May 2017

# QIAseq™ Targeted DNA Panel Handbook

Sample to Insight® solution for ultrasensitive targeted next-generation sequencing of DNA



QIAGEN

# Handbook Revision History

| Document | Cover date | Description of changes |
|---|---|---|
| QIAseq Targeted DNA Panel Handbook (R1) | July 2016 | Initial release. |
| QIAseq Targeted DNA Panel Handbook (R2) | May 2017 | Reagents added (use is highly recommended):<br>• FERA Solution (enhances efficiency)<br>• Ligation Buffer (enhances efficiency)<br>• FG solution (enables use of cfDNA containing cellular DNA)<br>Panel sizes added.<br>QIAseq Targeted DNA Panel Analysis pipeline portal updated.<br>Biomedical Genomics Workbench added as an analysis option.<br>Publication reference added for the QIAseq Targeted DNA Panel Analysis pipeline.<br>QIAxpert added as recommendation for assessing sample purity.<br>QIAxcel added as recommendation for assessing library quality.<br>Sample-specific reaction mixes and conditions added for procedures.<br>Fragmentation, end-repair and A-addition updated.<br>Volumes and bead ratios updated.<br>Adapter ligation procedures updated.<br>Library over-amplification addressed in Appendix E.<br>Figures and text updated. |

# Contents

# Kit Contents

| **QIAseq Targeted DNA Panel (including HC, Extended and Custom panels)** | | **(12)** | **(96)** |
|---|---|---|---|
| **Catalog no.** | | **333502 333512** | **333505 333515 333545 333525** |
| **Number of samples** | | **12** | **96** |
| One pool of region-specific primers | White | 75 µl | 600 µl |
| Fragmentation Buffer, 10x | Orange | 40 µl | 300 µl |
| Fragmentation Enzyme Mix | Brown | 75 µl | 600 µl |
| FERA Solution | Red | 15 µl | 110 µl |
| FG Solution | Yellow | 170 µl | 170 µl |
| Ligation Buffer, 5x | Clear | 160 µl | 1250 µl |
| DNA Ligase | Gray | 75 µl | 600 µl |
| Ligation Solution | Pink | 125 µl | 970 µl |
| Nuclease-Free Water | Blue | 1.5 ml | 10 ml |
| TEPCR Buffer, 5x | Violet | 60 µl | 500 µl |
| UPCR Buffer, 5x | Yellow | 60 µl | 500 µl |
| HotStarTaq® DNA Polymerase | Green | 30 µl | 240 µl |
| One bottle containing QIAseq Beads | – | 7 ml | 55 ml |

| **QIAseq Targeted DNA Booster Panel** | **(96)** |
|---|---|
| **Catalog no.** | **333535** |
| **Number of samples** | **96** |
| One pool of region-specific primers | 80 µl |

| **QIAseq 12-Index I**<br>**(12 sample index for 48 samples on Illumina® platform)** | **(48)** |
|---|---|
| **Catalog no.** | **333714** |
| **Number of samples** | **48** |
| IL-N7## Adapter contains 12 tubes of molecularly indexed adapters, with each tube corresponding to one sample index; each index can be used for up to 4 samples | 25 µl |
| IL-S502 Index Primer | 40 µl |
| IL-Forward Primer | 40 µl |
| IL-Universal Primer | 40 µl |
| QIAseq A Read1 Primer I (100 µM) | 24 µl |

| **QIAseq 96-Index A, B, C or D set I**<br>**(96 sample index for 384 samples on Illumina platform)** | **(384)** |
|---|---|
| **Catalog no.** | **333727**<br>**333737**<br>**333747**<br>**333757** |
| **Number of samples** | **384** |
| IL-N701-N715 (A or C set) or IL-N716-N729 (B or D set) Adapter Plate; each plate contains 12 molecularly indexed adapters, with each well corresponding to one sample index; each index can be used for up to 4 samples | 160 µl |
| 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 IL-S5## index primer and IL-Universal PCR primer pair for PCR amplification and sample indexing; kit can process up to 384 total samples | 4 |
| IL-Forward Primer | 310 µl |
| QIAseq A Read1 Primer I (100 µM) | 4 x 24 µl |
| 12-cap strips | 16 |

| **QIAseq 12-Index L (12 sample index for 48 samples on Ion Torrent™ platform)** | **(48)** |
|---|---|
| **Catalog no.** | **333764** |
| **Number of samples** | **48** |
| LT-BC# Adapter contains 12 tubes of molecularly indexed adapters, with each tube corresponding to one sample index; each index can be used for up to 4 samples | 25 µl |
| LT-P1 Primer | 40 µl |
| LT-Forward Primer | 40 µl |
| LT-Universal Primer | 40 µl |

| **QIAseq 12-Index L (96 sample index for 384 samples on Ion Torrent platform)** | **(384)** |
|---|---|
| **Catalog no.** | **333777** |
| **Number of samples** | **384** |
| LT-BC1-96 Adapter Plate; each plate contains 96 molecularly indexed adapters, each well corresponding to one sample index; kit can process up to 384 total samples | 20 µl per well |
| LT-P1 Primer | 310 µl |
| LT-Forward Primer | 310 µl |
| LT-Universal Primer | 310 µl |
| 12-cap strips | 16 |

| Cat. no. | Product name | Total number of primers* | Panel size (kb) |
|---|---|---|---|
| DHS-001Z | Human Breast Cancer Panel | 4831 | 370,942 |
| DHS-002Z | Human Colorectal Cancer Panel | 2929 | 215,328 |
| DHS-003Z | Human Myeloid Neoplasms Panel | 5887 | 436,672 |
| DHS-005Z | Human Lung Cancer Panel | 4149 | 318,059 |
| DHS-101Z | Human Actionable Solid Tumor Panel | 651 | 15,160 |
| DHS-102Z | Human BRCA1 and BRCA2 Panel | 223 | 16,405 |
| DHS-103Z | Human BRCA1 and BRCA2 Plus Panel | 348 | 25,590 |
| DHS-104Z | Human Pharmacogenomics Panel | 146 | 3,313 |
| DHS-105Z | Human Mitochondria Panel | 222 | 16,570 |
| DHS-3011Z | Human Inherited Disease Panel | 11,579 | 838,627 |
| DHS-3501Z | Human Comprehensive Cancer Panel | 11,311 | 836,670 |

* The number of primers in Custom, Extended and Booster panels is represented by the last digits of the catalog number. For example, a custom panel with catalog number CDHS-00100Z-1256 has 1256 primers.

# Storage

QIAseq Targeted DNA Panels (except QIAseq Beads) are shipped on dry ice and should be stored at –30°C to –15°C upon arrival. QIAseq Beads are shipped on cold packs and should be stored at 4°C. When stored properly, all reagents are stable for up to 6 months after delivery.

QIAseq Index kits are shipped on dry ice and should be stored at –30°C to –15°C upon arrival. When stored properly, QIAseq Index kits are stable for up to 6 months after delivery.

# Intended Use

QIAseq Targeted DNA Panels and QIAseq 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

To ensure consistent product quality, QIAseq Targeted DNA Panels are tested against predetermined specifications.

# Introduction

QIAseq Targeted DNA Panels enable sample-to-insight, targeted next-generation sequencing (NGS) of DNA. This highly optimized solution facilitates ultrasensitive variant detection using integrated unique molecular indices (UMIs) from cells, tissue and biofluids. The required amount of template for a single QIAseq Targeted sequencing reaction ranges from 10 to 40 ng for fresh DNA or 40 to 250 ng for FFPE DNA.

Next-generation sequencing of DNA is a powerful tool for the detection of genetic variations, including somatic mutations, single nucleotide polymorphisms (SNPs), copy number variation and small insertions/deletions. Target enrichment technology enhances DNA 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 cost. Many commercially available target enrichment, library preparation and sequencing methods all use DNA polymerase and amplification processes that introduce substantial bias and artifacts. This results in artifactual errors that greatly limit the detection of true low-frequency variants in heterogeneous samples, such as tumors. QIAseq Targeted DNA Panels overcome these biases/artifacts by utilizing a highly optimized reaction chemistry whereby UMIs are integrated into a single gene-specific, primer-based targeted enrichment process. QIAseq Targeted DNA Panels have also been optimized in combination with a specially formulated enrichment chemistry to achieve highly efficient enrichment on both regular and GC-rich regions at high multiplex levels. In addition, the panels are not platform-specific and are compatible with most medium- and high-throughput sequencers, including Illumina and Thermo Fisher Scientific systems.

Data analysis tools have been developed to perform all steps necessary to generate a DNA sequence variant report from NGS data. Collectively, QIAseq Targeted DNA Panels are a sample-to-insight solution for precision variant detection of targeted genomic regions using NGS (Figure 1).

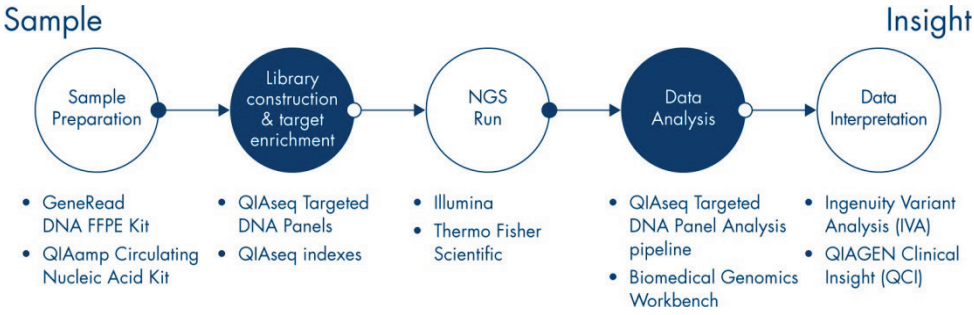



**Figure 1. Overview of the sample-to-insight NGS workflow with QIAseq Targeted DNA Panels.** The complete sample-to-insight procedure begins with DNA extraction. Next is library construction and target enrichment with QIAseq Targeted DNA Panels. Following NGS, data analysis is performed using the QIAseq Targeted DNA Panel Analysis Software pipeline or Biomedical Genomics Workbench. Ultimately, detected variants can be interpreted with the Ingenuity® Variant Analysis (IVA) tool or with QIAGEN Clinical Insight (QCI™).

## Principle and procedure

QIAseq Targeted DNA Panels are provided as single tube primer mixes, with up to 20,000 primers per panel. QIAseq Targeted DNA Panels are designed to enrich selected genes and regions using 10–40 ng fresh DNA or 40–250 ng FFPE DNA (Figure 2). Lower input amounts are possible; however, this will lead to fewer sequenced UMI and reduced variant detection sensitivity.

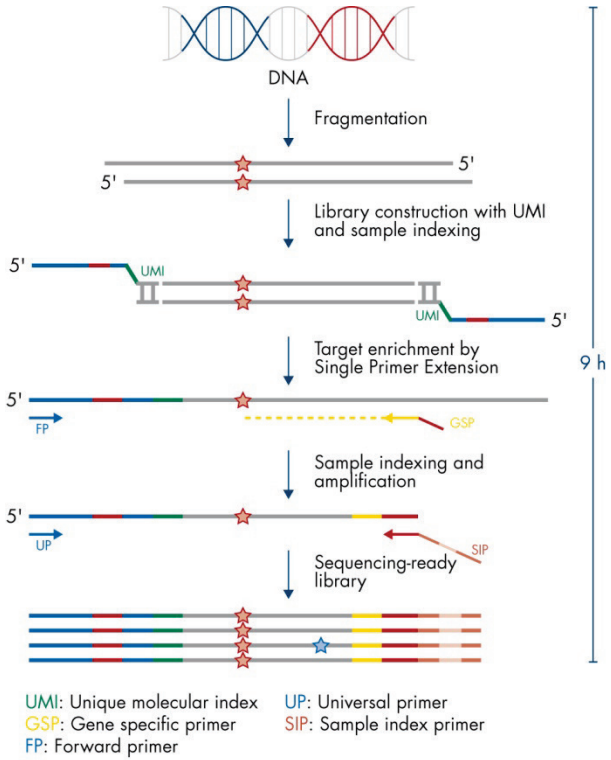



**Figure 2. QIAseq Targeted DNA Panels workflow.**

## Fragmentation

Genomic DNA samples are first fragmented, end repaired and A-tailed within a single, controlled multi-enzyme reaction. The prepared DNA fragments are then ligated at their 5' ends with a sequencing platform-specific adapter containing UMIs and sample index.

## UMI assignment

Prior to target enrichment and library amplification, each original DNA molecule is assigned a unique sequence or index, commonly referred to as a UMI. This assignment is accomplished by ligating fragmented DNA with an adapter containing a 12-base fully random sequence (i.e. the UMI). Statistically, this process provides $4^{12}$ 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.

## Target enrichment and final library construction

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 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 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 and GAIIx) and Thermo Fisher Scientific systems (Ion Personal Genome Machine® (PGM™), Ion Proton™ and Ion S5™). 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. When using Thermo Fisher Scientific systems 200 bp (or longer) single reads are recommended, except for the Human Mitochondria Panel where 300 bp (or longer) single reads are preferred.

## Principle of variant detection with UMIs

The principle of variant detection with UMIs is described in Figure 3. Due to intrinsic noise and sequence-dependent bias, indexed molecules may be amplified unevenly across the target regions. Target region coverage can be better achieved, however, by counting the number of UMIs rather than counting the number of total reads for each region. Sequence reads having different UMIs represent different original molecules, while sequence reads having the same UMIs are the result of PCR duplication from one original molecule. Errors from PCR amplification and from the sequencing process may also be present in final reads that lead to false positive variants in sequencing results. These artifactual variants can be greatly reduced by calling variants across all reads within a unique UMI instead of picking up variants at the original read level.

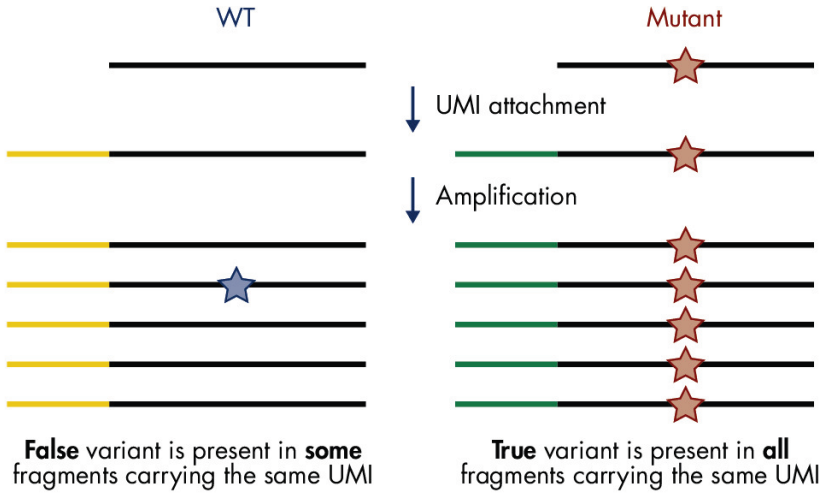



**Figure 3. Principle of variant detection with UMIs**. Each original molecule is tagged by a UMI. True variants are those mutations present in the majority of reads within a UMI, while false positives are mutations present in only one or a few reads within a UMI. Description of the variant calling algorithm can be found and downloaded from Xu, C, et al. BMC Genomics (2017) **18**, 5.

## Data analysis

The QIAseq Targeted DNA Panel Analysis pipeline is available at **http://www.qiagen.com/us/shop/genes-and-pathways/data-analysis-center-overview-page/**. The pipeline automatically performs all steps necessary to generate a DNA sequence variant report from your raw NGS data. An explanation of the principles of UMI -directed variant detection and the features of the primary sequence analysis output can be found and downloaded from Xu, C, et al. BMC Genomics (2017) **18**, 5. All detected variants can be further interpreted using Ingenuity Variant Analysis (IVA) and QIAGEN Clinical Insight (QCI).

# 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.

In addition to the QIAseq Targeted DNA Panels and the QIAseq Index Kit, the following are required.

- 80% ethanol (made fresh daily)*
- Nuclease-free pipet tips and tubes
- 1.5 ml LoBind tubes (Eppendorf, cat. no. 022431021)
- 0.2 ml PCR tubes, 96-well PCR plates or PCR strips and caps
- Ice
- Microcentrifuge
- Thermal cycler
- Multichannel pipets
- Single-channel pipets
- QIAxcel® or Agilent® 2100 Bioanalyzer®
- Agilent High Sensitivity DNA Kit (Agilent cat. no. 5067-4626)
- DynaMag™-96 Side Magnet (Thermo Fisher Scientific Inc., cat. no. 12331D)
- QIAseq DNA QuantiMIZE Kits, if using FFPE samples (QIAGEN, cat. no. 333404 or 333414)

* Do not use denatured alcohol, which contains other substances, such as methanol or methylethylketone.

# Important Notes

For optimal results, all DNA samples should demonstrate consistent quality according to the following criteria.

## DNA quality

The most important prerequisite for DNA sequence analysis is consistent, high-quality DNA from every experimental sample. Therefore, sample handling and DNA isolation procedures are critical to the success of the experiment. 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. 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, or alternatively in DNase-free 10 mM Tris buffer pH 8.0. **Important**: Do not use DEPC-treated water.

**Important**: Ensure that samples have been treated to remove RNA. RNA contamination will cause inaccuracies in DNA concentration measurements. Do not omit the recommended RNase treatment step to remove RNA.

**Note**: 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 genomic DNA**

| Kit | Starting material | Cat. no. |
|---|---|---|
| QIAamp® DNA Mini Kit | Small amounts of cells and tissue | 51304 |
| QIAamp DNA FFPE Tissue Kit | Animal/human tissues and cells | 56404 |
| GeneRead™ DNA FFPE Kit | Animal/human tissues and cells | 180134 |
| QIAamp Circulating Nucleic Acid Kit | Animal and human plasma and serum | 55114 |

For best results, all DNA samples should also demonstrate consistent quality according to the following criteria:

## 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 Bioanalyzer. Although DNA is enzymatically fragmented before target enrichment PCR, intact DNA generally yields better results than fragmented DNA due to tiling space between primers. Intact DNA usually has better coverage uniformity, more UMIs captured and more sensitive variant detection.

## Specific recommendations for FFPE DNA

If FFPE DNA is used for QIAseq Targeted DNA Panels, the QIAseq DNA QuantiMIZE Array or Assay Kit is strongly recommended for determining the optimal DNA amount for each FFPE DNA sample. Quantification based on mass calculations (OD, NanoDrop®) can be very inaccurate. Appendix B (page 59) provides detailed information for FFPE DNA quality assessment and input amount.

## 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 base position with specific fresh DNA input amounts at different coverage depths. Table 3 provides guidance on variant detection with fresh DNA amounts at different coverage depths.

**Table 2. Expected UMI Number to be sequenced, based on fresh DNA input and read depth***

| | 10 ng | | 20 ng | | 40 ng | |
|---|---|---|---|---|---|---|
| | **Mean UMI** | **Mean read** | **Mean UMI** | **Mean read** | **Mean UMI** | **Mean read** |
| 2.0 read pairs/UMI | 540 | 2160 | 910 | 3640 | 1940 | 7760 |
| 4.0 read pairs/UMI | 900 | 7200 | 1500 | 12000 | 3200 | 25600 |
| 8.6 read pairs/UMI | 1080 | 18576 | 1900 | 32680 | 4048 | 69626 |

* For example, if an average of 900 UMIs is required, 10 ng of DNA can be used at 4 read pairs per UMI read depth (7200x coverage). The expected UMI is related to sequencing depth when the same amount of DNA is used. With 10 ng DNA input, 2160x sequencing depth (2.0 read pairs/UMI) can have 540 UMI, while 7200x coverage (4.0 read pairs/UMI) will have 900 UMI sequenced.

**Table 3. Suggested fresh DNA input amount and sequencing depth for variant detection***

| Variant frequency | Input (ng) | Read pairs/UMI | Mean read |
|---|---|---|---|
| 5% | 10 | 4 | 7200 |
| 1% | 40 | 4 | 25600 |

* Variant detection is based on 90% sensitivity on the entire region of QIAseq Targeted DNA Panel.

## Variant detection

The number of UMIs sequenced directly impacts the variant detection sensitivity. Therefore, low-frequency mutation detection usually requires more DNA input and sequencing at deeper coverage to generate a sufficient amount of UMIs. Table 4 (next page) provides a general guideline for variant detection sensitivity with different numbers of UMIs sequenced at various read depths.

**Table 4. Estimated SNP/variant detection sensitivity with different numbers of UMIs at various read depths***

| Allele frequency | Mean UMI | 1.1 read pairs/UMI | 2.0 read pairs/UMI | 4.0 read pairs/UMI | 8.6 read pairs/UMI |
|---|---|---|---|---|---|
| 5% | 589 | 0.618 | 0.944 | 0.935 | 0.949 |
| 5% | 1178 | 0.855 | 0.963 | 0.960 | 0.965 |
| 5% | 2355 | 0.946 | 0.966 | 0.962 | 0.965 |
| 5% | 3533 | 0.952 | 0.965 | 0.966 | 0.967 |
| 1% | 722 | 0.318 | 0.565 | 0.619 | 0.628 |
| 1% | 1445 | 0.498 | 0.812 | 0.848 | 0.848 |
| 1% | 2167 | 0.668 | 0.852 | 0.906 | 0.915 |
| 1% | 3612 | 0.843 | 0.928 | 0.933 | 0.933 |

* For example, to achieve 90% sensitivity on 5% SNP variant detection, about 600 UMIs will be needed with two read pairs per UMI depth. From Table 2, 10 ng DNA with four read pairs/UMI depth (7200x coverage) or 20 ng DNA with two read pairs/UMI depth (3640x coverage) will give a sufficient number of UMIs and read depth for calling a 5% variant at 90% sensitivity.

## 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. For the Ion platforms, up to 96 sample indexes are available per run. General guidelines are provided for the number of samples that can be multiplexed in different sequencing platforms, based on panel size and read depth (Table 5, Table 6 and Table 7). Fine-tuning the read depth is possible after the first run.

**Table 5. Number of multiplexed samples based on panel size with 500x mean coverage***

| Instrument | Version | Capacity (paired-ends reads) | 1000 primers | 2500 primers | 5000 primers | 12,000 primers |
|---|---|---|---|---|---|---|
| MiniSeq | Mid output | 16 M | 32 | 12 | 6 | 2 |
| MiniSeq | High output | 50 M | 100 | 40 | 20 | 8 |
| MiSeq | v2 | 30 M | 60 | 24 | 12 | 5 |
| NextSeq 500 | Mid output | 260 M | 520 | 208 | 104 | 43 |
| NextSeq 500 | High output | 800 M | 1600 | 640 | 320 | 133 |
| HiSeq 2500 rapid run | Dual Flowcell v2 | 1200 M | 2400 | 960 | 480 | 200 |
| Ion PGM | 318 Chip v2 | 5 M† | 7 | 2 | 1 | N/A |
| Ion S5 | Ion 530 Chip | 15 M† | 21 | 8 | 4 | 1 |
| Ion S5 | Ion 540 Chip | 60 M† | 84 | 33 | 16 | 7 |
| Ion Proton | I Chip | 80 M† | 112 | 44 | 22 | 9 |

* Based on 2 x 151 bp paired-end read on Illumina platform and 200 bp read on Ion platform.

† Single reads only (specifically for Ion).

N/A: Not applicable, no samples can be run.

**Table 6. Number of multiplexed samples based on panel size with 2500x mean coverage***

| Instrument | Version | Capacity (paired-ends reads) | 1000 primers | 2500 primers | 5000 primers | 12,000 primers |
|---|---|---|---|---|---|---|
| MiniSeq | Mid output | 16 M | 6 | 2 | 1 | N/A |
| MiniSeq | High output | 50 M | 20 | 8 | 4 | 1 |
| MiSeq | v2 | 30 M | 12 | 4 | 2 | 1 |
| NextSeq 500 | Mid output | 260 M | 104 | 41 | 20 | 8 |
| NextSeq 500 | High output | 800 M | 320 | 128 | 64 | 26 |
| HiSeq 2500 rapid run | Dual Flowcell v2 | 1200 M | 480 | 192 | 96 | 40 |
| Ion Torrent PGM | 318 Chip v2 | 5 M† | 1 | N/A | N/A | N/A |
| Ion S5 | Ion 530 Chip | 15 M† | 4 | 1 | N/A | N/A |
| Ion S5 | Ion 540 Chip | 60 M† | 16 | 6 | 3 | 1 |
| Ion Proton | I Chip | 80 M† | 22 | 8 | 4 | 1 |

* Based on 2 x 151 bp paired-end read on Illumina platform and 200 bp read on Ion platform.

† Single reads only (specifically for Ion).

N/A: Not applicable, no samples can be run.

**Table 7. Number of multiplexed samples based on panel size with 20,000x mean coverage***

| Instrument | Version | Capacity (paired-ends reads) | 1000 primers | 2500 primers | 5000 primers | 12,000 primers |
|---|---|---|---|---|---|---|
| MiniSeq | Mid output | 16 M | N/A | N/A | N/A | N/A |
| MiniSeq | High output | 50 M | 2 | 1 | N/A | N/A |
| MiSeq | v2 | 30 M | 1 | N/A | N/A | N/A |
| NextSeq 500 | Mid output | 260 M | 13 | 5 | 2 | 1 |
| NextSeq 500 | High output | 800 M | 40 | 16 | 8 | 3 |
| HiSeq 2500 rapid run | Dual Flowcell v2 | 1200 M | 60 | 24 | 12 | 5 |
| Ion Torrent PGM | 318 Chip v2 | 5 M† | N/A | N/A | N/A | N/A |
| Ion S5 | Ion 530 Chip | 15 M† | N/A | N/A | N/A | N/A |
| Ion S5 | Ion 540 Chip | 60 M† | 2 | N/A | N/A | N/A |
| Ion Proton | I Chip | 80 M† | 2 | 1 | N/A | N/A |

* Based on 2 x 151 bp paired-end read on Illumina platform and 200 bp read on Ion platform.

† Single reads only (specifically for Ion).

N/A: Not applicable, no samples can be run.

## Next-generation sequencing read-length recommendations

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. When using Thermo Fisher Scientific systems 200 bp (or longer) single reads are recommended, except for the Human Mitochondria Panel where 300 bp (or longer) single reads are preferred.

# Protocol: QIAseq Targeted DNA Panel for Illumina Instruments

## Important points before starting

- This protocol covers all procedures required for the preparation of libraries for Illumina sequencers from "standard DNA" (i.e., cells or tissues), FFPE DNA and cfDNA.
- Before setting up the reaction, it is critical to accurately determine the amount of the input DNA (10–40 ng for standard DNA or cfDNA; up to 250 ng of FFPE DNA can be used, if QIAseq QuantiMIZE kits have been used. If an alternative method was used to determine the concentration of FFPE DNA, then up to 100 ng DNA can be used). Lower input amounts are possible; however, this will lead to fewer sequenced UMIs and reduced variant detection sensitivity.
- QIAseq Beads are used for all reaction cleanups.
- **Important**: Prepare fresh 80% ethanol daily.
- **Important**: The A, B, C or D IL-N7 Adapter Plate used in the adapter ligation reaction must be paired with the matching A, B, C or D IL-S5 Index Primer Plate used in the Universal PCR amplification reaction.
- Reaction and cleanup procedures can be performed in either PCR tubes or 96-well plate.
- Upon completion of the library preparation, the QIAseq Library Quant System can be used for library quantification.

## Fragmentation, end-repair and A-addition

1. On ice, prepare the fragmentation, end-repair and A-addition mix according to Table 8. Briefly centrifuge, mix by pipetting up and down 7–8 times and briefly centrifuge again. **Note**: In general, increasing the amount of DNA input will improve variant detection sensitivity – particularly for FFPE DNA. See Appendix B for more details.

**Table 8. Reaction mix for fragmentation, end-repair and A-addition**

| Component | Volume/reaction (Standard, FFPE or pure cfDNA) | Volume/reaction (cfDNA contaminated with cellular DNA) |
|---|---|---|
| DNA* | Variable | Variable |
| Fragmentation Buffer, 10x | 2.5 µl | 2.5 µl |
| FERA Solution | 0.75 µl | 0.75 µl |
| FG Solution | – | 1.25 µl |
| Nuclease-Free Water | Variable | Variable |
| **Total** | **20 µl** | **20 µl** |

* 10–40 ng for standard DNA or cfDNA. Use up to 250 ng of FFPE DNA if QIAseq QuantiMIZE kits were used, or up to 100 ng of FFPE DNA if an alternative method was used.

2. Add 5 µl Fragmentation Enzyme Mix to each reaction. Briefly centrifuge, mix by pipetting up and down 7–8 times (do not vortex) and briefly centrifuge again.

   **Important**: Keep the reaction tubes/plate on ice during the entire reaction setup.

3. Program the thermal cycler according to Table 9. Use the instrument's heated lid.

**Table 9. Cycling conditions for fragmentation, end-repair and A-addition***

| Step | Incubation temperature | Incubation time (standard DNA) | Incubation time (FFPE DNA) | Incubation time (cfDNA) |
|---|---|---|---|---|
| 1 | 4°C | 1 min | 1 min | 1 min |
| 2 | 32°C* | 24 min | 14 min | 14 min |
| 3 | 72°C | 30 min | 30 min | 30 min |
| 4 | 4°C | Hold | Hold | Hold |

* For Human Mitochondria Panel, use 8 min for both standard and FFPE DNA at 32°C incubation.

4. Before adding the tubes/plate to a thermal cycler, start the program. When the thermal cycler reaches 4°C, pause the program.

   **Important**: The thermal cycler must be pre-chilled and paused at 4°C.

5. Transfer the tubes/plate prepared in step 2 to the pre-chilled thermal cycler and resume the cycling program.
6. Upon completion, allow the thermal cycler to return to 4°C.
7. Place the samples on ice and immediately proceed with "Adapter ligation", below.

## Adapter ligation

8. Prepare the adapter ligation mix according to Table 10. Briefly centrifuge, mix by pipetting up and down 10–12 times and briefly centrifuge again.

   **Important**: Only one single-indexed adapter should be used per ligation reaction. Open one adapter tube at a time if using 12-index adapters and avoid cross-contamination. For 96-index adapters supplied in a plate (layout described in Figure 4), use a multichannel pipet to pipet the appropriate amount of adapters.

   **Important**: Pipet slowly to mix. The reaction mix is very viscous. Do not vortex.

**Table 10. Reaction mix for adapter ligation**

| Component | Volume/reaction, standard DNA | Volume/reaction, FFPE DNA | Volume/reaction, cfDNA |
|---|---|---|---|
| Fragmentation, end-repair and A-addition reaction (already in tube) | 25 µl | 25 µl | 25 µl |
| Ligation Buffer, 5x | 10 µl | 10 µl | 10 µl |
| IL-N7## adapter* | 2.8 µl | 2.8 µl | 0.5 µl |
| DNA Ligase | 5 µl | 5 µl | 5 µl |
| Ligation solution† | 7.2 µl | 7.2 µl | 7.2 µl |
| Nuclease-Free Water | – | – | 2.3 µl |
| **Total** | **50 µl** | **50 µl** | **50 µl** |

* This component applies to QIAseq IL-N7## adapters with up to a 12 sample index for QIAseq 12-index I or QIAseq 96-index I A, B, C or D set.

† Ligation solution is very viscous. It should be added into each reaction individually and not pre-mixed with other components for a master mix. Do not coat the outside of the pipet tip with ligation solution or excess volume may be added.

**IL-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 | | | | | | | | | | | | |
| F | | | | | | | | | | | | |
| G | | | | | | | | | | | | |
| H | | | | | | | | | | | | |

**IL-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 | | | | | | | | | | | | |
| F | | | | | | | | | | | | |
| G | | | | | | | | | | | | |
| H | | | | | | | | | | | | |

**Figure 4. Layout of sample adapters in QIAseq 96-Index I Set A, B, C or D**. Rows A through D of each plate have adapters. Rows E through H are empty. Each well in each row contains one sample adapter, and the amount of adapter in each well is enough for eight samples.

9. Program a thermal cycler to 20°C and incubate the reactions for 15 min.

   **Important**: Do not use heated lid.

10. Upon completion, place the reactions on ice and proceed with "Cleanup of adapter-ligated DNA", next page. Alternatively, the samples can be stored at –20°C in a constant-temperature freezer for up to 3 days.

## Cleanup of adapter-ligated DNA

11. For standard/FFPE samples, add 50 µl nuclease-free water to bring each sample to 100 µl. For cfDNA samples, add 30 µl nuclease-free water to bring each sample to 80 µl.
12. For standard/FFPE samples, add 100 µl QIAseq Beads. For cfDNA samples, add 112 µl QIAseq Beads. Mix well by pipetting up and down several times.
13. Incubate for 5 min at room temperature.
14. Place the tubes/plate on a magnetic rack for 10 min. Once the solution has cleared, with the beads still on the magnetic stand, carefully remove and discard the supernatant.

    **Important**: Do not discard the beads as they contain the DNA of interest.
15. With the beads still on the magnetic stand, add 200 µl 80% ethanol. Rotate the tube (2–3 times) or move the plate side-to-side between the two column positions of the magnet to wash the beads. Carefully remove and discard the wash.
16. Repeat the ethanol wash.

    **Important**: Completely remove all traces of the ethanol wash after this second wash. Remove the ethanol with a 200 µl pipet first, and then use a 10 µl pipet to remove any residual ethanol.
17. With the beads still on the magnetic stand, air dry at room temperature for 10 min.

    **Note**: Visually inspect that the pellet is completely dry.
18. Remove the beads from the magnetic stand, and elute the DNA from the beads by adding 52 µl nuclease-free water. Mix well by pipetting.
19. Return the tubes/plate to the magnetic rack until the solution has cleared.
20. Transfer 50 µl of the supernatant to clean tubes/plate.
21. For standard/FFPE samples, add 50 µl QIAseq Beads (for Human Mitochondria Panel use 35 µl). For cfDNA samples, add 70 µl QIAseq Beads. Mix well by pipetting up and down several times.
22. Incubate for 5 min at room temperature.

23. Place the tubes/plate on a magnetic rack for 5 min. Once the solution has cleared, with the beads still on the magnetic stand, carefully remove and discard the supernatant.

    **Important**: Do not discard the beads as they contain the DNA of interest.

24. With the beads still on the magnetic stand, add 200 µl 80% ethanol. Rotate the tube (2–3 times) or move the plate side-to-side between the two column positions of the magnet to wash the beads. Carefully remove and discard the wash.

25. Repeat the ethanol wash.

    **Important**: Completely remove all traces of the ethanol wash after this second wash. Remove the ethanol with a 200 µl pipet first, and then use a 10 µl pipet to remove any residual ethanol.

26. With the beads still on the magnetic stand, air dry at room temperature for 15 min.

    **Note**: Visually inspect that the pellet is completely dry. Ethanol carryover to the target enrichment PCR step will affect enrichment PCR efficiency.

27. Remove the beads from the magnetic stand, and elute the DNA from the beads by adding 12 µl nuclease-free water. Mix well by pipetting.

28. Return the tube/plate to the magnetic rack until the solution has cleared.

29. Transfer 9.4 µl of the supernatant to clean tubes or plate.

30. Proceed with "Target enrichment", next page. Alternatively, the samples can be stored at –20°C in a constant-temperature freezer for up to 3 days.

## Target enrichment

31. Prepare the target enrichment mix according to Table 11. Briefly centrifuge, mix by pipetting up and down 7–8 times and briefly centrifuge again.

**Table 11. Reaction mix for target enrichment**

| Component | Volume/reaction |
|---|---|
| Adapter-ligated DNA from "Cleanup of adapter-ligated DNA" | 9.4 µl |
| TEPCR buffer, 5x | 4 µl |
| QIAseq Targeted DNA Panel | 5 µl |
| IL-Forward primer | 0.8 µl |
| HotStarTaq DNA Polymerase | 0.8 µl |
| **Total** | **20 µl** |

32. Program a thermal cycler using the cycling conditions in Table 12 (panel with <1500 primers/tube) or Table 13 (panel with ≥1500 primers/tube).

**Table 12. Cycling conditions for target enrichment if number of primers <1500/tube**

| Step | Time | Temperature |
|---|---|---|
| Initial denaturation | 13 min | 95°C |
| | 2 min | 98°C |
| 8 cycles | 15 s | 98°C |
| | 10 min | 68°C |
| 1 cycle | 5 min | 72°C |
| Hold | 5 min | 4°C |
| Hold | ∞ | 4°C |

**Table 13. Cycling conditions for target enrichment if number of primers ≥1500/tube**

| Step | Time (1500–12,000 primers/tube) | Time (>12,000 primers/tube) | Temperature |
|---|---|---|---|
| Initial denaturation | 13 min | 13 min | 95°C |
| | 2 min | 2 min | 98°C |
| 6 cycles | 15 s | 15 s | 98°C |
| | 15 min | 30 min | 65°C |
| 1 cycle | 5 min | 5 min | 72°C |
| Hold | 5 min | 5 min | 4°C |
| Hold | ∞ | ∞ | 4°C |

33. Place the target enrichment reaction in the thermal cycler and start the run.
34. After the reaction is complete, place the reactions on ice and proceed with "Cleanup of target enrichment", below. Alternatively, the samples can be stored at –20°C in a constant-temperature freezer for up to 3 days.

## Cleanup of target enrichment

35. For standard/FFPE samples, add 80 µl nuclease-free water to bring each sample to 100 µl. For cfDNA samples, add 70 µl nuclease-free water to bring each sample to 90 µl.
36. For standard/FFPE samples, add 100 µl QIAseq Beads (for Human Mitochondria Panel use 70 µl). For cfDNA samples, add 108 µl QIAseq Beads. Mix well by pipetting up and down several times.
37. Incubate for 5 min at room temperature.
38. Place the tubes/plate on a magnetic rack for 5 min. After the solution has cleared, with the beads still on the magnetic stand, carefully remove and discard the supernatant.

    **Important**: Do not discard the beads as they contain the DNA of interest.

39. With the beads still on the magnetic stand, add 200 µl 80% ethanol. Rotate the tube (2–3 times) or move the plate side-to-side between the two column positions of the magnet to wash the beads. Carefully remove and discard the wash.
40. Repeat the ethanol wash.

    **Important**: Completely remove all traces of the ethanol wash after this second wash. Remove the ethanol with a 200 µl pipet first, and then use a 10 µl pipet to remove any residual ethanol.
41. With the beads still on the magnetic stand, air dry at room temperature for 10 min.

    **Note**: Visually inspect that the pellet is completely dry. Ethanol carryover to the next Universal PCR step will affect PCR efficiency.
42. Remove the beads from the magnetic stand, and elute the DNA from the beads by adding 16 µl nuclease-free water. Mix well by pipetting.
43. Return the tube/plate to the magnetic rack until the solution has cleared.
44. Transfer 13.4 µl of the supernatant to clean tubes/plate.
45. Proceed with "Universal PCR", below. Alternatively, the samples can be stored at –20°C in a constant-temperature freezer for up to 3 days.

## Universal PCR

46. Prepare the Universal PCR according to Table 14 or Table 15, depending in which index set is being used. Briefly centrifuge, mix by pipetting up and down 7–8 times and briefly centrifuge again.

    **Important**: If using QIAseq 96-index I Set A, B, C or D, mix components directly in IL-S5 Index Primer Plate A, B, C or D that contains pre-dispensed, dried index primer and Universal PCR primers. See Figure 5 for layout of index primer in the plate.

    **Important**: The A, B, C or D IL-N7 Adapter Plate used in the adapter ligation reaction must be paired with the matching A, B, C or D IL-S5 Index Primer Plate used in the Universal PCR amplification reaction.

**Table 14. Reaction mix for Universal PCR if using QIAseq 12-index I**

| Component | Volume/reaction |
|---|---|
| Target-enriched DNA from "Cleanup of target enrichment" | 13.4 µl |
| 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** |

**Table 15. Reaction Components for Universal PCR if using QIAseq 96-index I Set A, B, C or D***

| Component | Volume/reaction |
|---|---|
| Target-enriched DNA from "Cleanup of target enrichment" | 13.4 µl |
| UPCR Buffer, 5x | 4 µl |
| HotStarTaq DNA Polymerase | 1 µl |
| Nuclease-Free Water | 1.6 µl |
| **Total** | **20 µl** |

* Applies to QIAseq IL-S5 Index Primer Plate in A, B, C or D set. 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 A, B, C and D sets together.

**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 5. 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-N7 Adapter Plate in A, B, C or D set used in ligation must be paired with IL-S5 Index Primer Plate in A, B, C or D set, respectively.

47. Program a thermal cycler using the cycling conditions in Table 16 (cycling program) and Table 17 (cycle number).

**Table 16. Cycling conditions for Universal PCR**

| Step | Time | Temperature |
|---|---|---|
| Initial denaturation | 13 min | 95°C |
| | 2 min | 98°C |
| Number of cycles (see Table 17) | 15 sec | 98°C |
| | 2 min | 60°C |
| 1 cycle | 5 min | 72°C |
| Hold | 5 min | 4°C |
| Hold | ∞ | 4°C |

**Table 17. Amplification cycles for Universal PCR**

| Primers per pool | Cycle number, standard DNA | Cycle number, cfDNA | Cycle number, FFPE DNA |
|---|---|---|---|
| 6–24 | 26 | 28 | 30 |
| 25–96 | 24 | 26 | 28 |
| 97–288 | 22 | 24 | 26 |
| 289–1056 | 21 | 23 | 25 |
| 1057–1499 | 20 | 22 | 24 |
| 1500–3072 | 21 | 23 | 25 |
| 3073–4999 | 20 | 22 | 24 |
| 5000–12,000 | 19 | 21 | 23 |
| ≥12,001 | 18 | 20 | 22 |

48. After the reaction is complete, place the reactions on ice and proceed to "Cleanup of Universal PCR", next page. Alternatively, the samples can be stored at –20°C in a constant-temperature freezer for up to 3 days.

## Cleanup of Universal PCR

49. For standard/FFPE samples, add 80 µl nuclease-free water to bring each sample to 100 µl. For cfDNA samples, add 70 µl nuclease-free water to bring each sample to 90 µl.
50. For standard/FFPE samples, add 100 µl QIAseq Beads (for Human Mitochondria Panel use 70 µl). For cfDNA samples, add 108 µl QIAseq Beads. Mix well by pipetting up and down several times.
51. Incubate for 5 min at room temperature.
52. Place the tubes/plate on magnetic rack for 5 min to separate beads from supernatant. Once the solution has cleared, with the beads still on the magnetic stand, carefully remove and discard the supernatant.

    **Important**: Do not discard the beads as they contain the DNA of interest.
53. With the beads still on the magnetic stand, add 200 µl 80% ethanol. Rotate the tube (2–3 times) or move the plate side-to-side between the two column positions of the magnet to wash the beads. Carefully remove and discard the wash.
54. Repeat the ethanol wash.

    **Important**: Completely remove all traces of the ethanol wash after this second wash. Remove the ethanol with a 200 µl pipet first, and then use a 10 µl pipet to remove any residual ethanol.
55. With the beads still on the magnetic stand, air dry at room temperature for 10 min.

    **Note**: Visually inspect that the pellet is completely dry.
56. Remove the beads from the magnetic stand, and elute the DNA from the beads by adding 30 µl nuclease-free water. Mix well by pipetting.
57. Return the tubes/plate to the magnetic rack until the solution has cleared.
58. Transfer 28 µl supernatant to clean tubes or plate.
59. The library can be stored in a –20°C freezer prior to quantification using the QIAseq Library Quant System (see Appendix C, page 61). Amplified libraries are stable for several months at –20°C. Once quantification is performed proceed to "Protocol: Sequencing Setup on Illumina MiSeq and NextSeq", next page

# Protocol: 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 C, page 61).
- **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 DNA Panel on Illumina platform.
- Ensure libraries have been quantified using QIAseq Library Quant System (see Appendix C, page 61).
- 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.
- Instrument-specific imagery is included to aid in sequencing preparations.

## Sequencing Preparations for MiSeq

1. Set up the sample sheet with Custom Sequencing Read 1 primer using Illumina Experiment Manager v1.2, or later (Figure 6). Sample index of QIAseq Targeted DNA 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

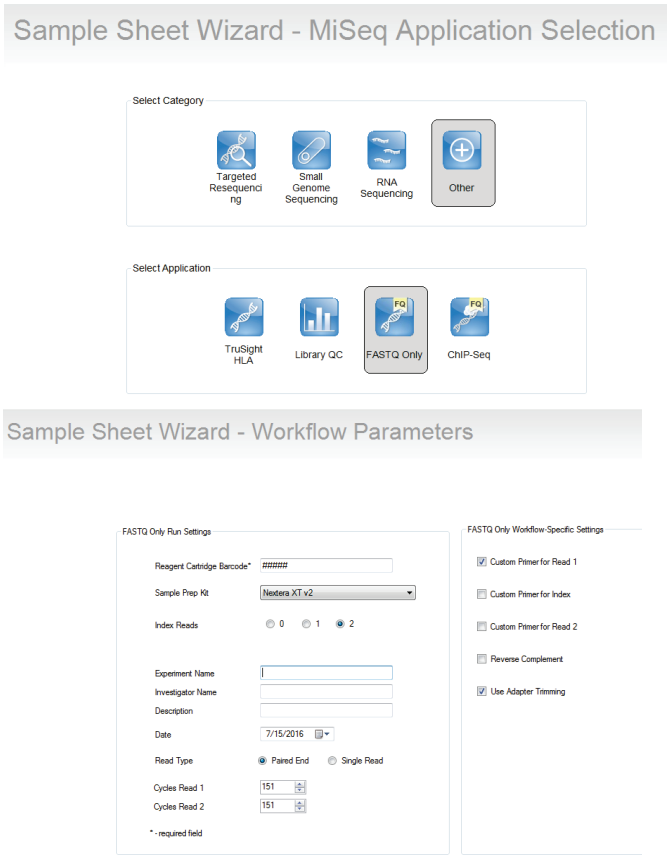



**Figure 6. Sample sheet using Illumina Experiment Manager.**

2. Dilute libraries to 2 nM or 4 nM for MiSeq. Then, 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 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.

3. Prepare and load the library onto a MiSeq according to the MiSeq System Denature and Dilute Libraries Guide. The final library concentration is 10–12 pM on the MiSeq.

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

4. 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 7). For more details, please refer to the Illumina protocol: miseq_using_custom_primers_15041638_b.pdf for the MiSeq.

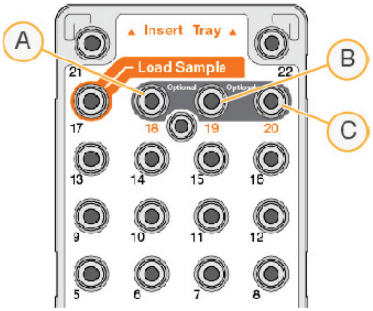
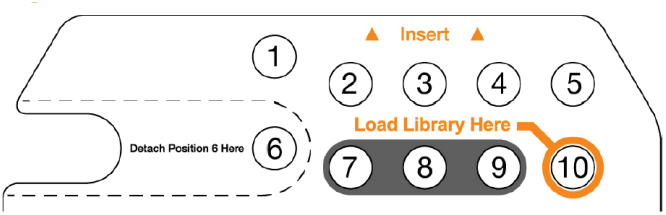



**Figure 7. Loading the QIAseq A Read 1 Primer I into position 18 (shown by 'A' in the figure; B and C are not relevant) of the MiSeq reagent cartridge.**

5. Upon completion of the sequencing run, proceed to "Appendix D: Data Analysis Using QIAGEN's QIAseq Targeted Sequencing Data Analysis Portal or the Biomedical Genomics Workbench", page 62.

### Sequencing Preparations for NextSeq

1. Use paired end read (151 cycles for read 1 and 2).
2. Use dual indexes (8 cycles of each).
3. Dilute libraries to 0.5, 1, 2 or 4 nM for NextSeq. Then, 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.
4. 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.
5. 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 8. NextSeq reagent cartridge.**

6. Upon completion of the sequencing run, proceed to "Appendix D: Data Analysis Using QIAGEN's QIAseq Targeted Sequencing Data Analysis Portal or the Biomedical Genomics Workbench", page 62.

# Protocol: QIAseq Targeted DNA Panel for Thermo Fisher Scientific Instruments

## Important points before starting

- This protocol covers all procedures required for the preparation of libraries for Thermo Fisher Scientific sequencers from "standard DNA" (i.e., cells or tissues), FFPE DNA and cfDNA.
- Before setting up the reaction, it is critical to accurately determine the amount of the input DNA (10–40 ng for standard DNA or cfDNA; up to 250 ng of FFPE DNA can be used, if QIAseq QuantiMIZE kits have been used. If an alternative method was used to determine the concentration of FFPE DNA, then up to 100 ng DNA can be used). Lower input amounts are possible; however, this will lead to fewer sequenced UMIs and reduced variant detection sensitivity.
- QIAseq Beads are used for all reaction cleanups.
- **Important**: Prepare fresh 80% ethanol daily.
- Reaction and cleanup procedures can be performed in either PCR tubes or 96-well plate.
- Upon completion of the library preparation, the QIAseq Library Quant System can be used for library quantification.

## Fragmentation, end-repair and A-addition

1. On ice, prepare the fragmentation, end-repair and A-addition mix according to Table 18. Briefly centrifuge, mix by pipetting up and down 7–8 times and briefly centrifuge again.

   **Note**: In general, increasing DNA input will improve variant detection sensitivity – particularly for FFPE DNA. See Appendix B (page 59) for more details.

**Table 18. Reaction mix for fragmentation, end-repair and A-addition**

| Component | Volume/reaction, standard, FFPE or pure cfDNA | Volume/reaction, cfDNA contaminated with cellular DNA |
|---|---|---|
| DNA* | Variable | Variable |
| Fragmentation Buffer, 10x | 2.5 µl | 2.5 µl |
| FERA Solution | 0.75 µl | 0.75 µl |
| FG Solution | – | 1.25 µl |
| Nuclease-Free Water | Variable | Variable |
| **Total** | **20 µl** | **20 µl** |

* 10–40 ng for standard DNA or cfDNA. Use up to 250 ng of FFPE DNA if QIAseq QuantiMIZE kits were used, or up to 100 ng of FFPE DNA if an alternative method was used.

2. Add 5 µl Fragmentation Enzyme Mix to each reaction. Briefly centrifuge, mix by pipetting up and down 7–8 times (do not vortex) and briefly centrifuge again.

   **Important**: Keep the reaction tubes/plate on ice during the entire reaction setup.

3. Program a thermal cycler according to Table 19. Use the instrument's heated lid.

**Table 19. Cycling conditions for fragmentation, end-repair and A-addition***

| Step | Incubation temperature | Incubation time (standard DNA) | Incubation time (FFPE DNA) | Incubation time (cfDNA) |
|---|---|---|---|---|
| 1 | 4°C | 1 min | 1 min | 1 min |
| 2 | 32°C* | 24 min | 14 min | 14 min |
| 3 | 72°C | 30 min | 30 min | 30 min |
| 4 | 4°C | Hold | Hold | Hold |

* For Human Mitochondria Panel, use 8 min for both standard and FFPE DNA at 32°C incubation.

4. Prior to adding the tubes/plate to a thermal cycler, start the program. When the thermal cycler reaches 4°C, pause the program.

   **Important**: The thermal cycler must be pre-chilled and paused at 4°C.

5. Transfer the tubes/plate prepared in step 2 to the pre-chilled thermal cycler and resume the cycling program.
6. Upon completion, allow the thermal cycler to return to 4°C.
7. Place the samples on ice, and immediately proceed with "Adapter ligation", below.

## Adapter ligation

8. Prepare the adapter ligation mix according to Table 20. Briefly centrifuge, mix by pipetting up and down 10–12 times and briefly centrifuge again.

   **Important**: Only one single-indexed adapter should be used per ligation reaction. Open one adapter tube at a time if using 12-index adapters and avoid cross-contamination. For 96-index adapters supplied in a plate (layout described in Figure 9) use a multichannel pipet to pipet the appropriate amount of adapters.
   Important: Pipet slowly to mix. The reaction mix is very viscous. Do not vortex.

**Table 20. Reaction mix for adapter ligation**

| Component | Volume/reaction, standard DNA | Volume/reaction, FFPE DNA | Volume/reaction, cfDNA |
|---|---|---|---|
| Fragmentation, end-repair and A-addition reaction (already in tube) | 25 µl | 25 µl | 25 µl |
| Ligation Buffer, 5x | 10 µl | 10 µl | 10 µl |
| LT-BC# adapter* | 2.8 µl | 2.8 µl | 0.5 µl |
| DNA Ligase | 5 µl | 5 µl | 5 µl |
| Ligation solution† | 7.2 µl | 7.2 µl | 7.2 µl |
| Nuclease-Free Water | 0 | 0 | 2.3 µl |
| **Total** | **50 µl** | **50 µl** | **50 µl** |

* This component applies to QIAseq LT-BC# adapters in QIAseq 12-index L and QIAseq 96-index L with up to 12 or 96 sample indices, respectively.

† The Ligation solution is very viscous. It should be added into each reaction individually and not pre-mixed with other components for a master mix. Do not coat the outside of the pipet tip with the ligation solution or excess volume may be added.

**LT-BC1-96 Adapter Plate**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | BC1 | BC2 | BC3 | BC4 | BC5 | BC6 | BC7 | BC8 | BC9 | BC10 | BC11 | BC12 |
| **B** | BC13 | BC14 | BC15 | BC16 | BC17 | BC18 | BC19 | BC20 | BC21 | BC22 | BC23 | BC24 |
| **C** | BC25 | BC26 | BC27 | BC28 | BC29 | BC30 | BC31 | BC32 | BC33 | BC34 | BC35 | BC36 |
| **D** | BC37 | BC38 | BC39 | BC40 | BC41 | BC42 | BC43 | BC44 | BC45 | BC46 | BC47 | BC48 |
| **E** | BC49 | BC50 | BC51 | BC52 | BC53 | BC54 | BC55 | BC56 | BC57 | BC58 | BC59 | BC60 |
| **F** | BC61 | BC62 | BC63 | BC64 | BC65 | BC66 | BC67 | BC68 | BC69 | BC70 | BC71 | BC72 |
| **G** | BC73 | BC74 | BC75 | BC76 | BC77 | BC78 | BC79 | BC80 | BC81 | BC82 | BC83 | BC84 |
| **H** | BC85 | BC86 | BC87 | BC88 | BC89 | BC90 | BC91 | BC92 | BC93 | BC94 | BC95 | BC96 |

**Figure 9. Layout of sample index adapters in QIAseq 96-Index L**. Each well contains one sample adapter. The amount of adapter in each well is sufficient for four samples.

9. Program a thermal cycler to 20°C, and incubate reactions 15 min.

   **Important**: Do not use heated lid.

10. Upon completion, place the reactions on ice and proceed with "Cleanup of adapter-ligated DNA", below. Alternatively, samples can be stored at –20°C in a constant-temperature freezer for up to 3 days.

## Cleanup of adapter-ligated DNA

11. For standard/FFPE samples, add 50 µl nuclease-free water to bring volume to 100 µl. For cfDNA samples, add 30 µl nuclease-free water to bring volume to 80 µl.
12. For standard/FFPE samples, add 100 µl QIAseq Beads. For cfDNA samples, add 112 µl QIAseq Beads. Mix well by pipetting up and down several times.
13. Incubate for 5 min at room temperature.

14. Place the tubes/plate on a magnetic rack for 10 min to separate beads from supernatant. Once the solution has cleared, with the beads still on the magnetic stand, carefully remove and discard the supernatant.

    Important: Do not discard the beads as they contain the DNA target of interest.

15. With the beads still on the magnetic stand, add 200 µl of 80% ethanol. Rotate the tube (2–3 times) or move the plate side-to-side between the two column positions of the magnet to wash the beads. Carefully remove and discard the wash.

16. Repeat the ethanol wash.

    **Important**: Completely remove all traces of the ethanol wash after this second wash. Remove the ethanol with a 200 µl pipet first, and then use a 10 µl pipet to remove any residual ethanol.

17. With the beads still on the magnetic stand, air dry at room temperature for 10 min.

    **Note**: Visually inspect that the pellet is completely dry.

18. Remove the beads from the magnetic stand, and elute the DNA from the beads by adding 52 µl nuclease-free water. Mix well by pipetting.

19. Return the tube/plate to the magnetic rack until the solution has cleared.

20. Transfer 50 µl of the supernatant to a clean tube or plate.

21. For standard/FFPE samples, add 65 µl QIAseq Beads (for Human Mitochondria Panel use 50 µl). For cfDNA samples, add 70 µl QIAseq Beads. Mix well by pipetting up and down several times.

22. Incubate for 5 min at room temperature.

23. Place the tubes/plate on a magnetic rack for 5 min to separate beads from supernatant. Once the solution has cleared, with the beads still on the magnetic stand, carefully remove and discard the supernatant.

    **Important**: Do not discard the beads as they contain the DNA target of interest.

24. With the beads still on the magnetic stand, add 200 µl of 80% ethanol. Rotate the tube (2–3 times) or move the plate side-to-side between the two column positions of the magnet to wash the beads. Carefully remove and discard the wash.

25. Repeat the ethanol wash.

    **Important**: Completely remove all traces of the ethanol wash after this second wash. Remove the ethanol with a 200 µl pipet first, and then use a 10 µl pipet to remove any residual ethanol.

26. With the beads still on the magnetic stand, air dry at room temperature for 15 min.

    **Note**: Visually inspect that the pellet is completely dry. Ethanol carryover to the target enrichment PCR step will affect enrichment PCR efficiency.

27. Remove the beads from the magnetic stand, and elute the DNA from the beads by adding 12 µl nuclease-free water. Mix well by pipetting.

28. Return the tubes/plate to the magnetic rack until the solution has cleared.

29. Transfer 9.4 µl of the supernatant to clean tubes or plate.

30. Proceed with "Target enrichment", below. Alternatively, the samples can be stored at –20°C in a constant-temperature freezer for up to 3 days.

## Target enrichment

31. Prepare the target enrichment mix according to Table 21. Briefly centrifuge, mix by pipetting up and down 7–8 times and briefly centrifuge again.

**Table 21. Reaction mix for target enrichment**

| Component | Volume/reaction |
|---|---|
| Adapter-ligated DNA from "Cleanup of adapter-ligated DNA" | 9.4 µl |
| TEPCR buffer, 5x | 4 µl |
| QIAseq Targeted DNA Panel | 5 µl |
| LT-Forward Primer | 0.8 µl |
| HotStarTaq DNA Polymerase | 0.8 µl |
| **Total** | **20 µl** |

32. Program a thermal cycler using the cycling conditions in Table 22 (<1500 primers/tube) or Table 23 (≥1500 primers/tube).

**Table 22. Cycling conditions for target enrichment if number of primers <1500/tube**

| Step | Time | Temperature |
|---|---|---|
| Initial denaturation | 13 min | 95°C |
| | 2 min | 98°C |
| 8 cycles | 15 sec | 98°C |
| | 10 min | 68°C |
| 1 cycle | 5 min | 72°C |
| Hold | 5 min | 4°C |
| Hold | ∞ | 4°C |

**Table 23. Cycling conditions for target enrichment if number of primers ≥1500/tube**

| Step | Time (1500–12,000 primers/tube) | Time (>12000 primers/tube) | Temperature |
|---|---|---|---|
| Initial denaturation | 13 min | 13 min | 95°C |
| | 2 min | 2 min | 98°C |
| 6 cycles | 15 s | 15 s | 98°C |
| | 15 min | 30 min | 65°C |
| 1 cycle | 5 min | 5 min | 72°C |
| Hold | 5 min | 5 min | 4°C |
| Hold | ∞ | ∞ | 4°C |

33. Place the target enrichment reaction in the thermal cycler and start the run.

34. After the reaction is complete, place the reactions on ice and proceed with "Cleanup of target enrichment", next page. Alternatively, the samples can be stored at –20°C in a constant-temperature freezer for up to 3 days.

## Cleanup of target enrichment

35. For all sample types, add 60 µl nuclease-free water to bring each sample to 80 µl.
36. For standard/FFPE samples, add 104 µl QIAseq Beads (for Human Mitochondria Panel use 80 µl). For cfDNA samples, add 112 µl QIAseq Beads. Mix well by pipetting up and down several times.
37. Incubate for 5 min at room temperature.
38. Place the tubes/plate on a magnetic rack for 5 min to separate beads from supernatant. Once the solution has cleared, with the beads still on the magnetic stand, carefully remove and discard the supernatant.

    **Important**: Do not discard the beads as they contain the DNA of interest.
39. With the beads still on the magnetic stand, add 200 µl 80% ethanol. Rotate the tube (2–3 times) or move the plate side-to-side between the two column positions of the magnet to wash the beads. Carefully remove and discard the wash.
40. Repeat the ethanol wash.

    **Important**: Completely remove all traces of the ethanol wash after this second wash Remove the ethanol with a 200 µl pipet first, and then use a 10 µl pipet to remove any residual ethanol.
41. With the beads still on the magnetic stand, air dry at room temperature for 10 min.

    **Note**: Visually inspect that the pellet is completely dry. Ethanol carryover to the next Universal PCR step will affect PCR efficiency.
42. Remove the beads from the magnetic stand, and elute the DNA from the beads by adding 16 µl nuclease-free water. Mix well by pipetting.
43. Return the tubes/plate to the magnetic rack until the solution has cleared.
44. Transfer 13.4 µl of the supernatant to clean tubes/plate.
45. Proceed with "Universal PCR", next page. Alternatively, the samples can be stored at –20°C in a constant-temperature freezer for up to 3 days.

## Universal PCR

46. Prepare the Universal PCR according to Table 24. Briefly centrifuge, mix by pipetting up and down 7–8 times and briefly centrifuge again.

**Table 24. Reaction mix for Universal PCR**

| Component | Volume/reaction |
|---|---|
| Target enriched DNA from "Cleanup of target enrichment" | 13.4 µl |
| UPCR buffer, 5x | 4 µl |
| LT-Universal Primer | 0.8 µl |
| LT-P1 Primer | 0.8 µl |
| HotStarTaq DNA Polymerase | 1 µl |
| **Total** | **20 µl** |

B24. Program a thermal cycler using the cycling conditions in Table 25 (cycling program) and Table 26 (number of cycles).

**Table 25. Cycling conditions for Universal PCR**

| Step | Time | Temperature |
|---|---|---|
| Initial denaturation | 13 min | 95°C |
| | 2 min | 98°C |
| Number of cycles (see Table 26) | 15 s | 98°C |
| | 2 min | 60°C |
| 1 cycle | 5 min | 72°C |
| Hold | 5 min | 4°C |
| Hold | ∞ | 4°C |

**Table 26. Amplification cycles for Universal PCR**

| Primers per pool | Number of cycles, standard DNA | Number of cycles, cfDNA | Number of cycles, FFPE DNA |
|---|---|---|---|
| 6–24 | 26 | 28 | 30 |
| 25–96 | 24 | 26 | 28 |
| 97–288 | 22 | 24 | 26 |
| 289–1056 | 21 | 23 | 25 |
| 1057–1499 | 20 | 22 | 24 |
| 1500–3072 | 21 | 23 | 25 |
| 3073–4999 | 20 | 22 | 24 |
| 5000–12,000 | 19 | 21 | 23 |
| ≥12,001 | 18 | 20 | 22 |

47. After the reaction is complete, place the reactions on ice and proceed with "Cleanup of Universal PCR", below. Alternatively, the samples can be stored at –20°C in a constant-temperature freezer for up to 3 days.

## Cleanup of Universal PCR

48. For all sample types, add 60 µl nuclease-free water to bring each sample to 80 µl.
49. For standard/FFPE samples, add 104 µl QIAseq Beads (for Human Mitochondria Panel use 80 µl). For cfDNA samples, add 112 µl QIAseq Beads. Mix well by pipetting up and down several times.
50. Incubate for 5 min at room temperature.
51. Place the tubes/plate on a magnetic rack for 5 min to separate beads from supernatant. Once the solution has cleared, with the beads still on the magnetic stand, carefully remove and discard the supernatant.

    **Important**: Do not discard the beads as they contain the DNA of interest.
52. With the beads still on the magnetic stand, add 200 µl 80% ethanol. Rotate the tube (2–3 times) or move the plate side-to-side between the two column positions of the magnet to wash the beads. Carefully remove and discard the wash.

53. Repeat the ethanol wash.

    **Important**: Completely remove all traces of the ethanol wash after this second wash. Remove the ethanol with a 200 µl pipet first, and then use a 10 µl pipet to remove any residual ethanol.

54. With the beads still on the magnetic stand, air dry at room temperature for 10 min.

    **Note**: Visually inspect that the pellet is completely dry.

55. Remove the beads from the magnetic stand, and elute the DNA from the beads by adding 30 µl nuclease-free water. Mix well by pipetting.

56. Return the tubes/plate to the magnetic rack until the solution has cleared.

57. Transfer 28 µl supernatant to clean tubes or plate.

58. The library can be stored in a –20°C freezer prior to quantification using the QIAseq Library Quant System (see "Appendix C: Library Quantification using the QIAseq Library Quant System", page 61). Amplified libraries are stable for several months at –20°C. Once quantification is performed, proceed with "Protocol: Sequencing Setup for Thermo Fisher Scientific Instruments", next page.

# Protocol: Sequencing Setup for Thermo Fisher Scientific Instruments

**Important**: Recommendations for library dilution concentrations and library loading concentrations are based on QIAseq Library Quant System (See Appendix C, page 61).

After the library is constructed, follow Appendix C (page 61) to determine the library dilution factor (which dilutes libraries to 4 pM), and dilute each individual library according to this factor.

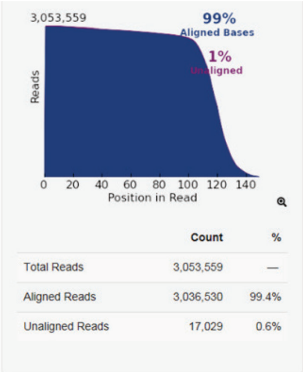
Libraries with a different sample index can be combined in equimolar amounts if similar sequencing depth is needed for each library. If combining libraries with the same number of primers, pool equal volumes of the individual libraries at 4 pM together. If combining libraries with different primer numbers, mix the libraries at a volume ratio according to their number of primers. For example, Library A has 5000 primers at 4 pM, and Library B has 600 primers at 4 pM; combining 50 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.

After combining libraries with different indexes, proceed to template preparation and sequencing according to the manufacturer instructions. The Sample index of QIAseq Targeted DNA Panels for the Ion Torrent is compatible with the Ion Xpress adapter sample index system. When using Thermo Fisher Scientific systems 200 bp (or longer) single reads are recommended, except for the Human Mitochondria Panel where 300 bp (or longer) single reads are preferred.

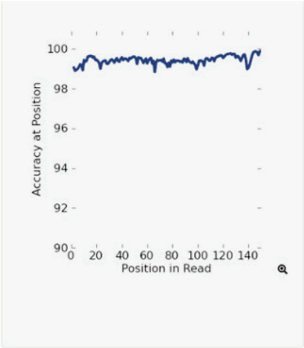
Upon completion of the sequencing run, proceed with "Protocol: Downloading Individual Unaligned .BAM File with a Multiplex Sample on Ion PGM Sequencer", next page.

# Protocol: Downloading Individual Unaligned .BAM File with a Multiplex Sample on Ion PGM Sequencer

1. Upon completion of the sequencing run, navigate to the report page on the Torrent Browser. Locate the “Output Files” section near the end of the report (Figure 10).




| | Count | % |
|---|---|---|
| Total Reads | 3,053,559 | — |
| Aligned Reads | 3,036,530 | 99.4% |
| Unaligned Reads | 17,029 | 0.6% |




**Alignment Quality**

| | AQ17 | AQ20 | Perfect |
|---|---|---|---|
| Total Number of Bases [bp] | 333 M | 315 M | 278 M |
| Mean Length [bp] | 114 | 110 | 99 |
| Longest Alignment [bp] | 167 | 166 | 166 |
| Mean Coverage Depth [x] | 0.1 | 0.1 | 0.1 |

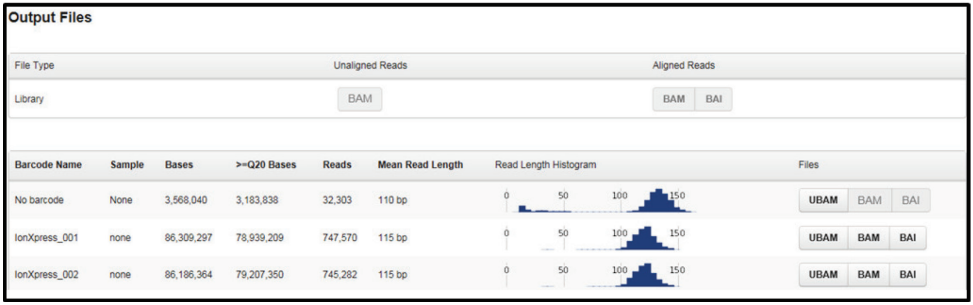



**Figure 10. Torrent Browser report page and output files.**

2. Click the "UBAM" button in the row corresponding to the individually indexed samples and column labeled "Files" in the table (Figure 11). These are the unaligned reads in BAM format, with the index separated for each sample. Save the .ubam file to your local disk. The file is usually several hundred megabytes to several gigabytes, depending on the size of the sequencing chip being used.

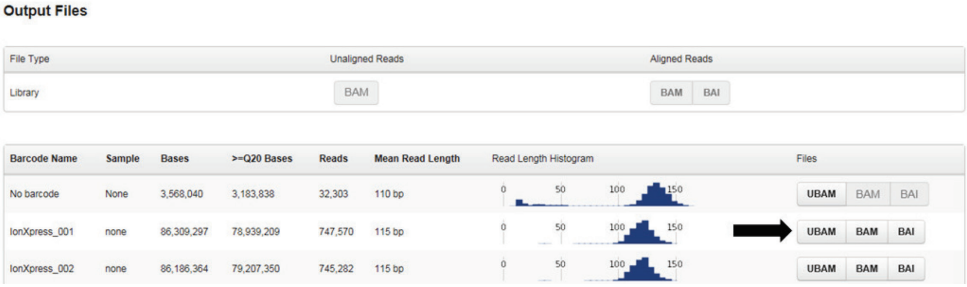



**Figure 11. Unaligned reads in BAM format.**

3. Locate the file that was just downloaded to the local disk. The individual .UBAM files will be needed to perform analysis using the QIAseq Targeted Sequencing Data Analysis Portal or the Biomedical Genomics Workbench.
4. Proceed to "Appendix D: Data Analysis Using QIAGEN's QIAseq Targeted Sequencing Data Analysis Portal or the Biomedical Genomics Workbench", page 62.

# 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 yield** | |
| a) Suboptimal reaction conditions due to low DNA quality | Make sure to use high-quality DNA to ensure optimal activity of the library enzymes. |
| b) Inefficient TEPCR or UPCR | QIAseq beads need to be completely dried before elution. Ethanol carryover to TEPCR and UPCR will affect PCR reaction efficiency. |
| **Unexpected signal peaks** | |
| a) Short peaks around 75 and 180 bp | These are primer–dimers from TEPCR (~75 bp) or UPCR (~180 bp). The presence of primer dimers indicates either not enough DNA input or inefficient PCR reactions. |
| b) Larger library fragments after UPCR | After the UPCR, library fragments are larger than the intended peak and can be a PCR artifact due to over-amplification of the DNA library. Over-amplification of the library won't affect the QIAseq Targeted DNA Panels sequencing performance. Decreasing the number of UPCR cycle numbers can reduce over-amplification. |
| **Sequencing issues** | |
| a) Too low or too high cluster density | Accurate library quantification is the key for optimal cluster density on any sequencing instrument. PCR based quantification method is recommended. Other methods may lead to the incorrect quantification of the library especially when there is over amplification. |
| b) Very low clusters passing filter | Make sure the library is accurately quantified and that the correct amount is loaded onto the sequencing instrument. In addition, the QIAseq A Read1 Primer I (100 µM) Custom Read 1 Sequencing primer MUST be used when sequencing on any Illumina platform. |

| | Comments and suggestions |
|---|---|
| **Variant detection issues** | |
| Known variants not detected | Variant detection sensitivity is directly related to the input DNA and read depth. Check Table 2, Table 3 and Table 4 to see if the required input DNA, UMI numbers and read depth are met for the specific variant detection application. |

# Appendix A: Combining an Existing Panel with a Booster Panel

If additional primers need to be added into an existing panel, a Booster Panel with up to 100 primers can be ordered. To combine the existing panel with a Booster Panel, follow the volume ratio indicated in Table 27.

**Table 27. Combining an existing panel (at 50 µl) with a booster panel**

| No. of primers in existing panel | Volume of existing panel to combine | Volume of booster panel to combine |
|---|---|---|
| 1–2000 | 50 µl | 5 µl |
| 2001–4000 | 50 µl | 3.75 µl |
| 4001–12,000 | 50 µl | 2.5 µl |
| 12,001–20,000 | 50 µl | 1.25 µl |

# Appendix B: FFPE DNA Quality and Quantity

Genomic DNA present in FFPE archives is usually damaged and fragmented to an uncertain extent. Commonly used DNA quantification methods, including spectrometers or fluorometers, do not differentiate between amplifiable and non-amplifiable DNA. Therefore, they cannot reliably measure the amplifiable amounts of DNA that are able to participate in the targeted enrichment step in the NGS workflow involving multiplex PCR, such as the QIAseq Targeted DNA Panels.

The QIAseq DNA QuantiMIZE System is a qPCR-based approach that determines the quantity and quality of the DNA amenable to PCR-based targeted enrichment prior to NGS. The system provides a cost-effective approach to qualify and quantify the DNA isolated from biological samples – mainly for FFPE samples. Please refer to the corresponding handbook for determining FFPE DNA quantity and quality with the QIAseq DNA QuantiMIZE System.

The QIAseq DNA QuantiMIZE System is recommended for determining FFPE DNA input for the QIAseq Targeted DNA Panels. If FFPE DNA is defined as high quality (quality control (QC) score ≤0.04) by QuantiMIZE, then up to 100 ng of DNA can be used. If the DNA is determined as low quality (QC score >0.04) then up to 250 ng of DNA can be used. The QC score of QuantiMIZE reflects the amount of amplifiable DNA present in the sample, therefore correlating with the number of UMIs that can be sequenced in the library (Figure 12, next page).

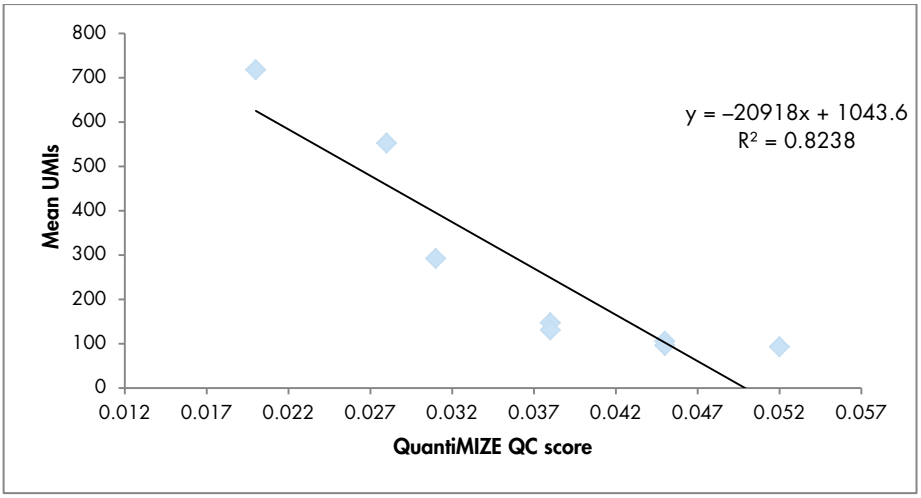



**Figure 12. Correlation between QIAseq QuantiMIZE QC Score and the number of UMIs.**

Compared to the same amount of fresh DNA, only 10–50% of UMIs can be captured from FFPE DNA, depending on the quality. This is due to a lower amplifiable DNA amount present in the FFPE samples. Therefore, a higher input amount is recommended for FFPE DNA samples to ensure that enough UMIs can be sequenced for variant detection.

However, if the quality of the FFPE DNA is not assessed by QIAseq QuantiMIZE kits, up to 100 ng can be used. If the FFPE DNA quality is high, an input of more than 100 ng will potentially overload the QIAseq Targeted DNA system.

# Appendix C: Library Quantification using the QIAseq Library Quant System

Due to the superior sensitivity of qPCR, we recommend quantifying the libraries using the QIAseq Library Quant System. With this system, the correct dilution of the library can be determined for sequencing. Please refer to the relevant handbook (available at **www.qiagen.com**) for library quantification.

# 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 sequencing data analysis portal. Our data analysis pipeline will perform mapping to the reference genome, UMI counting, read trimming (removing primer sequences), and variant identification. Alternatively, data from QIAseq Targeted DNA panels can be analyzed using the Biomedical Genomics Workbench, 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: **http://www.qiagen.com/us/shop/genes-and-pathways/data-analysis-center-overview-page/**.
3. Under "Choose format", select "NGS".
4. Under "Choose format", "Experiment Performed Using:" select "QIAseq Targeted DNA Panels" or "QIAseq Targeted DNA Custom Panels".
5. If a Catalog Panel was used then "Choose Array/Panel" that was used from the dropdown menu.
6. Under "Choose Instrument/Pack Size", select the pack size of the library kit used.
7. Under "Choose Instrument/Pack Size", select the instrument used for sequencing the libraries.
8. If a Custom Panel was used then specify the catalog number in the relevant box.
9. Click "Start Analysis".

10. On the "File Upload" tab, click "Add files" to upload sequencing files (Figure 13).

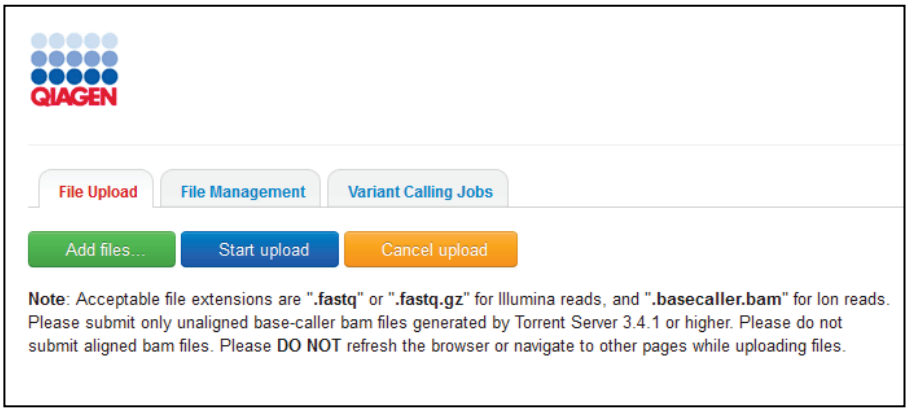



**Figure 13. File Upload tab of the QIAseq Targeted Sequencing Data Analysis Portal.**

11. Once the files have finished uploading, the files can be managed under the "File Management" tab.
12. Under the "File Management" tab, all files that have been uploaded to the portal are listed. Using this tab, it is possible to delete files that are no longer needed and share files with collaborators. Files can also be preselected for analysis by checking the boxes. The "Date From" and "Date To" fields can assist the selection of Preselect Files.
13. If "Preselect Files" has been selected in the "File Management" tab, the "Sample Read Files" will be already preselected in the "Variant Calling Jobs" tab.
14. Use the "Variant Calling Jobs" tab to set up the analysis.
    **QIAseq DNA Catalog #**: Select from dropdown menu if cataloged, enter manually if using a Custom, Extended or Booster panel.
    **Job type**: Single or matched tumor/normal
    **Copy Number Reference Job IDs**: For copy number analysis, normal sample(s) need to be analyzed with the portal before case samples are set up. Enter the job ID corresponding to your control samples for copy number analysis.

**File lanes**: For Illumina, choose 1-lane if you set up your runs using MiSeq/HiSeq/NextSeq concatenated, choose 4-lane if you set up your runs using NextSeq individual lane files. Choose 1-lane for Ion Torrent files.

**Select Read Files**: Select the files to be analyzed from the dropdown menu.

15. Click "Create jobs". The analysis job status changes from "Queued" to "In progress", then "Done successfully".
16. Once the analysis is completed, output files can be downloaded by clicking "Download".

# Appendix E: Analyze the Library Using Agilent 2100 Bioanalyzer

After the library is constructed and purified, the QIAxcel or Bioanalyzer can be used to check the fragment size and concentration with the High Sensitivity DNA Kit. Libraries prepared for Illumina instruments demonstrate a size distribution between 300–1000 bp (Figure 14 and Figure 15A, page 67). Library over-amplification is normal (Figure 15B, page 67) and this should not affect the sequencing results. Over-amplified libraries are usually single-stranded libraries with correct size, but appear as "larger fragments" due to secondary structure. Libraries prepared for Thermo Fisher Scientific instruments demonstrate a size distribution between 200–1000 bp (Figure 16, page 68). Amounts of DNA under the appropriate peaks can be used to quantify the libraries. However, due to the superior sensitivity of qPCR, we recommend quantifying the libraries using the QIAseq Library Quant System, especially when there are over-amplified libraries (See "Appendix C: Library Quantification using the QIAseq Library Quant System", page 61).

**Library (assessed using QIAxcel) prepared for Illumina instruments**

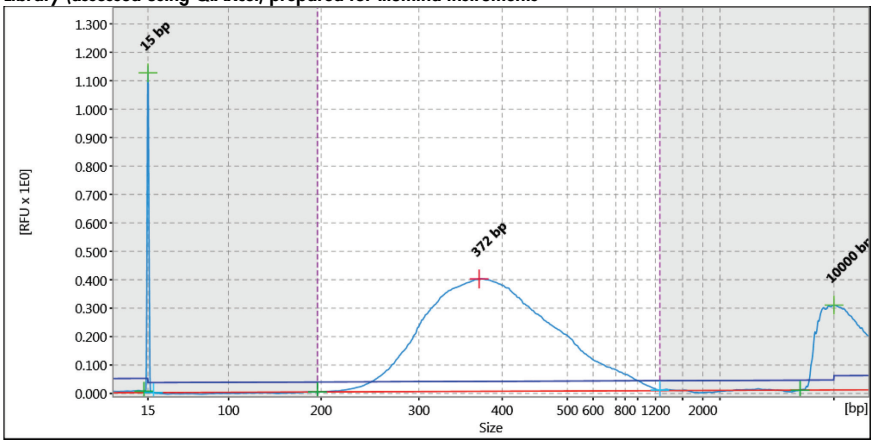



**Figure 14. Sample QIAxcel image of QIAseq Targeted DNA Panel libraries for Illumina instruments.** The size of the majority of the library fragments are between 300–1000 bp.

Recommended setting for checking QIAseq DNA Panel library on QIAxcel

**Cartridge**: DNA High Resolution

**Prepare libraries**: 1:5 dilution of libraries by using 1:1 mix of nuclease free water and QIAxcel dilution buffer.

**Size marker**: 100 bp – 2.5 kb, 2.5 ng

**Alignment marker**: 15 bp – 10 kb

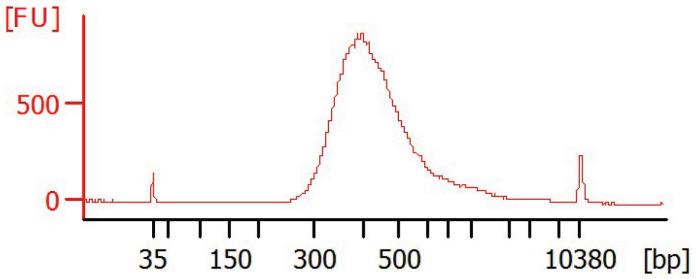
**Method**: OM500-AM10s (If library concentration is low, consider OL500)

**Injection time**: 40 seconds

**Analysis**: Default Smear DNA

**Peak calling**: 400 bp with 20% tolerance

**A: Library (without over-amplification) prepared for Illumina instruments**




**B: Library (with over-amplification) prepared for Illumina instruments**

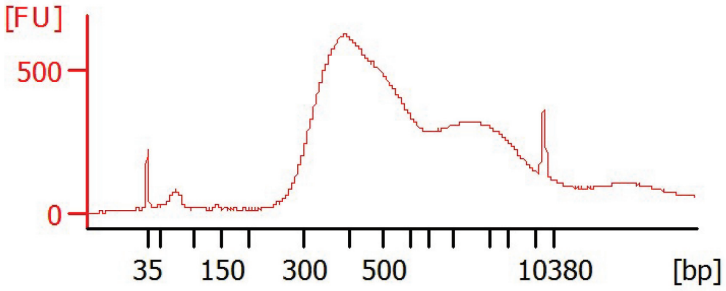



**Figure 15. Sample Bioanalyzer images of QIAseq Targeted DNA Panel libraries for Illumina instruments.** The size of the majority of the library fragments are between 300–1000 bp. **A** Library without over-amplification. **B** Library with over-amplification as indicated by the "larger fragment" peak.

**Library prepared for Thermo Fisher Scientific instruments**

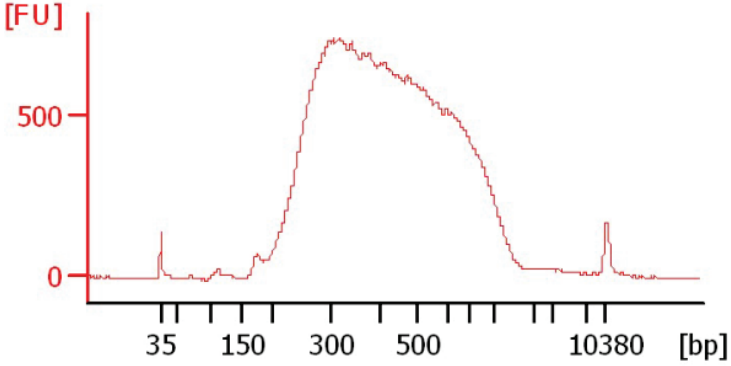



**Figure 16. Sample Bioanalyzer image of QIAseq Targeted DNA Panel libraries for Thermo Fisher Scientific instruments.** The size of the majority of the library fragments are between 200–1000 bp.

# Ordering Information

| Product | Contents | Cat. no. |
|---|---|---|
| QIAseq Targeted DNA Panel (12) | ALL reagents (except indexes) for targeted DNA sequencing; fixed panel for 12 samples; less than 200 genes | 333502 |
| QIAseq Targeted DNA Panel (96) | ALL reagents (except indexes) for targeted DNA sequencing; fixed panel for 96 samples; less than 200 genes | 333505 |
| QIAseq Targeted DNA HC Panel (12) | ALL reagents (except indexes) for targeted DNA sequencing; fixed panel for 12 samples; more than 200 genes | 333512 |
| QIAseq Targeted DNA HC Panel (96) | ALL reagents (except indexes) for targeted DNA sequencing; fixed panel for 96 samples; more than 200 genes | 333515 |
| QIAseq Targeted DNA Custom Panel (96) | ALL reagents (except indexes) for targeted DNA sequencing; Custom panel for 96 samples | 333525 |
| QIAseq Targeted DNA Extended Panel (96) | ALL reagents (except indexes) for targeted DNA sequencing; Extended panel for 96 samples | 333545 |
| QIAseq Targeted DNA Booster Panel (96) | Pool of primers used in combination with either cataloged or custom panels | 333535* |

* Visit **www.qiagen.com/GeneGlobe** to search for and order these products.

| Product | Contents | Cat. no. |
|---|---|---|
| QIAseq 12-Index I (48) | Box containing molecularly-indexed adapters and primers, enough for a total of 48 samples – for indexing up to 12 samples for targeted panel sequencing on Illumina platforms | 333714 |
| QIAseq 96-Index I Set A (384) | Box containing molecularly-indexed adapters and primers, enough for a total of 384 samples – for indexing up to 96 samples for targeted panel sequencing on Illumina platforms; one of four sets required for multiplexing 384 samples | 333727 |
| QIAseq 96-Index I Set B (384) | Box containing molecularly-indexed adapters and primers, enough for a total of 384 samples – for indexing up to 96 samples for targeted panel sequencing on Illumina platforms; two of four sets required for multiplexing 384 samples | 333737 |
| QIAseq 96-Index I Set C (384) | Box containing molecularly-indexed adapters and primers, enough for a total of 384 samples – for indexing up to 96 samples for targeted panel sequencing on Illumina platforms; three of four sets required for multiplexing 384 samples | 333747 |
| QIAseq 96-Index I Set D (384) | Box containing molecularly-indexed adapters and primers, enough for a total of 384 samples – for indexing up to 96 samples for targeted panel sequencing on Illumina platforms; four of four sets required for multiplexing 384 samples | 333757 |
| QIAseq 12-Index L (48) | Box containing molecularly-indexed adapters and primers, enough for a total of 48 samples – for indexing up to 12 samples for targeted panel sequencing on Ion Torrent platforms | 333764 |

| Product | Contents | Cat. no. |
|---|---|---|
| QIAseq 96-Index L (384) | Box containing molecularly-indexed adapters and primers in arrays, enough for a total of 384 samples – for indexing up to 96 samples for targeted panel sequencing on Ion Torrent platforms | 333777 |
| **Related products** | | |
| QIAseq Library Quant Array Kit | Reagents for quantification of libraries prepared for Illumina or Ion Torrent platforms; array format | 333304 |
| QIAseq Library Quant Assay Kit | Reagents for quantification of libraries prepared for Illumina or Ion Torrent platforms; assay format | 333314 |
| QIAseq DNA QuantiMIZE Array Kit | qPCR arrays for optimizing amount of input DNA and PCR cycling conditions for targeted enrichment of FFPE DNA | 333404 |
| QIAseq DNA QuantiMIZE Assay Kit | qPCR assays for optimizing amount of input DNA and PCR cycling conditions for targeted enrichment of FFPE DNA | 333414 |
| 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 Circulating Nucleic Acid Kit (50) | For 50 DNA preps: QIAamp Mini Columns, Tube Extenders (20 ml), QIAGEN Proteinase K, Carrier RNA, Buffers, VacConnectors, and Collection Tubes (1.5 ml and 2 ml) | 55114 |
| GeneRead DNA FFPE Kit (50) | QIAamp MinElute columns, Proteinase K, UNG, collection tubes (2 ml), buffers, Deparaffinization Solution, RNase A | 180134 |

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