October 2019

QIAseq® Targeted Methyl Panel Handbook

Targeted next-generation sequencing of methylated DNA

Sample to Insight

QIAGEN

Contents

Kit Contents

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

QIAseq Methyl DNA 8-Index Kit	**(8)**
Catalog no.	**335580**
No. of preps	**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”, page 52.

QIAseq Methyl DNA 96-Index A, B, C or D set	(96-384)
Catalog nos.	**335591**
	335592
	335593
	335594
No. of preps	**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

QIAseq Methyl DNA Panel

Catalog no.	Product name	Total number of primers*	Panel size (kb) (approximate)
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-001Z	Human T-cell Infiltration Panel	1415	283

* The number of primers in custom panels is represented by the last digits of the catalog number. For example, a custom panel with catalog number CMHS-10019Z-749 has 749 primers.

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 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 indices (UMIs) from cells, tissues, and biofluids. The required amount of template for a single QIAseq Targeted Methyl sequencing reaction ranges from 1 to 100 ng for fresh gDNA, 10 to 200 ng for FFPE DNA, or 10–100 ng for ccfDNA.

QIAseq targeted methylation sequencing, which combines bisulfite-mediated conversion of unmethylated cytosines to uracil and next generation sequencing (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 specimen for next-generation sequencing can be difficult to obtain or can yield limited amounts of nucleic acids. Therefore, researchers wish 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 since it allows complete conversion of unmethylated cytosines while avoiding fragmentation of the DNA during bisulfite treatment.

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).

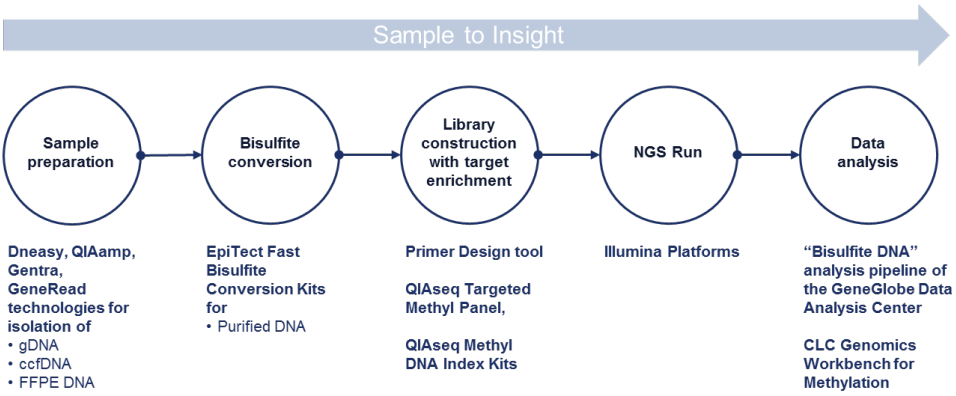


Figure 1. Targeted Methylation Sequencing Workflow.

Typical library concentrations from a QIAseq Methyl 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 ccfDNA.

Principle and procedure

QIAseq Targeted Methyl Panels are provided as single-tube primer mixes with up to 3,000 primers per panel. QIAseq Targeted Methyl DNA Panels are designed to enrich selected genes and regions using 1–100 ng fresh DNA, 10–200 ng FFPE DNA, or 10-200 ng ccfDNA. 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.

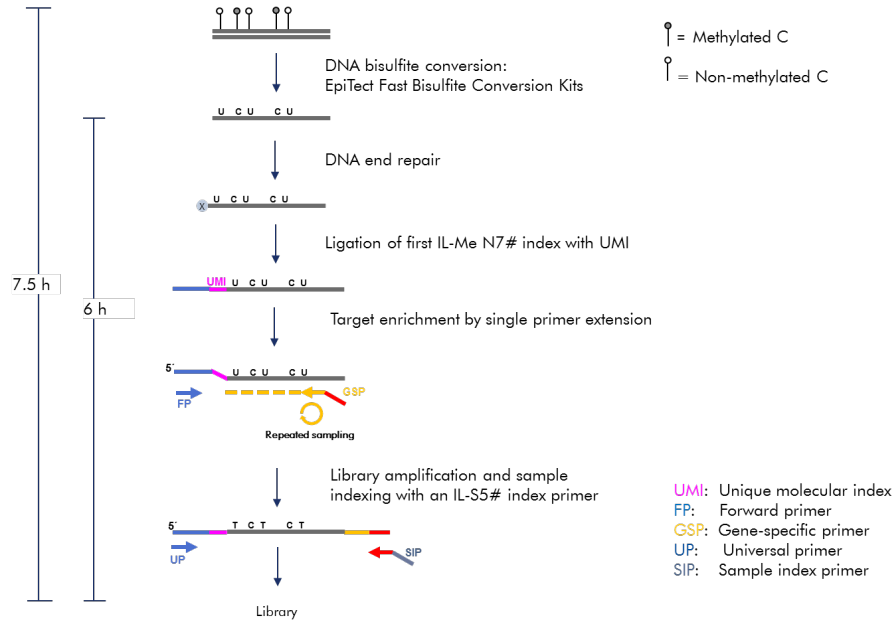


Figure 2. QIAseq Targeted Methyl Panel workflow.

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 indices 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. Non ligated 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 indices.

Next-generation sequencing

QIAseq Targeted DNA Panels are platform agnostic and are compatible with most medium- and high-throughput sequencers, including Illumina NGS systems (MiniSeq®, MiSeq® Personal Sequencer, NextSeq® 500, HiSeq® 1000, HiSeq 1500, HiSeq 2000, HiSeq 2500). When using Illumina NGS systems, QIAseq Targeted DNA 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 fresh gDNA and 151 bp PE reads if starting with ccfDNA and FFPE-DNA.

Data analysis

The QIAseq Targeted Methyl Panel Analysis pipeline is available at **ngsdataanalysis2.qiagen.com/QIAseqMethylDNA/analysisjob**. 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 12.0.2 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 ccfDNA using EpiTect Fast Bisulfite Conversion kits (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 or 59824)
- PCR tubes or plates
- LoBind® tubes for storage of generated libraries (Eppendorf, cat. nos. 0030108094, 0030108116, or 0030108132)
- Pipettes and pipette tips
- Magnetic racks for magnetic bead separation (e.g., DynaMag™-2 /96 Magnet, Thermo Fisher Scientific, cat. no. 12321D*)
- Thermocycler
- Microcentrifuge
- Vortexer
- NanoDrop® UV-Vis spectrophotometer for DNA- and bisulfite-treated DNA quantification
- Ice
- 96–100% ethanol
- QIAxpert®
- QIAxcel 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 Assay Kit (QIAGEN, cat. no. 333314) for library quantification

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

Important Notes

DNA preparation and quality control

High-quality DNA is essential for obtaining good bisulfite conversion, library generation, and sequencing results. The most important prerequisite for any DNA sequence analysis experiment is consistent, high-quality DNA from every experimental sample. 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.

Important:

- Do not use DEPC-treated water.
- 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.
- If genomic DNA samples must be harvested from biological samples for which kits are not available, please contact 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
GeneRead® DNA FFPE Kit	Formalin-fixed, paraffin-embedded tissues	180134
QIAamp Circulating Nucleic Acid Kit	Animal and human plasma and serum	55114
EpiTect Fast DNA Bisulfite Kit	Purified DNA	59824

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 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, since 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 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 that 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. When using Illumina NGS systems, QIAseq Targeted DNA libraries require a custom sequencing primer for Read 1 (QIAseq A Read1 Primer I) and 151 bp paired end reads. If the sample integrity will allow 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 ends reads**	**100**	**1000**	**2000**
MiniSeq	Mid output	16 M	80	8	4
MiniSeq	High output	50 M	250	25	13
MiSeq	V2	30 M	150	15	8
MiSeq	V3	50 M	225	25	13
NextSeq 500	Mid output	260 M	1300	131	70
NextSeq 500	High output	800 M	4000	406	216
HiSeq 2500	Rapid Run v2 with OBCG	600 M	3000	304	162

* 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 pipet tips that are certified sterile, DNase- and RNase-free.
- Before starting, wipe down work area and pipets with an RNase and DNA cleaning product such as RNase Away® (Sigma-Aldrich) or LookOut® DNA Erase (Sigma-Aldrich).
- For consistent bisulfite conversion, library construction and amplification, ensure the thermal cycler used in the protocol is in good working order and has been calibrated to the manufacturer's specifications.
- Read the entire protocol before beginning. Take note of stopping points where samples can be frozen at –30 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 if 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 homogenously without any visual clumps.

Recommended library quantification method

QIAGEN's QIAseq Library Quant Assay Kit (cat. no. 333314), which contains laboratory-verified forward and reverse primers together with a DNA standard, is highly recommended for accurate quantification of the prepared library. Alternatively, QIAxcel System or Agilent Bioanalyzer may be also used for quantification and qualification of the amplified libraries.

Protocol: Bisulfite Conversion of Unmethylated Cytosines in DNA using EpiTect Fast DNA Kits

This protocol enables bisulfite conversion of DNA amounts of 1 ng–2 µg in a volume of up to 20 µl (high concentration protocol).

The QIAseq targeted methyl Panel Library was optimized for 1–100 ng gDNA,10–200 ng FFPE DNA and 10-200 ng ccfDNA 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 further recommendations in handling of reagents and background information.
- 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 16 of the EpiTect Fast Bisulfite Conversion Handbook.
- 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.

1. Prepare the bisulfite reactions in 200 µl PCR tubes (not provided) according to Table 4. Add each component in the order listed.

Table 4. Bisulfite reaction components.

Component	Volume/reaction
DNA	Variable (maximum 20 µl)
Nuclease-free water	Variable*
Bisulfite Solution	85 µl
DNA Protect Buffer	35 µl
Total reaction volume	**140 µl**

***** The combined volume of DNA- and Nuclease-free water must total 20 µl

2. 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.

3. Perform the bisulfite DNA conversion using a thermal cycler. Program the thermal cycler according to Table 5. The complete cycle should take approximately 30 min.

   Optional: In some cases, it may be necessary to extend the 60°C cycle time to 20 min to achieve complete bisulfite DNA conversion.

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. BisU DNA repair cycling conditions

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

* In some cases, it may be necessary to extend the 60°C cycle time up to 20 min to achieve complete bisulfite DNA conversion.

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

- **IMPORTANT**: Since 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

1. 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.

2. 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*) 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 ccfDNA, adding Carrier RNA to experiments with input less than 40 ng will decrease DNA loss and increase coverage.

3. 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.
4. Place the necessary number of MinElute DNA spin columns and collection tubes in a suitable rack. Transfer the entire mixture from each tube from Step 7 into the corresponding MinElute DNA spin column.
5. Centrifuge the spin columns at maximum speed for 1 min. Discard the flow-through and place the spin columns back into the collection tubes.
6. 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.
7. 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.
8. Centrifuge the spin columns at maximum speed for 1 min. Discard the flow-through and place the spin columns back into the collection tubes.
9. 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.
10. Repeat Step 13 once.
11. Add 250 µl ethanol (96–100%) to each spin column and centrifuge at maximum speed for 1 min.
12. 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.
13. 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 no heating block is available repeat the spin columns step using a fresh collection tube to avoid any residual ethanol carry over.

14. Place the spin columns into clean 1.5 ml microcentrifuge tubes (not provided) and add 22 µl Buffer EB (elution buffer) directly onto the center of each spin-column membrane and close the lids gently.
15. Incubate the spin columns at room temperature for 1 min.
16. Centrifuge for 1 min at 15,000 x *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 2 weeks for DNA input in bisulfite treatment less than 200 ng.

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 EpiTect Fast Bisulfite Conversion kits but can be used with bisulfite conversion methods that generate single-stranded DNA with fragment mean size between 200–2000 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 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.
- 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 (20) 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 reaction on ice.

Table 6. Bisulfite converted DNA repair reaction mix setup

Component	Volume/reaction
TM-repair buffer, 10x	3 µl
Nuclease-free water	6 µl
T4 Polynucleotide Kinase	1 µl
Bisulfite converted DNA	20 µl
Total reaction volume	30 µl

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

Table 7. Bisulfite converted DNA repair cycling conditions

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

Note: 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.

4. Transfer reaction mix from step 2 to the thermocycler and start the bisulfite converted DNA repair cycling program (Table 7). Place 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 pipet carefully into the tube ensuring the accurate amount of buffer.

Table 8. Ligation mix

Component	Volume/reaction
Ligase Buffer, 2x	45 µl
Nuclease-free water	6 µl
DNA Ligase	4 µl
Total reaction volume	**55 µl**

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 described in Figure 3), use a multichannel pipette to pipet the appropriate amount of adapters and change pipette tips after each pipetting step to avoid cross-contamination. Spin down plate to collect liquid on bottoms of well 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 C set

	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-N716-N729 Adapter Plate in QIAseq 96-Index I Set B or D set

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

Table 9. Ligation cycling conditions

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

Note: Use a thermocycler with heated lid off. Ensure that lid is cooled down to room temperature.

10. Place ligation mixes from step 8 in the thermocycler 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 resuspended homogenously 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. Ensure that supernatant is clear by visual control.
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.
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 carryover and inhibition in 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 to –30°C if needed.

Target enrichment:

20. Thaw DNA from Step 17 if stored at –15 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/reaction*
Nuclease-free water	1.6 µl
GeneRead DNAseq Panel 5x PCR Buffer	8 µl
dNTP Mix (10 mM)	0.8 µl
QIAseq Targeted Methyl Panel	8 µl
IL Forward Primer	1.6 µl
HotStarTaq DNA Polymerase	3 µl
Total	**23 µl**

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

22. Mix carefully 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.

Table 11. Target enrichment cycling conditions

Step	Temperature	Incubation time <2000 primer/tube	Incubation time >2000 primer/tube
1	95°C	13 min	13 min
2	98°C	2 min	2 min
3 (8 cycles)	98°C 65°C	15 sec 10 min	15 sec 15 min
4	72°C	5 min	5 min
5	4°C	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 to –30°C, if needed.

Cleanup of the target enrichment reaction

27. For sample purification, mix carefully 42 µl (1x) QIAseq Beads with each sample by pulse-vortexing. Ensure that the beads are resuspended homogenously 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 is depending 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 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: Beads need to be completely dried to avoid ethanol carryover and inhibition in 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 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

	10-150 primer per pool		150-2000 primer per pool	
Input	**gDNA**	**FFPE-DNA**	**gDNA**	**FFPE-DNA**
1 ng	26	28	25	27
10 ng	23	26	22	25
50 ng	21	24	20	23
100 ng	19	21	18	20

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

35. Thaw DNA from Step 34 and amplification reagents on ice.
36. Prepare a reaction mix by adding the components in the order according to Table 13 if working with QIAseq Methyl DNA 8-index Kit and according to Table 14 if using the QIAseq Methyl DNA 96-index I Set A, B, C, or D. Track the number of the used indexes.

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

Component	Volume/reaction
DNA of step 31	13.4 µl
QIAseq UPCR Buffer 5x	4 µl
IL Universal primer	0.8 µl
IL-S502 Index Primer	0.8 µl
HotStarTaq DNA Polymerase	1 µl
Total	**20 µl**

37. Mix by pulse vortexing and spin down and place on ice.

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

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

Component	Volume/reaction
Nuclease-free water	1.6 µl
QIAseq UPCR Buffer 5x	4 µl
HotStarTaq DNA Polymerase	1 µl
Total	**6.6 µl**

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.

38. If working with QIAseq Methyl DNA 96-index I Set A, B, C, or D, add 13.4 µl of the 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.

IL-S502-S511 Index Primer Plate in QIAseq 96-index I Set A or B set

	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 C or D set

	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 pre-dispensed 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.

39. 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: The total volume of the amplification reaction is 20 µl.

40. Place the tubes or plates with the reaction mixes from step 37 and 39 in the cycler and start the cycling program as outlined in Table 15.

Table 15. Library amplification cycling conditions (UPCR)

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

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

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

Clean up of amplified library:

42. Add 80 µl of ice cold nuclease-free water to the 20 µl sample from Step 41 and mix.
43. Add 100 µl (1x) QIAseq Beads to each sample and mix thoroughly by pulse vortexing.
44. Incubate for 5 min at room temperature. Immobilize beads on a magnet and discard the clear supernatant.
45. Add 200 µl fresh 70% ethanol to each bead pellet immobilized on the magnet.
46. Discard the supernatant. Carefully remove all remaining ethanol droplets from the tube inner walls.
47. 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.

48. 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 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 300–1000 bp. The median fragment size can be used for subsequent qPCR-based quantification methods.

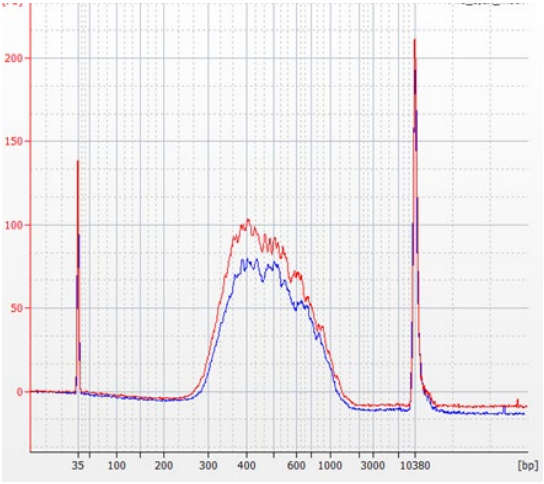


Figure 5. Capillary electrophoresis device trace of generated libraries. Capillary electrophoresis device trace data showing the correct size distribution of library fragments. Shown are replicate libraries starting from 40 ng bisulfite-converted DNA. Libraries were generated using a 750 primer-pool. Libraries were diluted 1:5 and loaded on an Agilent High Sensitivity DNA chip.

Protocol: Library Generation from Bisulfite-Converted ccfDNA

This procedure describes the NGS library preparation starting from bisulfite-converted, single-stranded ccfDNA and include 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 ccfDNA using EpiTect Fast Bisulfite Conversion kits, but can be used with bisulfite conversion methods that generate single-stranded DNA with fragment mean size of approximately 170 bp.
- Samples of 10–200 ng ccfDNA 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 ccfDNA.
- Avoid DNA contamination of reagents by using separate laboratory equipment (e.g., pipets, filter pipet 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 ccfDNA:

1. Thaw bisulfite converted ccfDNA from step 20 page 21 (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. Setup the bisulfite converted ccfDNA 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 ccfDNA repair reaction mix setup

Component	Volume/reaction
TM-repair buffer, 10x	3 µl
Nuclease-free water	6 µl
T4 Polynucleotide Kinase	1 µl
Bisulfite converted ccfDNA	20 µl
Total reaction volume	30 µl

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

Table 17. Bisulfite converted ccfDNA repair cycling conditions

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

Note: 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

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

Adapter ligation:

5. During bisulfite converted ccfDNA 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. Avoid carryover of big droplets with the pipette tip and pipet carefully into the tube ensuring the accurate amount of buffer.

Table 18. Ligation mix

Component	Volume/reaction
Ligase Buffer, 2x	45 µl
Nuclease-free water	6 µl
DNA Ligase	4 µl
Total reaction volume	**55 µl**

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 ccfDNA 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 described in Figure 3), use a multichannel pipette to pipet the appropriate amount of adapters. Spin down plate to collect liquid on bottoms of well 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.

Table 19. Ligation cycling conditions

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

Note: Use a thermocycler with heated lid off. Ensure that lid is cooled down to room temperature.

10. Place ligation mixes from step 8 in the thermocycler 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 144 µl (1.6x) QIAseq Beads with each sample by pulse vortexing. Ensure that beads 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 beads on a magnet for approximately 5 min and discard the clear supernatant.

    Note: Time for immobilization is dependent on the type of magnet. Ensure that supernatant is clear by visual control.
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.
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 83.2 µl (1.6x) 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 to –30°C if needed.

Target enrichment:

20. Thaw DNA from Step 19 if stored at –15 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 20. Add each component in the order listed in this table.

Table 20. Target enrichment reaction mix

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

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

22. Mix carefully 17 µl of ligated and purified DNA from Step 17 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.

Table 21.Target enrichment cycling conditions

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

24. Place the PCR tubes in the thermal cycler and start the preprogrammed 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 samples back on ice.

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

Cleanup of the target enrichment reaction

27. For sample purification, carefully mix 67.2 µl (1.6x) QIAseq Beads with each sample by pulse vortexing. Ensure that beads are completely mixed and 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: Time for immobilization is dependent 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 5–10 min until the beads are dry. Over-drying may result in lower DNA recovery; visual control 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 83.2 µl (1.6x) 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 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 beads carry over. Store at –15 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	10–200 primers per pool	201–2000 primers per pool
1 ng ccfDNA	24	22
10 ng ccfDNA	21	19
50 ng ccfDNA	20	18
100 ng ccfDNA	19	17

35. Thaw DNA from Step 34 and amplification reagents on ice.
36. Prepare a reaction mix by adding the components in the order according to Table 23 if working with QIAseq Methyl DNA 8-index Kit and according to Table 24 if using the QIAseq Methyl DNA 96-index I Set A, B, C, or D. Track the number of the used indexes

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

Component	Volume/reaction
DNA of step 31	13.4 µl
QIAseq UPCR Buffer 5x	4 µl
IL Universal primer	0.8 µl
IL-S502 Index Primer	0.8 µl
HotStarTaq DNA Polymerase	1 µl
Total	**20 µl**

37. Mix by pulse vortexing and spin down and place on ice.

Note: Total volume of reaction is 20 µl.

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

Component	Volume/reaction
Nuclease-free water	1.6 µl
QIAseq UPCR Buffer 5x	4 µl
HotStarTaq DNA Polymerase	1 µl
Total	**6.6 µl**

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.

38. If working with QIAseq Methyl DNA 96-index I Set A, B, C, or D, add 13.4 µl of the DNA from Step 32 to one well of the QIAseq IL-S5 Index Primer Plate in Set A, B, C or D, as illustrated in Figure 4.

39. 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: The total volume of the amplification reaction is 20 µl.

40. Place the tubes or plates with the reaction mixes from step 37 and 39 in the cycler and start the cycling program as outlined in Table 25.

Table 25. Library amplification cycling conditions (UPCR)

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

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

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

Clean up of amplified library:

41. Add 80 µl of ice cold water to the 20 µl sample from Step 36 and mix by pulse vortexing.
42. Add 160 µl (1.6x) QIAseq Beads to each sample and mix thoroughly by pulse vortexing. Ensure that the beads are resuspended homogenously without any visual clumps.
43. Incubate for 5 min at room temperature. Briefly spin down, immobilize beads on a magnet until supernatant is clear and discard the clear supernatant.
44. Add 200 µl fresh 70% ethanol to each bead pellet immobilized on the magnet.
45. Discard the supernatant. Carefully remove all remaining ethanol droplets from the tube inner walls.
46. 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.

47. 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 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 200–600 bp. The median fragment size can be used for subsequent qPCR-based quantification methods.

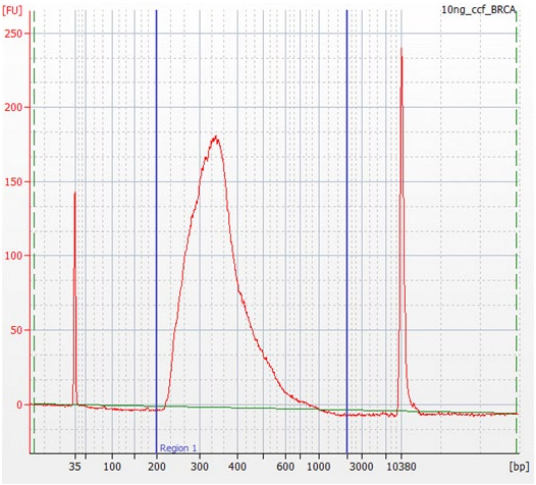


Figure 6. Capillary electrophoresis device trace of generated libraries. Capillary electrophoresis device trace data showing the correct size distribution of library fragments. Shown is a library starting from 10 ng bisulfite-converted ccfDNA. Libraries were generated using a 1400 primer-pool. Libraries were diluted 1:2 and loaded on an Agilent High Sensitivity DNA chip. The mean library fragment size of 350 bp represent exactly the size of ccfDNA of approximately 170 bp plus the adapter length.

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.
d)	Sub-optimal reaction conditions during enzymatic reactions	Ensure that cycling conditions and incubation temperatures are programmed correctly.
e)	Inefficient library amplification	Inefficient adapter–dimer removal using QIAseq Beads. Mix beads thoroughly and use the recommended amount for purification. Beads are overdried. Make visual control during drying of beads, 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 remove carefully before drying the beads.

		Comments and suggestions
Unexpected signal peaks in capillary electrophoresis device traces		
a)	Presence of shorter peaks between 90 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 case of DNA, the DNA was very strong fragmented and short fragments have been overamplified. Perform a second round of QIAseq Beads clean-up with 0.8x QIAseq beads for gDNA and FFPE preparations and 1.4x QIAseq Beads for ccfDNA 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 3 cycles and repeat bead clean-up.

Appendix A: Sequencing Setup on Illumina MiSeq and NextSeq

Important points before starting

- **Important**: Recommendations for library dilution concentrations and library loading concentrations are based on QIAseq Library Quant System (See "Appendix B: Adapter Bar Codes for QIAseq Targeted Methyl Panel DNA Libraries", page 52).
- **Important**: QIAseq A Read1 Primer I (Custom Read 1 Sequencing Primer) **MUST** be used when performing sequencing on Illumina platform.
- **Important**: QIAseq A Read1 Primer I (the Custom Read 1 Sequencing Primer) goes into the following specific reagent cartridge positions:

  MiniSeq Position #15

  MiSeq Position #18

  NextSeq Position #7
- **Important**: Paired-end sequencing **SHOULD** be used for QIAseq Targeted Methyl Panel on Illumina platform.
- Ensure libraries have been quantified using QIAseq Library Quant System (See "Appendix B: Adapter Bar Codes for QIAseq Targeted Methyl Panel DNA Libraries", page 52).
- For complete instructions on how to denature sequencing libraries, prepare custom index primers, and set up a sequencing run, please refer to the system-specific Illumina documents.

Sequencing Preparations for MiSeq

- Set up the sample sheet with Custom Sequencing Read 1 primer using Illumina Experiment Manager v1.2, or later (Figure 7). Sample index of QIAseq Targeted Methyl Panel is compatible with Illumina Nextera® XT v2 adapter sample index system. Set the parameters as follows:

  Category: Select Other

  Select Application: Check FASTQ Only

  Sample Prep Kit: Select Nextera XT v2

  Index Reads: Select 2

  Read Type: Select Paired End Read

  Cycles for both Read 1 and 2: Select 151

  Important: Check Custom Primer for Read 1

  Important: Check Use Adapter Trimming

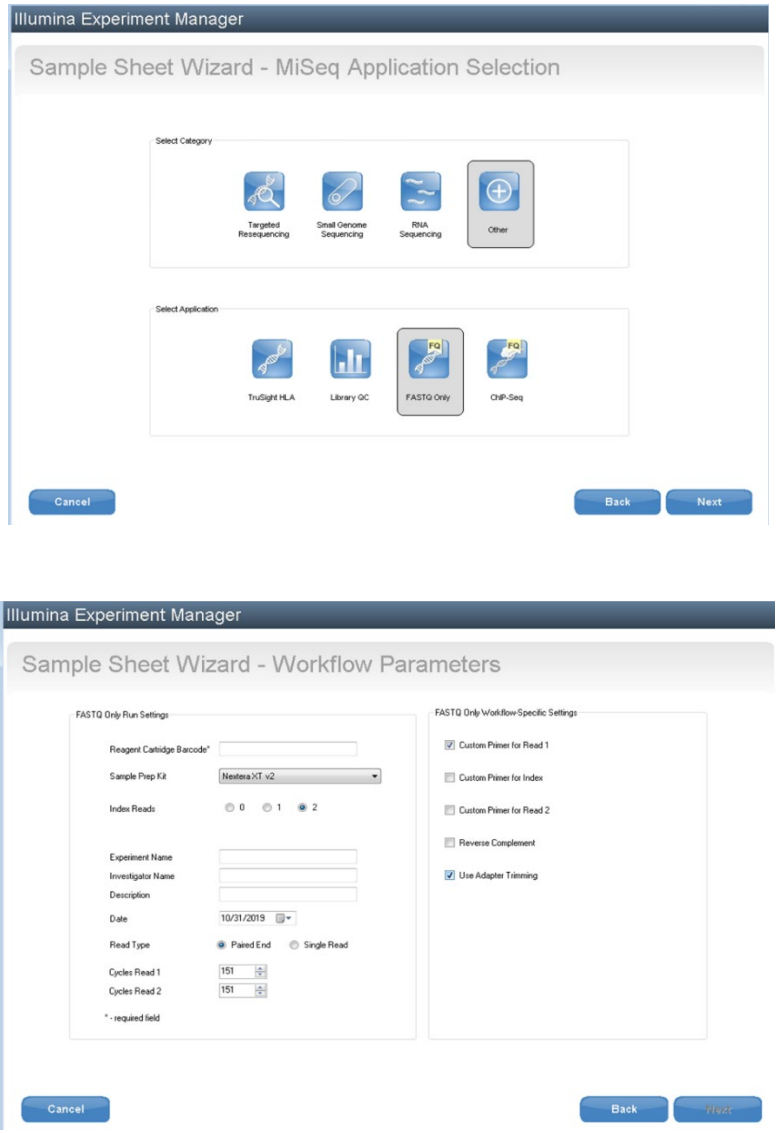


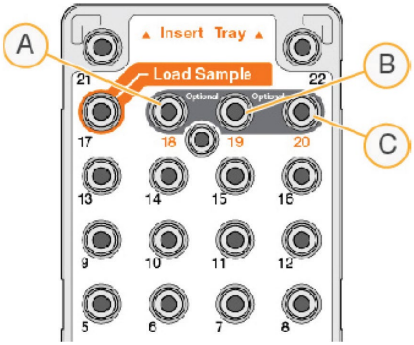
Figure 7 Sample sheet using Illumina Experiment Manager.

- Dilute libraries to 1.8 - 2 nM for MiSeq. Combine libraries with different sample indexes in equimolar amounts if 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.
- 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.

  Note: Recommendations for library loading concentrations are based on QIAseq Library Quant System.
- 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). For more details, please refer to the "Illumina protocol: miseq_using_custom_primers_15041638_b.pdf for the MiSeq".



A Position 18 for Read 1 Custom Primer

Figure 8. Loading the QIAseq A Read 1 Primer I into position 18 (shown by 'A' in the figure; B and C are not relevant)

- 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", page 53.

Sequencing Preparations for NextSeq

- Use paired end read (151 cycles for read 1 and 2).
- Use dual indexes (8 cycles of each).
- Set up the sample sheet with Custom Sequencing Read 1 primer using Illumina Experiment Manager v1.2, or later (Figure 9) Sample index of QIAseq Targeted Methyl Panel is compatible with Illumina Nextera XT v2 adapter sample index system. Set the parameters as follows:

  Category: Select Other

  Select Application: Check FASTQ Only

  Sample Prep Kit: Select Nextera XT v2

  Index Reads: Select 2

  Read Type: Select Paired End Read

  Cycles for both Read 1 and 2: Select 151

  Important: Check Use Adapter Trimming
- Dilute libraries to 0.5, 1, 2, or 4 nM for NextSeq. Combine libraries with different sample indexes in equimolar amounts if 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 4 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 5000 primers at 4 nM, and Library B has 600 primers at 4 nM; combining 50 µl Library A with 6 µl Library B will result in similar coverage depth for both Libraries A and B in the same sequencing run.

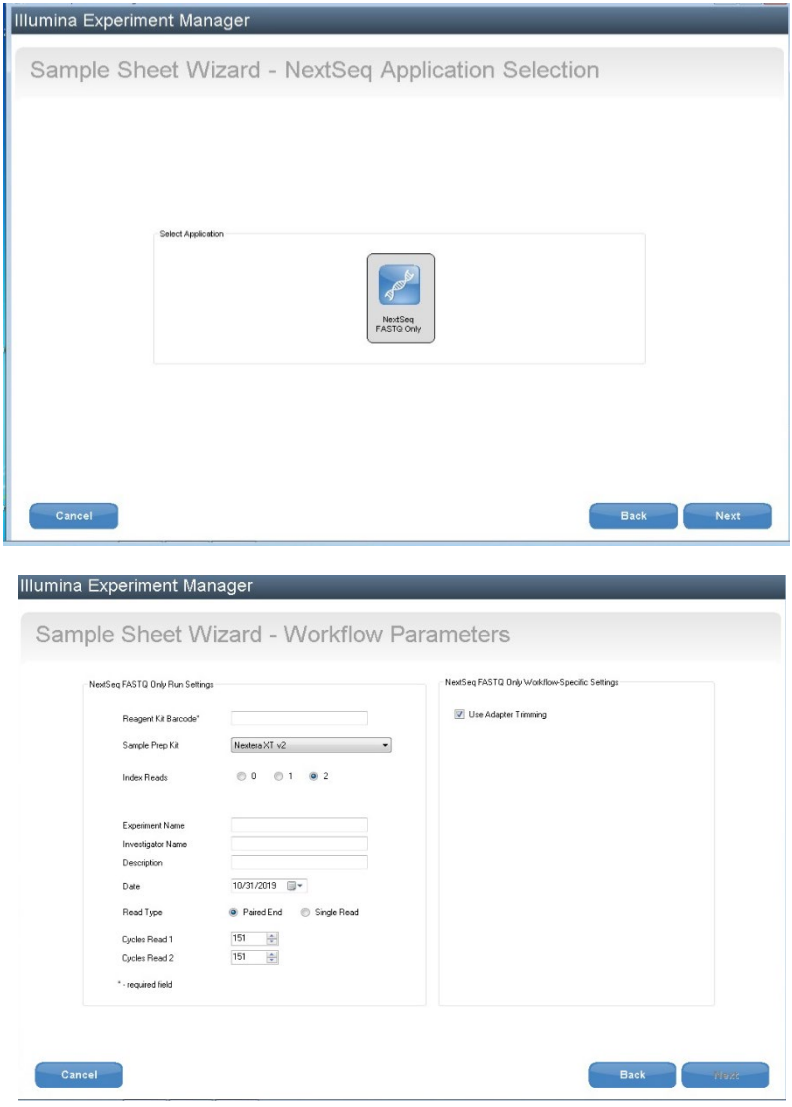


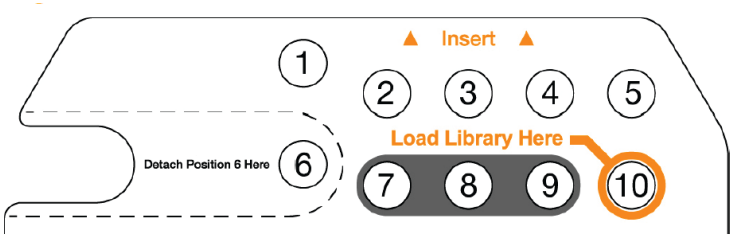
Figure 9.Sample sheet using Illumina Experiment Manager.

- Prepare and load the library onto a NextSeq according to the *NextSeq System Denature and Dilute Libraries Guide*. The final library concentration is 1.2–1.5 pM on the NextSeq.

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

- Use 1994 µl HT1 (Hybridization Buffer) to dilute 6 µl QIAseq A Read 1 Primer I (provided) to obtain a final concentration of 0.3 µM. Load 2 ml of the diluted QIAseq A Read 1 Primer I to position 7 of the NextSeq reagent cartridge.

  Note: All other steps refer to run setup workflow as described in the *NextSeq 500 SystemGuide* (part #15046563) or *NextSeq 550 System Guide* (part #15069765-02).



Position #	Custom Primer
7	Custom Read 1 primer

Figure 10. NextSeq reagent cartridge.

- 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", page 53 (**geneglobe.qiagen.com/analyze**)

Appendix B: 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-N721	GCAGCGTA		
IL-N722	CTGCGCAT		
IL-N723	GAGCGCTA		
IL-N724	CGCTCAGT		
IL-N726	GTCTTAGG		
IL-N727	ACTGATCG		
IL-N728	TAGCTGCA		
IL-N729	GACGTCGA		

Appendix C: 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 mapping to the reference genome, UMI counting, read trimming (removing primer sequences), and methylation calling.

Alternatively, data from QIAseq Targeted DNA 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 dropdown menu and click on "Start Analysis".
6. Upload or manage your data in the tab "Read Files". Upload FASTQ files using the button "Upload New Files".

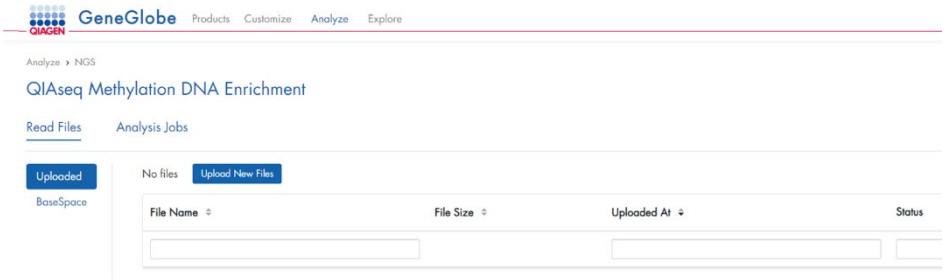


Figure 11. File Upload tab of the QIAseq Targeted Sequencing Data Analysis Portal.

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 click "Analyze" to start the job. Once analysis is completed, download the zip folder.

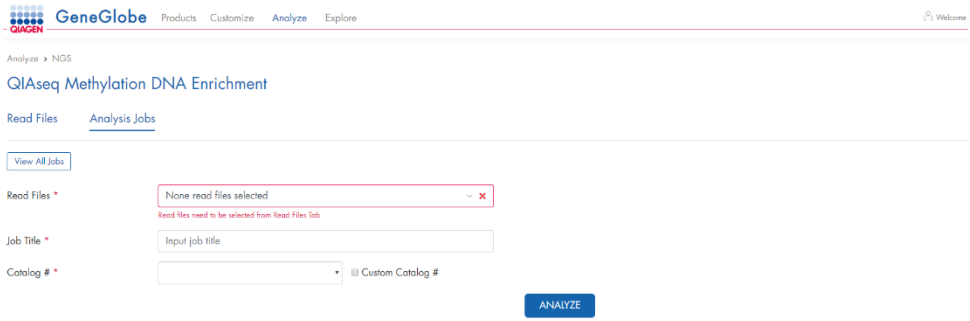


Figure 12. Analysis job creation tab of the QIAseq Targeted Sequencing Data Analysis Portal.

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
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
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
GeneRead DNA FFPE Kit (50)*	QIAamp MinElute columns, Proteinase K, UNG, collection tubes (2 ml), buffers, Deparaffinization Solution, RNase A	180134

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

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

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

Notes

Handbook Revision History

Date	Revision
10/2019	Initial release

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10/2019