vortexing (8–10 times) and spin down. Keep the reaction on ice.

Table 39. Bisulfite-converted DNA repair reaction mix for 96 reactions

Component	Volume (µL) per 96 reactions plus 10%
TM-repair buffer, 10x	316.8
Nuclease-free Water	633.6
T4 Polynucleotide Kinase	105.6

3. Using a microdispensing pipette, add 10 µL of the master mix of the table above in each well of a 96 0.2 mL-well PCR plate.
4. Add the 20 µL bisulfite-converted DNA from step 16 to the master mix plate. Total volume is 30 µL.
5. Seal the plate with an adhesive foil or cap-strips and mix by repeatedly vortexing 8–10 times. Make sure that all wells of the plates are mixed, and then spin down.
6. Program a thermal cycler with the protocol described in Table 40 on the next page.

   Important: Use a thermal cycler with heated lid at 75°C. If not possible, run incubation at 37°C with an open lid, and then close the heated lid for incubation at 65°C.

Table 40. Bisulfite-converted DNA repair cycling conditions

Step	Temperature (°C)	Incubation time (min)
1	37	30
2	65	20
3	4	Hold

7. Transfer the reaction mix from step 2 to the thermal cycler and start the bisulfite converted DNA repair cycling program (Table 40 above). Place samples on ice after cycling completion.

Adapter ligation

8. During bisulfite-converted DNA repair cycling, prepare the ligation mix according to Table 41 below. Mix thoroughly by pulse vortexing, and then spin down.

   Note: Ligase Buffer 2x should be equilibrated at room temperature. The ligase buffer has a high density. Mix well by pulse vortexing before pipetting. Avoid carryover of big droplets with the pipette tip and pipette carefully into the tube, ensuring the accurate amount of buffer.

Table 41. Ligation mix

Component	Volume (µL) per 96 reactions plus 10%
Ligase Buffer, 2x	4752
Nuclease-free Water	633.6
DNA Ligase	422.4
Total master mix volume	**55**

9. Add 55 µL ligation master mix to each 30 µL end-repaired DNA sample from the previous step and mix by pulse vortexing 4 times and then spin down.

10. Add 5 µL of IL-Me-N7## adapter to the ligation mixes from the previous step and track the used adapters.

    Important: Only one single-indexed adapter should be used per ligation reaction. Open one adapter tube at a time if using 8-index adapters and avoid cross-contamination.

    For 96-index adapters supplied in a plate (layout described in Figure 3), use a multichannel pipette to transfer the appropriate amount of adapters. Spin down the plate to collect liquid on the bottoms of the well before pipetting.

    Note: The foil covering the 96-index adapters is pierceable with a pipette tip.

11. After adding the adapters, mix by short vortexing, spin down, and place samples on ice.

12. Program a thermal cycler with the protocol described in Table 42 below.

    Important: Use a thermal cycler with heated lid off. Ensure that lid is cooled down to room temperature.

Table 42. Ligation cycling conditions

Step	Temperature (°C)	Incubation time
1	25	15 min
2	4	Hold

13. Place the ligation mixes from step 10 in the thermal cycler and run the ligation cycling program (Table 42 above).

14. After cycling is complete, proceed directly with cleanup of the ligated fragments.

Cleanup of ligated fragments

Use 8-channel pipettes to transfer and mix the samples when working with plates. To avoid cross contamination, change pipette tips after each completion of mixing step. Before

pipetting, check if all pipetting tip are fitted well on the pipette. Visually inspect if all pipette tips have the same volume. Try to avoid the formation of bubbles during pipetting.

15. Fill 15 mL volume of beads in a reagent reservoir. Ensure that the beads are resuspended homogeneously without any visible clumps.
16. For sample purification, mix 108 µL (1.2x) QIAseq Beads with each sample by pulse vortexing. Ensure that beads are equilibrated at room temperature and are completely resuspended without any visible clumps.
17. Seal the plate and incubate for 5 min at room temperature. Pulse spin the plate to collect all liquid on the bottom, immobilize beads on a 96-magnetic stand for approximately 5 min, and discard the clear supernatant using the multichannel pipette.

    Note: Time for immobilization is dependent on the type of magnet. Ensure that supernatant is clear by visual inspection.
18. Fill 25 mL of freshly prepared 70% ethanol in a reagent reservoir and pipette 180 µL of 70% ethanol to each bead pellet immobilized on the magnet.
19. Discard the supernatant. Carefully remove all remaining ethanol droplets from the tube inner walls. Do not disturb the immobilized magnetic bead pellet
20. Incubate on the magnetic stand for 5–10 min until the beads are dry. Over-drying may result in lower DNA recovery. Remove from the magnetic stand.
21. Elute by carefully resuspending in 55 µL of Nuclease-free Water. To pipette Nuclease-free Water, use a repeated pipettor or microdispensing pipette. Incubate for 5 min at room temperature. Immobilize beads and transfer 52 µL supernatant to a new tube.
22. Mix 63 µL (1.2x) QIAseq Beads with each sample by pulse-vortexing and repeat steps 17–20.

    Note: Visually inspect that the pellet is completely dry to avoid ethanol carryover and inhibition in the next reaction.

23. Elute by resuspending beads in 20 µL of Nuclease-free Water. Incubate for 5 min at room temperature. Immobilize the beads and transfer 17 µL of supernatant into a new PCR plate. Avoid any magnetic beads carryover with the ligated DNA fragments. Ligated DNA may be stored overnight at –15°C to –30°C if needed.

Target enrichment

24. Thaw DNA from step 23 if stored at –15°C to –30°C and amplification reagents on ice. Mix all reagents gently, spin down, and place on ice.

25. Prepare a reaction mix according to Table 43 below. Add each component in the order listed in this table.

Table 43. Target enrichment reaction mix

Component	Volume (µL) per 96 reactions plus 10%
Nuclease-free Water	169
GeneRead DNAseq Panel 5x PCR Buffer	844.8
dNTP Mix (10 mM)	84.5
QIAseq Targeted Methyl Panel	844.8
IL Forward Primer	169
HotStarTaq DNA Polymerase	316.8
Total	**2428.8**

26. Using a microdispensing pipette, add 23 µL of the target enrichment reaction mix to 17 µL of ligated and purified DNA from step 23. Mix by pipetting up and down 10x, spin down, and place on ice.

27. Program a thermal cycler with the protocols described in the table below. Set the cycler heated lid on.

Table 44. Target enrichment cycling conditions

		Incubation time	
Step	**Temperature (°C)**	**<2000 primer/tube**	**>2000 primer/tube**
1	95	13 min	13 min
2	98	2 min	2 min
3 (8 cycles)	98 65	15 s 10 min	15 s 15 min
4	72	5 min	5 min
5	4	Hold	Hold

28. Place the PCR plate in the thermal cycler and start the preprogrammed target enrichment cycling with the conditions outlined in Table 44 above.
29. After cycling is complete, **quickly** transfer samples on ice.
30. Add 2 µL of ice cold TM Stop Solution to the 40 µL sample, seal the plate, mix by pulse vortexing 10 times, and place samples back on ice. Work quickly with a microdispensing pipette.

    Note: After the addition of TM Stop Solution, DNA can be stored overnight at –15°C to –30°C, if needed.

Cleanup of the target enrichment reaction

31. Add 12 mL of well mixed beads into a reagent reservoir.
32. For sample purification, carefully mix 50.4 µL (1.2x) QIAseq Beads with each sample by pipetting up and down 10 times using a multichannel pipette. Ensure that the beads are resuspended homogeneously without visible clumps.
33. Seal the plate and incubate for 5 min at room temperature. Briefly spin down and collect all liquid on the plate bottom. Immobilize beads on a 96-magnetic rack for approximately

5 min and discard the clear supernatant.

Note: Time for immobilization depends on the type of magnet. Ensure that supernatant is clear by visual inspection.

34. Fill 25 mL of freshly prepared 70% ethanol in a reagent reservoir and pipette 180 µL of 70% ethanol to each bead pellet immobilized on the magnet.
35. Discard the supernatant. Carefully remove all remaining ethanol droplets from the tube inner walls.
36. Incubate on the magnetic stand for 5–10 min until the beads are dry. Over-drying may result in lower DNA recovery; visual inspection is strongly recommended. Remove from the magnetic stand.
37. Elute by carefully resuspending in 55 µL Nuclease-free Water. Incubate for 5 min at room temperature. Immobilize beads and transfer 52 µL supernatant to a new tube.
38. Mix 62.4 µL (1.2x) QIAseq Beads with each sample by pulse vortexing and repeat steps 28–31.

    Note: Beads need to be completely dried to avoid ethanol carryover and inhibition in the next reaction. Remove any ethanol droplets with a pipette.
39. Elute by resuspending the beads in 20 µL Nuclease-free Water. Incubate for 5 min at room temperature. Immobilize the beads and transfer 17 µL of supernatant into a new tube. Avoid any magnetic beads carry over. Store at –15°C to –30°C.

Library amplification

This step will amplify the library and add the second index.

For library amplification, use the number of cycles as indicated in Table 45 on the next page.

Table 45. Typical required cycles for library amplification

Input cfDNA (ng)	50–150 primers per pool	151–2000 primers per pool	2001–5000 primers per pool
50	27	26	25
100	25	24	23
200	24	23	22

40. Thaw DNA (from step 39 on the previous page) and amplification reagents on ice.
41. Prepare a reaction mix by adding the components in the order according to Table 46 below.
42. Mix by pulse vortexing, and spin down. Place on ice.

    Note: Total volume of the amplification reaction is 20 µL.

Table 46. Universal PCR mix if using QIAseq Methyl DNA 96-index I Set A, B, C, or D

Component	Volume (µL) per 96 reactions plus 10%
Nuclease-free Water	169
QIAseq UPCR Buffer 5x	422.4
HotStarTaq DNA Polymerase	105.6
Total	**697**

Note: Applies to QIAseq IL-S5 Index Primer Plate in Set A, B, C, or D. The final library dual sample index is determined by the combination of the IL-N7 Adapter Plate and the QIAseq IL-S5 Index Primer Plate. Total sample index level can be up to 384-plex if using QIAseq 96-index Set A, B, C, and D together.

43. Add 13.4 µL of the DNA from step 39 to one well of the QIAseq IL-S5 Index Primer Plate in Set A, B, C, or D, as illustrated in Figure 4.
44. Add 6.6 µL of the universal PCR mix prepared according to Table 24 to each well of the adapter plate already including the DNA. Seal the plate, mix, spin down, and place on ice.

    Note: Total volume of reaction is 20 µL.

45. Place the plate with the reaction mixes from step 44 in the cycler and start the cycling program as outlined below.

Table 47. Library amplification cycling conditions (UPCR)

Step	Temperature (°C)	Incubation time
1	95	13 min
2	98	2 min
3 (Y-cycles*)	98	15 s
	62	2 min
4	72	5 min
5	4	Hold

* Y-cycles: cycle number extracted from Table 45 on the previous page.

46. After cycling completion, proceed with library purification. Alternatively, the amplified library can be stored at –30°C to –15°C.

Cleanup of amplified library

47. Add 13 mL of well-mixed beads into a reagent reservoir.
48. Using a microdispensing pipette, add 80 µL of ice-cold Nuclease-free Water to the 20 µL sample from step 46 and mix.
49. Add 120 µL (1.2x) QIAseq Beads to each sample and mix thoroughly by pipetting up and down 10 times.
50. Incubate for 5 min at room temperature. Immobilize beads on a magnet and discard the clear supernatant.
51. Fill 25 mL of freshly prepared 70% ethanol in a reagent reservoir and pipette 180 µL of 70% ethanol to each bead pellet immobilized on the magnet.

52. Discard the supernatant. Carefully remove all remaining ethanol droplets from the tube inner walls.

53. Incubate on the magnetic stand for 5–10 min until the beads are dry. Over-drying may result in lower DNA recovery. Remove from the magnetic stand.

54. Elute by carefully resuspending in 25 µL Nuclease-free Water. Incubate for 5 min at room temperature. Immobilize beads on a magnet and transfer 20 µL supernatant to a new LoBind tube.

The libraries can be stored at –30°C to –15°C prior to quantification using the QIAseq Library Quant System.

Assess the quality of the library using a capillary electrophoresis device or comparable method. Check for the correct size distribution of library fragments. The majority of the library fragments are between 300–1000 bp. The median fragment size can be used for subsequent qPCR-based quantification methods.

Ordering Information

Product	Contents	Cat. no.
QIAseq Targeted Methyl Panel (8)	Enzymes, Buffers, Reagents, and Magnetic Beads for generation of 8 libraries including enrichment primer panel. Generated libraries are for use with Illumina Instruments.	335501
QIAseq Targeted Methyl Panel (96)	Enzymes, Buffers, Reagents, and Magnetic Beads for generation of 96 libraries including enrichment primer panel. Generated libraries are for use with Illumina Instruments.	335511
QIAseq Custom Targeted Methyl Panel (96)	Enzymes, Buffers, Reagents, and Magnetic Beads for generation of 96 libraries including enrichment primer panel. Generated libraries are for use with Illumina Instruments.	335602
QIAseq Custom Targeted Methyl Panel (384)	Enzymes, Buffers, Reagents, and Magnetic Beads for generation of 384 libraries including enrichment primer panel. Generated libraries are for use with Illumina Instruments.	333603
QIAseq Methyl DNA 8-Index Kit	Contains all required adapters and primers for the library generation and the library amplification including 8 single use tubes of molecularly indexed adapters IL-Me-N7##, IL-S502 Index Primer, IL-Forward Primer, IL-Universal Primer, and QIAseq A Read 1 Primer I (100 µM).	335580
QIAseq Methyl DNA 96-Index A, B, C, or D set	Contains all required adapters and primers for the library generation and the library amplification in plate format and allow generation of 96-384 libraries, including IL-Me-N701-N715 (A or C) or IL-Me-N716-N729 (B or D) adapter plate; IL-S502-S511 (A or B set) or IL-S513-S522 (C or D set) Primer Plate, IL-Forward Primer, and QIAseq A Read 1 Primer I (100 µM).	335591 335592 335593 335594
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
QIAseq Universal Normalizer Kit	Enzymes, buffers and reagents to allow for the normalization of 24 or 96 Illumina-compatible libraries	180613 180615
EpiTect Fast DNA Bisulfite Kit (10)	Trial kit for 10 preps: Bisulfite Solution, DNA Protect Buffer, MinElute DNA Spin Columns, Carrier RNA, and Buffers	59802

Product	Contents	Cat. no.
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 DNA Bisulfite Kit (200)	For 200 preps: Lysis Buffer, Proteinase K, Bisulfite Solution, DNA Protect Buffer, MinElute DNA Spin Columns, Carrier RNA, and Buffers	59826
QIAamp DNA Mini Kit (50)*	For 50 DNA preps: 50 QIAamp Mini Spin Columns, QIAGEN Proteinase K, Collection Tubes (2 mL), reagents, and buffers	51304
QIAamp DNA FFPE Advanced Kit (50)*	QIAamp UCP MinElute Columns, collection tubes, Deparaffinization Solution, Proteinase K, RNase A, RNase-free Water, and buffers	56604
QIAamp MinElute cfDNA 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
QIAseq DNA QuantiMIZE Assay Kit	Assay 100 and Assay 200 for 400 x 25 µL reactions (400 µL) each; QuantiMIZE Control gDNA (50 µL); RNase- and DNase-free Water (1 mL x 4); GeneRead qPCR SYBR Green Mastermix (1.35 mL x 4)	333414
QIAseq DNA QuantiMIZE Array Kit	qPCR arrays for optimizing amount of input DNA and PCR cycling conditions for targeted enrichment of FFPE DNA	333404

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Document Revision History

Date	Description
10/2019	Initial release
09/2025	Updated screenshots for LRM. Added appendices: procedures for PhiX, Normalizer protocol, and EpiTect Fast96 conversion procedure with library prep. Typographical and formatting changes.

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