July 2025

QIAseq® Targeted cfDNA Ultra Handbook

For ultrasensitive targeted next-generation sequencing (NGS) of cfDNA for Illumina® NGS systems

Sample to Insight

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Kit Contents

QIAseq Targeted cfDNA Ultra Catalog no. No. of samples	334051 12	334055 96
One pool of region-specific primers	60 µL	480 µL
ERA Buffer, 10x	60 µL	480 µL
ERA Enzyme	60 µL	480 µL
UPH Ligation Buffer, 2.5x	350 µL	2 x 1250 µL
DNA Ligase	75 µL	600 µL
HiFi Ultra Buffer, 5x	144 µL	2 x 768 µL
HiFi Ultra Polymerase	40 µL	315 µL
Nuclease-free Water	1.5 mL	10 mL
QIAseq Bead Binding Buffer (one bottle)	7 mL	54 mL
QIAseq Beads (one bottle)	10 mL	38.4 mL

QIAseq Targeted cfDNA Ultra Booster Catalog no. No. of samples	334085 96
One pool of region-specific primers	80 µL

Cat. no.	Product name	Total number of primers*	Panel size (bases)
YHS-001Z	Breast Cancer Ultra Panel	1045	21,960
YHS-002Z	Colorectal Cancer Ultra Panel	529	6,618
YHS-005Z	Lung Cancer Ultra Panel	948	26,448
YHS-101Z	Actionable Solid Tumor Ultra Panel	2284	44,012
YHS-003Z	Myeloid Neoplasms Ultra Panel	2302	32,716

* The number of primers in Custom and Booster panels is represented by the last digits of the catalog number. For example, a booster panel with catalog number SYHS-00100Z-48 has 48 primers.

QIAseq cfDNA Ultra Reagent Kit Catalog no. No. of samples	 334455 96
ERA Buffer, 10x	480 µL
ERA Enzyme	480 µL
UPH Ligation Buffer, 2.5x	2 x 1250 µL
DNA Ligase	600 µL
HiFi Ultra Buffer, 5x	2 x 768 µL
HiFi Ultra Polymerase	315 µL
Nuclease-free Water	10 mL
QIAseq Bead Binding Buffer (one bottle)	54 mL
QIAseq Beads (one bottle)	38.4 mL

QIAseq Targeted cfDNA Ultra 96-Unique Dual Indices

QIAseq Targeted cfDNA Ultra UDI Set* Catalog no. No. of samples	Set A 334165 96	Set B 334175 96	Set C 334185 96	Set D 334195 96
QUDI-96AA †	9 µL	N/A	N/A	N/A
QUDI-96BA †	N/A	9 µL	N/A	N/A
QUDI-96CA †	N/A	N/A	9 µL	N/A
QUDI-96DA †	N/A	N/A	N/A	9 µL
AdP-DP-Phased Adapter	144 µL	144 µL	144 µL	144 µL
SmP-IL5 TEPCR-F Primer	250 µL	250 µL	250 µL	250 µL

* 10 bp dual indices.

† Index Primer Plate (DNA Ultra UDI Set A, B, C and D Final Plate); each plate contains 96 pairs of sample index primers plus universal primers, with each well corresponding to one pair of UDI sample index; each index is single-use.

QIAseq Targeted cfDNA Ultra 12-Unique Dual Indices

QIAseq Targeted cfDNA Ultra UDI (12)*	
Catalog no.	**334151**
No. of samples	**12**
QUDI-12A†	9 µL
AdP-DP-Phased Adapter	18 µL
SmP-IL5 TEPCR-F Primer	31 µL

* 10 bp dual indices.

† Index Primer Plate (DNA Ultra UDI 12 Index Final Plate); each plate contains 12 pairs of sample index primers plus universal primers, with each well corresponding to one pair of UDI sample index; each index is single-use.

Shipping and Storage

The QIAseq Targeted cfDNA Ultra Kits (except QIAseq Beads and QIAseq Bead Binding Buffer) are shipped on dry ice and should be stored at –30°C to –15°C in a constant-temperature freezer upon arrival. The QIAseq Beads and QIAseq Bead Binding Buffer are shipped on cold packs and should be stored at 2–8°C upon arrival.

The QIAseq Targeted cfDNA Ultra Index Kits are shipped on dry ice and should be stored at –30°C to –15°C upon arrival.

When stored correctly, the QIAseq Targeted cfDNA Ultra Kits are good until the expiration date printed on the kit label.

Intended Use

The QIAseq Targeted cfDNA Ultra 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 the QIAseq Targeted cfDNA Ultra is tested against predetermined specifications, to ensure consistent product quality.

Introduction

The QIAseq Targeted cfDNA Ultra enables streamlined Sample to Insight®, targeted next-generation sequencing (NGS) of cell-free DNA (cfDNA). This highly optimized, automation-friendly solution facilitates ultrasensitive variant detection down to 0.1% by using integrated unique molecular indices (UMIs) and high-fidelity chemistry from cfDNA in biofluids within 8 hours and is coupled with an error-correction data analysis. The required amount of template for a single QIAseq Targeted cfDNA Ultra sequencing reaction ranges from 5 to 80 ng of cfDNA.

The NGS of DNA is a powerful tool for the detection of genetic variations, including somatic mutations, single-nucleotide polymorphisms, copy-number variation, and small insertions and 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 use regular 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 below 0.5% in heterogeneous samples, such as cfDNA. The QIAseq Targeted cfDNA Ultra overcomes these biases and artifacts with the use of a highly optimized, high-fidelity reaction chemistry and by incorporating UMIs into a single gene-specific, primer-based targeted enrichment process.

The QIAseq Targeted cfDNA Ultra has been optimized 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 QIAseq Targeted cfDNA Ultra library can be sequenced with Illumina default sequencing primers and is compatible with most medium- and high-throughput Illumina sequencers.

Our data analysis tools have been developed to perform all steps necessary to generate a DNA sequence variant report from NGS data. Collectively, the QIAseq Targeted cfDNA Ultra is a Sample to Insight solution for precision variant detection of targeted genomic regions using NGS (Figure 1).

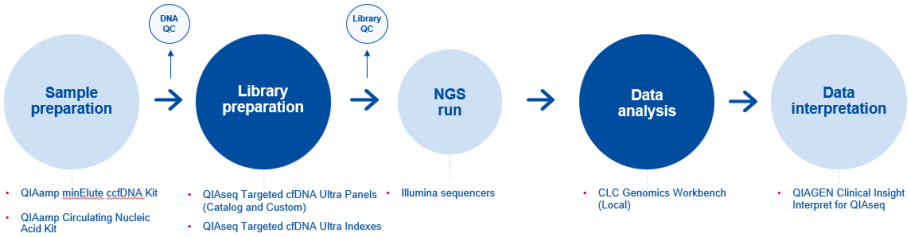

Figure 1. Overview of the Sample to Insight NGS workflow for cell-free DNA with the QIAseq Targeted cfDNA Ultra. This complete Sample to Insight procedure begins with DNA extraction followed by library construction and target enrichment with the QIAseq Targeted cfDNA Ultra. After NGS, data analysis is performed using the QIAGEN CLC Genomics Workbench. Ultimately, detected variants can be interpreted with the QIAGEN Clinical Insight Interpret for clinical relevant information.

Principle and Procedure

The QIAseq Targeted cfDNA Ultra Kits are provided as single-tube primer mixes, with up to 20,000 primers per panel. The QIAseq Targeted cfDNA Ultra Kit is designed to enrich selected genes and regions using 5–80 ng cfDNA (Figure 2). Lower input amounts are possible; however, this will lead to fewer sequenced UMIs and reduced variant detection sensitivity.

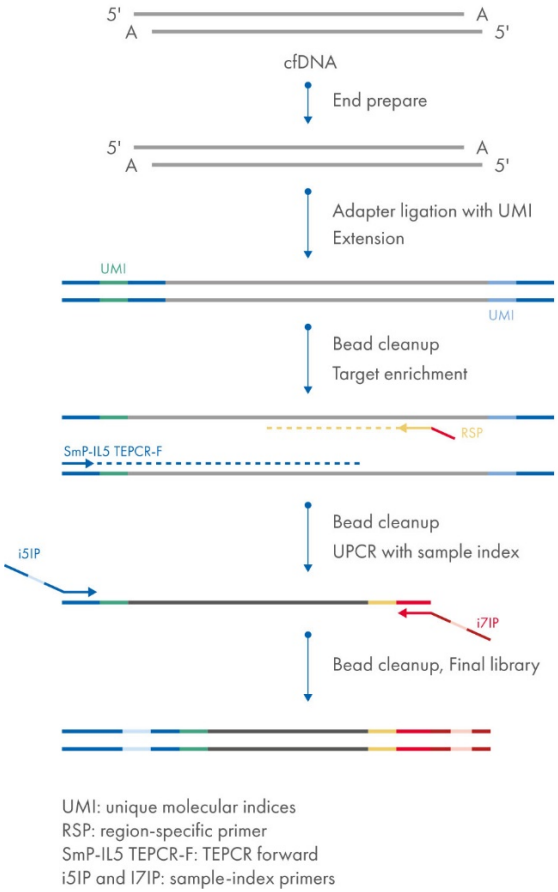

Figure 2. QIAseq Targeted cfDNA Ultra workflow.

End Preparation

cfDNA samples are first end repaired and A tailed within a single, controlled multienzyme reaction. The prepared DNA fragments are then ligated with a sequencing platform-specific adapter containing UMIs.

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.

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.

NGS adapter and index technologies

The QIAseq Targeted cfDNA Ultra Panels use unique dual index (UDI) primers for sample indexing. The UDI primers significantly reduce the risk of index-bleeding issues associated with different Illumina sequencing instruments, as well as reducing the impact of low-level contamination during oligo synthesis, and kit manufacturing, as well as carry over on the Illumina sequencing instrument itself. Hence, each sample will be assigned two unique indices to mitigate errors from image analysis, sequencing error, demultiplexing, and oligo synthesis contamination, reducing reads mis-assignment to wrong samples.

Next-generation sequencing

The QIAseq Targeted cfDNA Ultra is compatible with most medium- and high-throughput sequencers including Illumina NGS systems (MiniSeq®, MiSeq®, NextSeq® 500/550, NextSeq 1000/2000, NovaSeq® 6000, and NovaSeq® X).

Principle of variant detection with UMIs

The principle of variant detection with UMIs is described in Figure 3. Indexed molecules may be amplified unevenly across the target regions due to intrinsic noise and sequence-dependent bias. However, target region coverage can be better achieved 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.

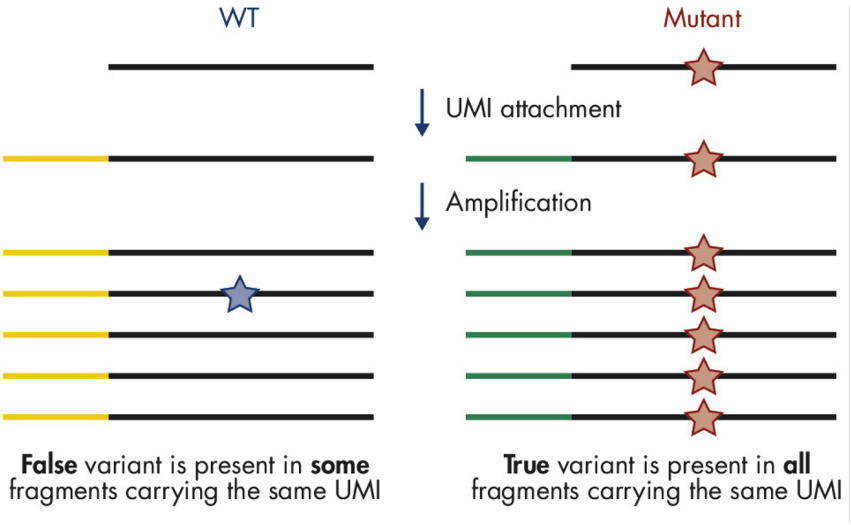

Figure 3. Principle of variant detection with UMIs. Each original molecule is tagged by a UMI. True variants are mutations present in the majority of reads within a UMI, while false positives are mutations present in only one or a few reads within a UMI.

Data analysis

The data for the QIAseq Targeted cfDNA Ultra can be analyzed using the Biomedical Genomics Analysis plugin to the QIAGEN CLC Genomics Workbench. The plugin provides workflows and tools for all steps from the initial data processing and quality assurance through data analyses, annotation, and reporting. A detailed guide to UMI-directed variant detection in CLC Genomics Workbench can be found in *Biomedical Genomics Analysis Plugin User Manual*.

All detected variants can be further interpreted using QIAGEN's Clinical Insight (QCI®) Interpret.

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 cfDNA Ultra and Index Kit, the following are required:

- DynaMag™-96 Side Magnet (Thermo Fisher Scientific Inc., cat. no. 12331D)
- QIAxpert® Instrument (cat. no. 9002340), Qubit™ 4 Fluorometer (Thermo Fisher Scientific Inc., cat. no. Q33238), or Qubit Flex Fluorometer (Thermo Fisher Scientific Inc., cat. no. Q33327)
- Ethanol, 80% (made fresh each day before use)*
- Nuclease-free pipette tips and tubes
- 1.5 mL DNA LoBind® tubes (Eppendorf®, cat. no. 022431021)
- PCR tubes and caps (0.2 mL individual PCR tubes [VWR, cat. no. 20170-012], tube and caps 8 strips [VWR, cat. no. 93001 118]) or 96-well PCR plates and caps
- Ice
- Microcentrifuge
- Thermal cycler
- Multichannel pipettes
- Single-channel pipettes
- QIAxcel® Connect System (cat. no. 9003110), QIAxcel Advanced System (cat. no. 9001941), or Agilent® 2100 Bioanalyzer® (Agilent, cat. no. G2939AAR)
- QIAxcel DNA High Sensitivity Kit (cat. No. 929012), QIAxcel DNA High Resolution Kit (QIAGEN, cat. no. 929002), or Agilent High Sensitivity DNA Kit (Agilent, cat. no. 5067-4626)

*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 isolation and quality check

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 System.

The QIAGEN kits listed in Table 1 are recommended for the preparation of cfDNA from serum or 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.

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

Table 1. Recommended kits for purification of cfDNA

Kit	Starting material	Cat. no.
QIAamp® Circulating Nucleic Acid Kit	Up to 5 mL of serum or plasma	55114
QIAamp MinElute ccfDNA Mini Kit	Up to 4 mL of serum or plasma	55204
QIAamp MinElute ccfDNA Midi Kit	Up to 10 mL of serum or plasma	55284
EZ1&2 ccfDNA Kit	Automated extraction from up to 8 mL serum or plasma	954854

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

cfDNA quality

cfDNA quality can be checked using the QIAxcel System, Agilent Bioanalyzer or Agilent TapeStation. Good cfDNA samples should have a distinct cfDNA peak and a minimal amount of cellular genomic DNA that is of high-molecular weight.

cfDNA quantification

If the cfDNA is free of high-molecular weight cellular DNA, its concentration can be measured by fluorescence-based quantification, such as with the Qubit Fluorometer. If a significant amount of cellular DNA is present in cfDNA sample, it is recommended to measure the amount based on the cfDNA peak with either the QIAxcel System or the Bioanalyzer.

DNA input amount and sequencing depth

The number of UMIs captured from the original DNA sample is related to the DNA input amount and sequencing depth. Adequate sequencing of captured UMIs is necessary for UMI-based variant detection and requires relatively deep sequencing coverage. Table 2 provides guidance on variant detection with cfDNA amounts at different depths of coverage.

Table 2. Suggested cfDNA input amount and sequencing depth for variant detection*

Variant frequency (%)	Input (ng)	Read pairs per UMI	Mean read
0.5	30	4	24,000
0.2	30	4	24,000
0.1	60	4	48,000

* Variant detection is based on 80–90% sensitivity on the entire panel region of the QIAseq Targeted cfDNA Ultra.

Variant detection

The number of UMIs sequenced directly impacts the sensitivity of variant detection. Therefore, low-frequency mutation detection usually requires more DNA input and sequencing at deeper coverage (i.e., more reads per UMI) to generate a sufficient amount of UMIs.

Sequencing capacity and sample multiplex level

Sample multiplexing is one of the most important NGS tools for increasing throughput and reducing costs. It works by combining multiple samples to be processed together in a single sequencing run; as a consequence, sequencing reads need to be demultiplexed by reassigning each single read to its original source library. This is facilitated by the integration of sample index sequences into the individual library molecules.

The QIAseq Targeted cfDNA Ultra Panels are paired with a fully compatible UDI sample-indexing solution. Each QIAseq Targeted cfDNA Ultra UDI Kit includes one of the following:

- QIAseq Targeted cfDNA Ultra UDI Set A (96): DNA Ultra UDI Set A index primer plate, QUDI-96AA
- QIAseq Targeted cfDNA Ultra UDI Set B (96): DNA Ultra UDI Set B index primer plate, QUID-96BA
- QIAseq Targeted cfDNA Ultra UDI Set C (96): DNA Ultra UDI Set C index primer plate, QUDI-96CA
- QIAseq Targeted cfDNA Ultra UDI Set D (96): DNA Ultra UDI Set D index primer plate, QUDI-96DA
- QIAseq Targeted cfDNA Ultra UDI (12): DNA Ultra UDI 12 Index primer plate, QUDI-12A

The QIAseq Ultra UDI Kits use a fixed combination of two unique bar code motives per sample-index primer pair. Therefore, each single-index motive is only used once on any UDI index primer.

Use of UDI indexes effectively mitigates the risk of read misassignment due to index hopping, which is important for detecting variants at very low frequency. By filtering misassigned reads during the demultiplexing of individual samples, highly accurate output data is generated.

To multiplex more than 96 libraries in a single sequencing run, combine kits with different sets, QIAseq Targeted cfDNA Ultra UDI Set A, B, C or D. For example, combining the unique dual QIAseq Targeted cfDNA Ultra UDI Set A and B (96) kits will allow the generation of 192 libraries with different unique dual sample indexes for 192-plex sequencing.

Sample multiplexing level is determined by the size of the panel, required depth of coverage and sequencing platform read capacity. General guidelines are provided for the number of samples that can be multiplexed with different sequencing platforms, based on panel size and read depth (Table 3 and Table 4). Read depth can be fine-tuned after the first run.

Table 3. Number of multiplexed samples based on panel size with 25,000x mean coverage*

Instrument	Version	Capacity (paired-ends reads)	500 primers	1000 primers	2500 primers	5000 primers	10,000 primers
iSeq	v2 reagents	8 M	N/A	N/A	N/A	N/A	N/A
MiniSeq	Mid output	16 M	1	N/A	N/A	N/A	N/A
MiniSeq	High output	50 M	4	2	N/A	N/A	N/A
MiSeq	v2 reagents	30 M	2	1	N/A	N/A	N/A
MiSeq	v3 reagents	50 M	4	2	N/A	N/A	N/A
NextSeq 500	Mid output	260 M	20	10	4	2	1
NextSeq 500	High output	800 M	64	32	12	6	3
NextSeq 1000/2000	P1 flow cell	200 M	16	8	3	1	N/A
NextSeq 1000/2000	P2 flow cell	800 M	64	32	12	6	3
NextSeq 2000	P3 flow cell	2.4 B	192	96	38	19	9
NextSeq 2000	P4 flow cell	3.6B	288	144	57	28	13
NovaSeq 6000	SP (2 lanes per flow cell)	1.6 B	128	64	25	12	6
NovaSeq 6000	S1 (2 lanes per flow cell)	3.2 B	256	128	51	25	12
NovaSeq 6000	S2 (2 lanes per flow cell)	8.2 B	656	328	131	65	32
NovaSeq 6000	S4 (4 lanes per flow cell)	20 B	1600	800	320	160	80
NovaSeq X	1.5B flow cell	3.2 B	256	128	51	25	12
NovaSeq X	10B flow cell	20 B	1600	800	320	160	80
NovaSeq X	25B flow cell	52 B	4160	2080	832	416	208

* Based on 2 x 149 bp paired-end reads.

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

Table 4. Number of multiplexed samples based on panel size with 50,000x mean coverage*

Instrument	Version	Capacity (paired-ends reads)	500 primers	1000 primers	2500 primers	5000 primers	10,000 primers
iSeq	v2 reagent	8 M	N/A	N/A	N/A	N/A	N/A
MiniSeq	Mid output	16 M	N/A	N/A	N/A	N/A	N/A
MiniSeq	High output	50 M	2	1	N/A	N/A	N/A
MiSeq	v2 reagents	30 M	1	N/A	N/A	N/A	N/A
MiSeq	v3 reagents	50 M	2	1	N/A	N/A	N/A
NextSeq 500	Mid output	260 M	10	5	2	1	N/A
NextSeq 500	High output	800 M	32	16	6	3	1
NextSeq 1000/2000	P1 flow cell	200 M	8	4	1	N/A	N/A
NextSeq 1000/2000	P2 flow cell	800 M	32	16	6	3	1
NextSeq 2000	P3 flow cell	2.4 B	96	48	19	9	4
NextSeq 2000	P4 flow cell	3.6B	144	72	28	13	6
NovaSeq 6000	SP (2 lanes per flow cell)	1.6 B	64	32	12	6	3
NovaSeq 6000	S1 (2 lanes per flow cell)	3.2 B	128	64	25	12	6
NovaSeq 6000	S2 (2 lanes per flow cell)	8.2 B	328	164	65	32	16
NovaSeq 6000	S4 (4 lanes per flow cell)	20 B	800	400	160	80	40
NovaSeq X	1.5B flow cell	3.2 B	128	64	25	12	6
NovaSeq X	10B flow cell	20 B	800	400	160	80	40
NovaSeq X	25B flow cell	52 B	2080	1040	416	208	104

* Based on 2 x 149 bp paired-end reads for.

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

NGS read-length recommendations

When using Illumina NGS systems, the QIAseq Targeted cfDNA Ultra UDI libraries require 149 bp paired-end reads and dual 10 bp indices.

Protocol: DNA End Prepare

Important points before starting

- This protocol covers all procedures required for the preparation of libraries for Illumina sequencers from cfDNA.
- Before setting up the reaction, it is critical to accurately determine the amount of the input cfDNA. When cfDNA is contaminated with cellular genomic DNA, measuring the concentration of the cfDNA peak with the QIAxcel, Bioanalyzer or similar methods is much more accurate than measuring the concentration of the whole sample.
- Reaction and cleanup procedures can be performed in either PCR tubes or 96-well plates.
- Set up reactions on ice.
- Do not vortex any reagents or reactions.

Procedure: end prepare

1. Thaw nucleic acid samples on ice. Gently mix, briefly centrifuge to collect residual liquid from the sides of the tubes, and then return to ice.
2. Prepare the reagents required for preparing the ends.
    a. Thaw 10x ERA Buffer on ice or, if required, at room temperature (15–25°C). Immediately upon thawing, place the buffer on ice. Keep ERA Enzyme on ice.
    b. Mix all reagents by flicking the tube, and centrifuge briefly.
3. On ice, prepare the end prepare mix according to Table 5. Briefly centrifuge, mix by pipetting up and down at least 12 times with pipetting volume close to 25 µL and briefly centrifuge again.

    Note: In general, increasing the amount of DNA input will improve variant detection sensitivity.

    Important: Keep the reaction tubes or plates on ice during the entire reaction setup.

Note: If setting up more than one reaction, prepare a volume of master mix 10% greater than what is required for the total number of reactions.

Table 5. Reaction mix for end prepare

Component	Volume per reaction
cfDNA*	Variable
10x ERA Buffer	2.5 µL
ERA Enzyme	5 µL
Nuclease-free Water	Variable
Total	**25 µL**

* For cfDNA, add 5–80 ng. We recommend 30 ng or more for detection of variants below 0.5%.

4. Program the thermal cycler according to Table 6. Use the instrument's heated lid.
5. Before adding the tubes or plate to a thermal cycler, start the program. When the thermal cycler reaches 4°C, pause the program.

   Important: The thermal cycler must be prechilled and paused at 4°C.

   Important: Do not use the heated lid during the 20°C incubation step. Alternatively, the lid temperature can be set at 65°C.
6. Transfer the tubes or plate prepared in step 3 to the prechilled thermal cycler and resume the cycling program.

Table 6. Incubation conditions for fragmentation and end prepare

Step	Incubation temperature (°C)	Incubation time
1	4	1 min
2	20	15 min
3	65	15 min
4	4	Hold

7. Upon completion, allow the thermal cycler to return to 4°C.
8. Place the samples on ice and immediately proceed to "Protocol: Adapter Ligation" on the next page.

Protocol: Adapter Ligation

Important points before starting

- The 25 µL product from "Protocol: DNA End Prepare" on page 23 is the starting material for this protocol.
- Set up reactions on ice.
- Do not vortex any reagents or reactions.
- The QIAseq Beads are used for ligation reaction cleanup. There is no need to bring beads to room temperature before use.
- Prepare fresh 80% ethanol each day before use.
- Ensure that the QIAseq Beads are thoroughly mixed at all times. This necessitates working quickly and resuspending the beads immediately before use. If a delay in the protocol occurs, simply vortex the beads.

Procedure: adapter ligation

1. Prepare the reagents required for the DNA ligation.
    a. Thaw AdP-DP-Phased Adapter on ice. Thaw UPH Ligation Buffer, 2.5x, on ice or at room temperature. Immediately upon thawing, place the buffer on ice. Keep DNA Ligase on ice.
    b. Mix by flicking the tube, and then centrifuge briefly.
2. Prepare the adapter ligation mix according to Table 7. Briefly centrifuge, mix by pipetting up and down at least 12 times with pipetting volume close to 50 µL and briefly centrifuge again.

    Important: The AdP-DP-Phased Adapter does not contain a sample index; hence, one single adapter is used for all samples.

Note: If setting up more than one reaction, prepare a volume of master mix 10% greater than what is required for the total number of reactions.

Table 7. Reaction mix for adapter ligation

Component	Volume/reaction, <10 ng cfDNA	Volume/reaction, 10–50 ng cfDNA	Volume/reaction, >50 ng cfDNA
End prepare reaction (already in tube)	25 µL	25 µL	25 µL
UPH Ligation Buffer, 2.5x	20 µL	20 µL	20 µL
AdP-DP-Phased Adapter	0.5 µL	1 µL	1.5 µL
DNA Ligase	5 µL	5 µL	5 µL
Total	**50.5 µL**	**51 µL**	**51.5 µL**

3. Program the thermal cycler according to Table 8.

   Important: Do not use the heated lid during the 20°C incubation step. Alternatively, the lid temperature can be set at 65°C.

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

5. Transfer the tubes or plate prepared in step 2 to the prechilled thermal cycler and resume the cycling program.

Table 8. Incubation conditions for DNA ligation

Step	Incubation temperature (°C)	Incubation time
1	4	1 min
2	20	15 min
3	65	15 min
4	4	Hold

6. Upon completion, allow the thermal cycler to return to 4°C.
7. Place the samples on ice and immediately proceed to "Procedure: ligation cleanup" (below). Alternatively, the samples can be stored at –20°C in a constant-temperature freezer for up to 3 days.

Procedure: ligation cleanup

8. Add 40 µL Nuclease-free Water to bring each sample to approximately 90 µL.
9. Add 108 µL QIAseq Beads. Mix well by vortexing or pipetting up and down at least 15 times with pipetting volume close to 190 µL. Incubate for 5 min at room temperature.
10. Place the tubes or plate on a magnetic rack for 10 min (tube) or 15 min (plate). 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.
11. With the beads still on the magnetic stand, add 80 µL water to the beads, then add 80 µL QIAseq bead binding buffer.
12. Take the tubes or plate off the magnetic stand, mix well by vortexing or pipetting up and down at least 15 times with pipetting volume close to 160 µL.
13. Return the tubes or plate to the 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.
14. With the beads still on the magnetic stand, add 200 µL 80% ethanol and leave beads in ethanol for 30 seconds. Carefully remove and discard the ethanol.
15. Repeat the ethanol wash.

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

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

    Note: Visually inspect that the pellet is completely dry. Ethanol carryover to the target enrichment PCR step will significantly impact enrichment PCR efficiency. Over drying beads will not affect DNA elution.

17. 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.

18. Return the tubes or plate to the magnetic rack until the solution has cleared.

19. Transfer 10 µL of the supernatant to clean tubes or plate.

    Important: Make sure no beads remain in the elution. Bead carryover to the target enrichment PCR step will significantly impact enrichment PCR efficiency.

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

Protocol: Target Enrichment

Important points before starting

- The 10 µL cleaned ligation reaction from "Protocol: Adapter Ligation" on page 26 is the starting material for this protocol.
- Set up reactions on ice.
- Do not vortex any reagents or reactions.
- The QIAseq Beads are used for target enrichment PCR cleanup. There is no need to bring beads to room temperature before use.
- Prepare fresh 80% ethanol each day before use.
- Ensure that the QIAseq Beads are thoroughly mixed at all times. This necessitates working quickly and resuspending the beads immediately before use. If a delay in the protocol occurs, simply vortex the beads.

Procedure: target enrichment

1. Prepare the reagents required for the Target Enrichment PCR (TEPCR).
    a. Thaw HiFi Ultra Buffer, 5x; QIAseq Targeted cfDNA Ultra Panel; and SmP-IL5 TEPCR-F Primer on ice or at room temperature. Immediately upon thawing, place the buffer on ice. Keep HiFi Ultra Polymerase on ice.
    b. Mix all reagents by flicking the tube, and then centrifuge briefly.
2. Prepare the target enrichment mix according to Table 9. Briefly centrifuge, mix by pipetting up and down at least 12 times with pipetting volume close to 20 µL, and briefly centrifuge again.

    Note: If setting up more than one reaction, prepare a volume of master mix 10% greater than what is required for the total number of reactions.

Important: Do not add more HiFi Ultra Polymerase than it is indicated in Table 9. Adding more could result in low enrichment specificity.

Table 9. Reaction mix for Target Enrichment

	Volume per reaction			
Component	**<=1100 primers**	**1101–5000 primers**	**5001–10,000 primers**	**>10,000 primers**
Cleaned Adapter-ligated DNA (already in tube) from "Procedure: ligation cleanup"	10 µL	10 µL	10 µL	10 µL
HiFi Ultra Buffer, 5x	4 µL	4 µL	4 µL	4 µL
QIAseq Targeted cfDNA Ultra Panel	5 µL	5 µL	5 µL	5 µL
SmP-IL5 TEPCR-F Primer	2 µL	2 µL	2 µL	2 µL
HiFi Ultra Polymerase*	–	0.25 µL	0.5 µL	0.75 µL
HiFi Ultra Polymerase (10x diluted)* †	0.6 µL	–	–	–
Total	**21.6 µL**	**21.25 µL**	**21.5 µL**	**21.75 µL**

* It is recommended to make a master mix instead of pipetting small amount of HiFi Ultra Polymerase enzyme.

† Immediately before setting up the reaction, dilute HiFi Ultra Polymerase 10 folds with Nuclease-free Water. There is enough amount of HiFi Ultra Polymerase in the kit for the dilution.

3. Program a thermal cycler using the cycling condition in Table 10 (panel with <=1100 primers/tube), Table 11 (panel with 1101–10,000 primers/tube) or Table 12 (panel with ≥10,001 primers/tube). Use the instrument's heated lid.

Table 10. Cycling conditions for target enrichment if number of primers <=1100/tube

Step	Time (<400 primers)	Time (400-1100 primers)	Temperature (°C)
Initial denaturation	2 min	2 min	98
8 cycles	20 s 5 min	20 s 10 min	98 70
1 cycle	3 min	3 min	72
Hold	∞	∞	4

Table 11. Cycling conditions for target enrichment if number of primers 1101–10000/tube

Step	Time	Temperature (°C)
Initial denaturation	2 min	98
8 cycles	20 s 12 min	98 69
1 cycle	3 min	72
Hold	∞	4

Table 12. Cycling conditions for target enrichment if number of primers ≥10001/tube

Step	Time	Temperature (°C)
Initial denaturation	2 min	98
8 cycles	20 s 10 min	98 68
1 cycle	3 min	72
Hold	∞	4

4. Place the target enrichment reaction in the thermal cycler and start the run.
5. After the reaction is complete, place the reactions on ice and proceed to "Procedure:

TEPCR cleanup" (next page). Alternatively, the samples can be stored at –20°C in a constant-temperature freezer for up to 3 days.

Procedure: TEPCR cleanup

1. Add 70 µL Nuclease-free Water to bring each sample to approximately 90 µL.
2. Add 108 µL QIAseq Beads. Mix well by vortexing or pipetting up and down at least 15 times with pipetting volume close to 190 µL. Incubate for 5 min at room temperature.
3. Place the tubes or plate on a magnetic rack for 5 min (tube) or 10 min (plate). 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.
4. With the beads still on the magnetic stand, add 200 µL 80% ethanol and leave beads in ethanol for 30 s. Carefully remove and discard the ethanol.
5. Repeat the ethanol wash.

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

   Note: Visually inspect that the pellet is completely dry. Ethanol carryover to the next universal PCR step will significantly impact PCR efficiency. Over drying beads will not affect DNA elution.
7. 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.
8. Return the tubes or plate to the magnetic rack until the solution has cleared.

9. Transfer 50 µL of the supernatant to clean tubes or plate. Transfer 5 µL to a clean tube or plate for universal PCR setup.

   Important: Make sure no beads transfer to the elution. Bead carryover to the universal PCR step will significantly impact PCR efficiency.

   Note: Only 5 µL of cleaned TEPCR volume will be used in the universal PCR. This volume is sufficient, as the DNA molecules were amplified in multiple cycles during TEPCR. The rest of the TEPCR volume can be stored at –20°C if needed.

10. Proceed with "Protocol: Universal PCR". Alternatively, the samples can be stored at –20°C in a constant-temperature freezer for up to 3 days.

Protocol: Universal PCR

Important points before starting

- The 5 µL cleaned product from "Protocol: Target Enrichment" on page 30 is the starting material for this protocol.
- Set up reactions on ice.
- Do not vortex any reagents or reactions.
- The QIAseq Targeted cfDNA Ultra UDI (12) or QIAseq Targeted cfDNA Ultra UDI Set A (96), Set B (96), Set C (96) or Set D (96) is used for sample indexing.
- The Index Primer Plate contains predispensed index primer pairs and the universal PCR primer and is sealed with pierceable aluminum heat sealing film.
- Puncture the film using standard 200 µL pipette tips to transfer the appropriate amount of index primer to the tubes or plate for the universal PCR.
- The QIAseq Beads are used for universal PCR cleanup. There is no need to bring beads to room temperature before use.
- Prepare fresh 80% ethanol each day before use.
- Ensure that the QIAseq Beads are thoroughly mixed at all times. This necessitates working quickly and resuspending the beads immediately before use. If a delay in the protocol occurs, simply vortex the beads.

Procedure: universal PCR

1. Prepare the reagents required for the universal PCR.
    a. Thaw HiFi Ultra Buffer (5x), DNA Ultra UDI 12, and 96-Index Plate on ice or at room temperature. Immediately upon thawing, place the items on ice. Keep HiFi Ultra

Polymerase on ice.

b. Mix by flicking the tube, and then centrifuge briefly.

2. Prepare the universal PCR in the tubes or plate containing the cleaned target-enriched from TEPCR cleanup reaction.

   Note: If setting up more than one reaction, prepare a volume of master mix 10% greater than what is required for the total number of reactions.

3. For the QIAseq Targeted cfDNA Ultra UDI plates, pierce the foil seal associated with each well that will be used, and transfer 2 µL (each well contains a forward primer and a reverse primer, each with a unique index) to the cleaned target-enriched DNA from "Procedure: TEPCR cleanup" sample tubes or plate according to Table 13. Mix by pipetting up and down at least 12 times with pipetting volume close to 40 µL and briefly centrifuge again.

   Important: Only one UDI pair should be used per universal PCR.

   Important: The QIAseq Targeted cfDNA Ultra UDI index plates are stable for a maximum of 10 freeze–thaw cycles. If all 96 wells have not been used, cover the used wells with foil and return to the freezer. Do not reuse wells from the QIAseq Targeted cfDNA Ultra UDI index plates once the foil seals have been pierced. Reusing wells would risk significant cross-contamination.

Table 13. Reaction components for universal PCR if using QIAseq Targeted cfDNA Ultra UDI (12) or QIAseq Targeted cfDNA Ultra UDI Set A, B, C, or D (96)

Component	Volume/reaction (µL)
Cleaned target-enriched DNA from "Procedure: TEPCR cleanup"	5
HiFi Ultra Buffer, 5x	8
Index primers from QIAseq Targeted cfDNA Ultra UDI index plate*	2
HiFi Ultra Polymerase	2
Nuclease-free water	23
Total	**40**

* Applies to QIAseq Targeted cfDNA Ultra UDI (12) or QIAseq Targeted cfDNA Ultra UDI Set A, B, C, and D (96).

DNA Ultra UDI 12 Index Plate (QUDI-12A) in QIAseq Targeted cfDNA Ultra UDI (12)

	1	2	3	4	5	6	7	8	9	10	11	12
A	IL5-QUDI-1 IL7-QUDI-1	IL5-QUDI-9 IL7-QUDI-9										
B	IL5-QUDI-2 IL7-QUDI-2	IL5-QUDI-10 IL7-QUDI-10										
C	IL5-QUDI-3 IL7-QUDI-3	IL5-QUDI-11 IL7-QUDI-11										
D	IL5-QUDI-4 IL7-QUDI-4	IL5-QUDI-12 IL7-QUDI-12										
E	IL5-QUDI-5 IL7-QUDI-5											
F	IL5-QUDI-6 IL7-QUDI-6											
G	IL5-QUDI-7 IL7-QUDI-7											
H	IL5-QUDI-8 IL7-QUDI-8											

DNA Ultra UDI Set A Plate (QUDI-96AA) in QIAseq Targeted cfDNA Ultra UDI Set A (96)

	1	2	3	4	5	6	7	8	9	10	11	12
A	IL5-QUDI-1 IL7-QUDI-1	IL5-QUDI-9 IL7-QUDI-9	IL5-QUDI-17 IL7-QUDI-17	IL5-QUDI-25 IL7-QUDI-25	IL5-QUDI-33 IL7-QUDI-33	IL5-QUDI-41 IL7-QUDI-41	IL5-QUDI-49 IL7-QUDI-49	IL5-QUDI-57 IL7-QUDI-57	IL5-QUDI-65 IL7-QUDI-65	IL5-QUDI-73 IL7-QUDI-73	IL5-QUDI-81 IL7-QUDI-81	IL5-QUDI-89 IL7-QUDI-89
B	IL5-QUDI-2 IL7-QUDI-2	IL5-QUDI-10 IL7-QUDI-10	IL5-QUDI-18 IL7-QUDI-18	IL5-QUDI-26 IL7-QUDI-26	IL5-QUDI-34 IL7-QUDI-34	IL5-QUDI-42 IL7-QUDI-42	IL5-QUDI-50 IL7-QUDI-50	IL5-QUDI-58 IL7-QUDI-58	IL5-QUDI-66 IL7-QUDI-66	IL5-QUDI-74 IL7-QUDI-74	IL5-QUDI-82 IL7-QUDI-82	IL5-QUDI-90 IL7-QUDI-90
C	IL5-QUDI-3 IL7-QUDI-3	IL5-QUDI-11 IL7-QUDI-11	IL5-QUDI-19 IL7-QUDI-19	IL5-QUDI-27 IL7-QUDI-27	IL5-QUDI-35 IL7-QUDI-35	IL5-QUDI-43 IL7-QUDI-43	IL5-QUDI-51 IL7-QUDI-51	IL5-QUDI-59 IL7-QUDI-59	IL5-QUDI-67 IL7-QUDI-67	IL5-QUDI-75 IL7-QUDI-75	IL5-QUDI-83 IL7-QUDI-83	IL5-QUDI-91 IL7-QUDI-91
D	IL5-QUDI-4 IL7-QUDI-4	IL5-QUDI-12 IL7-QUDI-12	IL5-QUDI-20 IL7-QUDI-20	IL5-QUDI-28 IL7-QUDI-28	IL5-QUDI-36 IL7-QUDI-36	IL5-QUDI-44 IL7-QUDI-44	IL5-QUDI-52 IL7-QUDI-52	IL5-QUDI-60 IL7-QUDI-60	IL5-QUDI-68 IL7-QUDI-68	IL5-QUDI-76 IL7-QUDI-76	IL5-QUDI-84 IL7-QUDI-84	IL5-QUDI-92 IL7-QUDI-92
E	IL5-QUDI-5 IL7-QUDI-5	IL5-QUDI-13 IL7-QUDI-13	IL5-QUDI-21 IL7-QUDI-21	IL5-QUDI-29 IL7-QUDI-29	IL5-QUDI-37 IL7-QUDI-37	IL5-QUDI-45 IL7-QUDI-45	IL5-QUDI-53 IL7-QUDI-53	IL5-QUDI-61 IL7-QUDI-61	IL5-QUDI-69 IL7-QUDI-69	IL5-QUDI-77 IL7-QUDI-77	IL5-QUDI-85 IL7-QUDI-85	IL5-QUDI-93 IL7-QUDI-93
F	IL5-QUDI-6 IL7-QUDI-6	IL5-QUDI-14 IL7-QUDI-14	IL5-QUDI-22 IL7-QUDI-22	IL5-QUDI-30 IL7-QUDI-30	IL5-QUDI-38 IL7-QUDI-38	IL5-QUDI-46 IL7-QUDI-46	IL5-QUDI-54 IL7-QUDI-54	IL5-QUDI-62 IL7-QUDI-62	IL5-QUDI-70 IL7-QUDI-70	IL5-QUDI-78 IL7-QUDI-78	IL5-QUDI-86 IL7-QUDI-86	IL5-QUDI-94 IL7-QUDI-94
G	IL5-QUDI-7 IL7-QUDI-7	IL5-QUDI-15 IL7-QUDI-15	IL5-QUDI-23 IL7-QUDI-23	IL5-QUDI-31 IL7-QUDI-31	IL5-QUDI-39 IL7-QUDI-39	IL5-QUDI-47 IL7-QUDI-47	IL5-QUDI-55 IL7-QUDI-55	IL5-QUDI-63 IL7-QUDI-63	IL5-QUDI-71 IL7-QUDI-71	IL5-QUDI-79 IL7-QUDI-79	IL5-QUDI-87 IL7-QUDI-87	IL5-QUDI-95 IL7-QUDI-95
H	IL5-QUDI-8 IL7-QUDI-8	IL5-QUDI-16 IL7-QUDI-16	IL5-QUDI-24 IL7-QUDI-24	IL5-QUDI-32 IL7-QUDI-32	IL5-QUDI-40 IL7-QUDI-40	IL5-QUDI-48 IL7-QUDI-48	IL5-QUDI-56 IL7-QUDI-56	IL5-QUDI-64 IL7-QUDI-64	IL5-QUDI-72 IL7-QUDI-72	IL5-QUDI-80 IL7-QUDI-80	IL5-QUDI-88 IL7-QUDI-88	IL5-QUDI-96 IL7-QUDI-96

DNA Ultra UDI Set B Plate (QUDI-96BA) in QIAseq Targeted cfDNA Ultra UDI Set B (96)

	1	2	3	4	5	6	7	8	9	10	11	12
A	IL5-QUDI-97 IL7-QUDI-97	IL5-QUDI-105 IL7-QUDI-105	IL5-QUDI-113 IL7-QUDI-113	IL5-QUDI-121 IL7-QUDI-121	IL5-QUDI-129 IL7-QUDI-129	IL5-QUDI-137 IL7-QUDI-137	IL5-QUDI-145 IL7-QUDI-145	IL5-QUDI-153 IL7-QUDI-153	IL5-QUDI-161 IL7-QUDI-161	IL5-QUDI-169 IL7-QUDI-169	IL5-QUDI-177 IL7-QUDI-177	IL5-QUDI-185 IL7-QUDI-185
B	IL5-QUDI-98 IL7-QUDI-98	IL5-QUDI-106 IL7-QUDI-106	IL5-QUDI-114 IL7-QUDI-114	IL5-QUDI-122 IL7-QUDI-122	IL5-QUDI-130 IL7-QUDI-130	IL5-QUDI-138 IL7-QUDI-138	IL5-QUDI-146 IL7-QUDI-146	IL5-QUDI-154 IL7-QUDI-154	IL5-QUDI-162 IL7-QUDI-162	IL5-QUDI-170 IL7-QUDI-170	IL5-QUDI-178 IL7-QUDI-178	IL5-QUDI-186 IL7-QUDI-186
C	IL5-QUDI-99 IL7-QUDI-99	IL5-QUDI-107 IL7-QUDI-107	IL5-QUDI-115 IL7-QUDI-115	IL5-QUDI-123 IL7-QUDI-123	IL5-QUDI-131 IL7-QUDI-131	IL5-QUDI-139 IL7-QUDI-139	IL5-QUDI-147 IL7-QUDI-147	IL5-QUDI-155 IL7-QUDI-155	IL5-QUDI-163 IL7-QUDI-163	IL5-QUDI-171 IL7-QUDI-171	IL5-QUDI-179 IL7-QUDI-179	IL5-QUDI-187 IL7-QUDI-187
D	IL5-QUDI-100 IL7-QUDI-100	IL5-QUDI-108 IL7-QUDI-108	IL5-QUDI-116 IL7-QUDI-116	IL5-QUDI-124 IL7-QUDI-124	IL5-QUDI-132 IL7-QUDI-132	IL5-QUDI-140 IL7-QUDI-140	IL5-QUDI-148 IL7-QUDI-148	IL5-QUDI-156 IL7-QUDI-156	IL5-QUDI-164 IL7-QUDI-164	IL5-QUDI-172 IL7-QUDI-172	IL5-QUDI-180 IL7-QUDI-180	IL5-QUDI-188 IL7-QUDI-188
E	IL5-QUDI-101 IL7-QUDI-101	IL5-QUDI-109 IL7-QUDI-109	IL5-QUDI-117 IL7-QUDI-117	IL5-QUDI-125 IL7-QUDI-125	IL5-QUDI-133 IL7-QUDI-133	IL5-QUDI-141 IL7-QUDI-141	IL5-QUDI-149 IL7-QUDI-149	IL5-QUDI-157 IL7-QUDI-157	IL5-QUDI-165 IL7-QUDI-165	IL5-QUDI-173 IL7-QUDI-173	IL5-QUDI-181 IL7-QUDI-181	IL5-QUDI-189 IL7-QUDI-189
F	IL5-QUDI-102 IL7-QUDI-102	IL5-QUDI-110 IL7-QUDI-110	IL5-QUDI-118 IL7-QUDI-118	IL5-QUDI-126 IL7-QUDI-126	IL5-QUDI-134 IL7-QUDI-134	IL5-QUDI-142 IL7-QUDI-142	IL5-QUDI-150 IL7-QUDI-150	IL5-QUDI-158 IL7-QUDI-158	IL5-QUDI-166 IL7-QUDI-166	IL5-QUDI-174 IL7-QUDI-174	IL5-QUDI-182 IL7-QUDI-182	IL5-QUDI-190 IL7-QUDI-190
G	IL5-QUDI-103 IL7-QUDI-103	IL5-QUDI-111 IL7-QUDI-111	IL5-QUDI-119 IL7-QUDI-119	IL5-QUDI-127 IL7-QUDI-127	IL5-QUDI-135 IL7-QUDI-135	IL5-QUDI-143 IL7-QUDI-143	IL5-QUDI-151 IL7-QUDI-151	IL5-QUDI-159 IL7-QUDI-159	IL5-QUDI-167 IL7-QUDI-167	IL5-QUDI-175 IL7-QUDI-175	IL5-QUDI-183 IL7-QUDI-183	IL5-QUDI-191 IL7-QUDI-191
H	IL5-QUDI-104 IL7-QUDI-104	IL5-QUDI-112 IL7-QUDI-112	IL5-QUDI-120 IL7-QUDI-120	IL5-QUDI-128 IL7-QUDI-128	IL5-QUDI-136 IL7-QUDI-136	IL5-QUDI-144 IL7-QUDI-144	IL5-QUDI-152 IL7-QUDI-152	IL5-QUDI-160 IL7-QUDI-160	IL5-QUDI-168 IL7-QUDI-168	IL5-QUDI-176 IL7-QUDI-176	IL5-QUDI-184 IL7-QUDI-184	IL5-QUDI-192 IL7-QUDI-192

Figure 4. Layout of DNA Ultra UDI Index Primer Plate in QIAseq Targeted cfDNA Ultra UDI (12) and QIAseq Targeted cfDNA Ultra UDI Sets A and B (96). Each well contains one pair of predispensed sample index primers plus universal primers for a single reaction in "Protocol: Universal PCR".

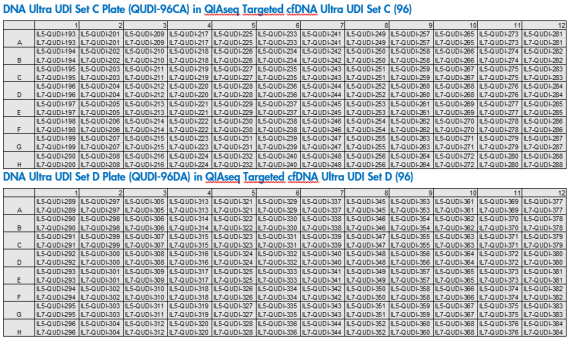

Figure 5. Layout of DNA Ultra UDI Index Primer Plate in QIAseq Targeted cfDNA Ultra UDI Sets C and D (96). Each well contains one pair of predispensed sample index primers plus universal primers for a single reaction in "Protocol: Universal PCR".

4. Program a thermal cycler using the cycling conditions in Table 14 and Table 15 indicates the cycle number to use dependent on the number of primers in the pool. Use the instrument's heated lid.

Table 14. Incubation conditions for universal PCR

Step	Time	Temperature (°C)
Initial denaturation	2 min	98
Number of cycles (see Table 15)	20 s 1 min	98 60
1 cycle	3 min	72
Hold	∞	4

Table 15. Amplification cycles for universal PCR

Primers per pool	Cycle number
6–24	26
25–96	24
97–288	22
289–1056	21
1057–3072	20
3073–5999	18
6000–12,000	17
≥12,001	16

5. After the reaction is complete, place the reactions on ice and proceed to "Procedure: cleanup of universal PCR" (below). Alternatively, the samples can be stored at –20°C in a constant-temperature freezer for up to 3 days.

Procedure: cleanup of universal PCR

6. Add 50 µL Nuclease-free Water to bring each sample to 90 µL.
7. Add 108 µL QIAseq Beads to the completed universal PCR; mix well by vortexing or pipetting up and down at least 15 times with pipetting volume close to 190 µL.
8. Incubate for 5 min at room temperature.
9. Place the tubes or plate on magnetic rack for 5 min (tube) or 10 min (plate) to separate beads from supernatant. Once the solution has cleared, with the tubes or plate still on the magnetic stand, carefully remove and discard the supernatant.

   Important: Do not discard the beads as they contain the DNA of interest.
10. With the tubes or plate still on the magnetic stand, add 200 µL 80% ethanol and leave beads in ethanol for 30 seconds. Carefully remove and discard the ethanol.

11. Repeat the ethanol wash.

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

12. With the tubes or plate still on the magnetic stand, air dry at room temperature for at least 10 min.

    Note: Visually inspect that the pellet is completely dry. Over drying the beads will not affect DNA elution.

13. Remove the tubes or plate from the magnetic stand, and elute the DNA from the beads by adding 30 µL Nuclease-free Water. Mix well by pipetting or vortexing.

14. Return the tubes or plate to the magnetic rack until the solution has cleared.

15. Transfer 28 µL supernatant to clean tubes or plate.

16. Proceed to "Recommendations: Library QC and Quantification" (next page). Alternatively, the library can be stored at –30°C to –15°C in a constant-temperature freezer. Amplified libraries are stable for several months at –30°C to –15°C. Once quantification is performed, proceed to "Protocol: Sequencing Setup on Illumina MiSeq, NextSeq 500/550, NextSeq 1000/2000, MiniSeq and NovaSeq" on page 44

Recommendations: Library QC and Quantification

NGS library QC

After the library is constructed and purified, QC can be performed with QIAGEN's QIAxcel Systems or Agilent's Bioanalyzer or TapeStation to check for the correct size distribution of the library fragments and for the absence of primer dimers (approximately <200 bp) and concentration. Majority library fragments prepared for Illumina instruments demonstrate a size distribution between 200 and 400 bp (Figure 6 and Figure 7). Library overamplification is normal (Figure 7B), and this should not affect the sequencing results. Overamplified libraries are usually single-stranded libraries with correct size but appear as "larger fragments" due to secondary structures. 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 overamplified libraries (See "Preferred library quantification method").

Recommended setting for checking QIAseq cfDNA Ultra library on QIAxcel Advanced and Connect systems

QIAxcel Connect

- QIAxcel DNA High Sensitivity Kit (1200) (cat. no. 929012)
- Use method Default High Sensitivity with 1–10 µL library

QIAxcel Advanced or QIAxcel Connect

- QIAxcel DNA High Resolution Kit (cat. no. 929002)
- QX Alignment Marker: 15 bp/5 kb (cat. no. 929524)

- QX DNA Size Marker: 100 bp – 2.5 kb (cat. no. 929559)
- Use the *Application Guide for Low-Concentration Libraries*. To access guides and system files for Library QC, contact QIAGEN Technical Services.

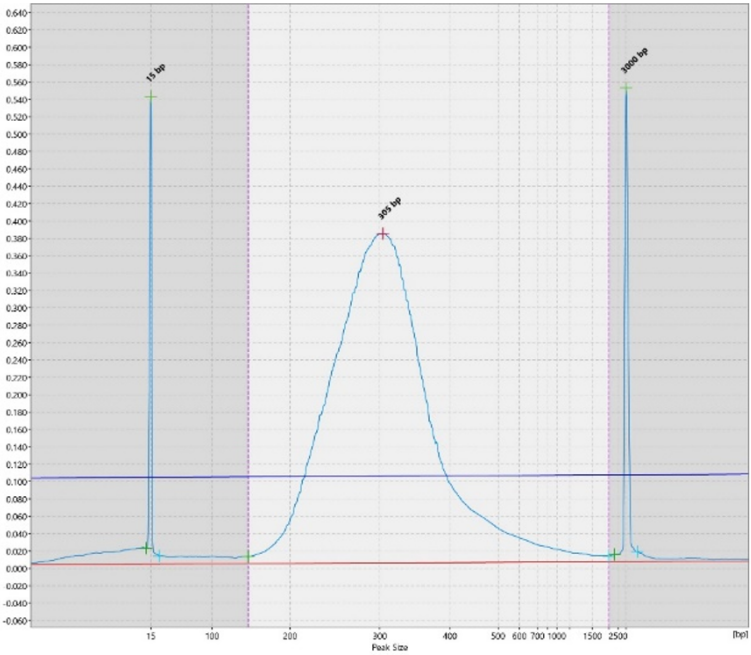

Figure 6. Sample QIAxcel image of QIAseq Targeted cfDNA Ultra libraries for Illumina instruments. The library assessed using QIAxcel illustrates the size of the majority of the library fragments are between 200 and 400 bp.

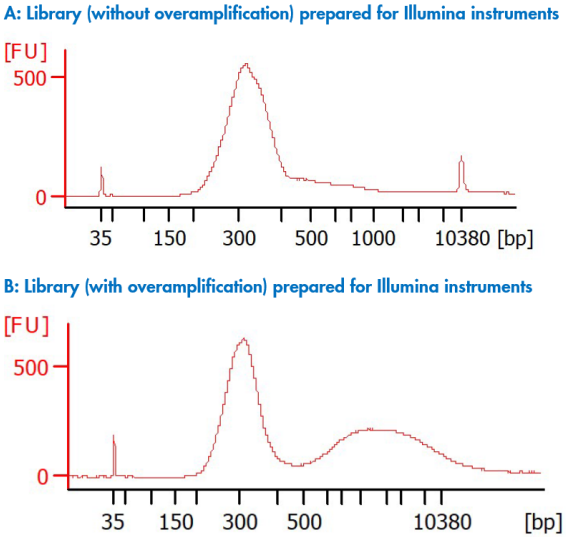

Figure 7. Sample Bioanalyzer images of QIAseq Targeted cfDNA Ultra libraries for Illumina instruments. The size of the majority of the library fragments are between 200 and 400 bp. **A**: Library without overamplification. **B**: Library with overamplification as indicated by the "larger fragment" peak.

Preferred library quantification method

The library yield measurements from the Bioanalyzer or TapeStation rely on fluorescence dyes that intercalate into DNA. These dyes cannot discriminate between molecules with or without adapter sequences, yet only complete QIAseq Targeted cfDNA Ultra libraries with full adapter sequences will be sequenced. Due to the superior sensitivity of qPCR, we recommend quantifying the libraries using, QIAGEN's QIAseq Library Quant Assay Kit (cat. no. 333314), which contains laboratory-verified forward and reverse primers, together with a DNA standard. 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.

Protocol: Sequencing Setup on Illumina MiSeq, NextSeq 500/550, NextSeq 1000/2000, MiniSeq and NovaSeq

Important points before starting

- Recommendations for library dilution concentrations and library loading concentrations are based on QIAseq Library Quant System (see "Preferred library quantification method" on the previous page). If using Qubit for library quantification, load 50% less as starting point to avoid over clustering and adjust accordingly after the first run.
- Paired-end sequencing should be used for the QIAseq Targeted cfDNA Ultra on Illumina platform.
- To make sequencing preparation more convenient, download Illumina-compatible sample sheets for different sequencing instruments on **www.qiagen.com**, from the Resources tab of the QIAseq Targeted cfDNA Ultra page.
- Paired-end sequencing of 149 bp should be used for QIAseq Targeted cfDNA Ultra UDI libraries and dual 10 bp indices on Illumina platforms.
- For two-channel sequencing chemistry platforms such as MiniSeq, NextSeq, and NovaSeq, 10% PhiX can be included in the run to improve sequencing quality. For complete instructions on how to denature sequencing libraries, and set up a sequencing run, please refer to the system-specific Illumina documents.

Sequencing preparations for MiSeq with QIAseq Targeted cfDNA Ultra UDI Sets

1. When working with the QIAseq Targeted cfDNA Ultra UDI Sets, use Local Run Manager (LRM) v2 or later on the instrument to upload a sample sheet (see the Resources tab of the QIAseq Targeted cfDNA Ultra and download the appropriate template) and proceed with

sequencing: Read 1 is 149 bp, Read 2 is 149 bp, and each Index Read is 10 bp.

2. **Sample dilution and pooling**: Dilute libraries to 2 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. **Library preparation and loading**: 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. Upon completion of the sequencing run, proceed to "Appendix B: Data Analysis Using QIAGEN's CLC Genomic Workbench" (page "Appendix B: Data Analysis Using QIAGEN's CLC Genomic Workbench" on page 51).

Sequencing preparations for MiniSeq, NextSeq 500/550 and NextSeq1000/2000 with QIAseq Targeted cfDNA Ultra UDI Sets

1. When working with the QIAseq Targeted cfDNA Ultra UDI Sets, use LRM v2 or later on the instrument to upload a sample sheet (see the Resources tab of the QIAseq Targeted cfDNA Ultra page and download the appropriate template) and proceed with

sequencing: Read 1 is 149 bp, Read 2 is 149 bp and each Index Read is 10 bp.

2. **Sample dilution and pooling**: Dilute libraries to 1 nM for MiniSeq, 0.5, 1, 2 or 4 nM for NextSeq 500/550 and 2 nM for NextSeq 1000/2000. 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.

3. **Library preparation and loading**: Prepare and load the library onto a MiniSeq, NextSeq 500/550 or NextSeq 1000/2000 according to the MiniSeq or NextSeq System Denature and Dilute Libraries Guide. The final library concentration is 1.0–1.2 pM on the MiniSeq or NextSeq 500/550 and 650 pM for NextSeq 1000/2000 onboard denature and dilute.

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

   Note: For P3/P4 flow cell with XLEAP-SBS chemistry kit of NextSeq2000, load 488 pM that’s about 25% less compared to standard SBS according to Illumina recommendation.

4. Upon completion of the sequencing run, proceed to ""Appendix B: Data Analysis Using QIAGEN's CLC Genomic Workbench" on page 51".

Sequencing preparations for NovaSeq with QIAseq Targeted cfDNA Ultra UDI Index Sets

1. When working with the QIAseq Targeted cfDNA Ultra UDI Sets, upload a sample sheet (see the Resources tab of the QIAseq Targeted DNA Panel page and download the appropriate template) and proceed with sequencing: Read 1 is 149 bp, Read 2 is 149 bp and each Index Read is 10 bp.
2. Sample dilution and pooling: Dilute libraries to 4 nM for NovaSeq. 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.
3. Library preparation and loading: Prepare and load the library onto a NovaSeq according to the NovaSeq 6000 or NovaSeq x Sequencing System Guide. The final pooled library concentration recommendation is between 2–4 nM yielding a final loading concentration of between 140–200 pM on the NovaSeq.

   Note: Recommendations for library loading concentrations are based on QIAseq Library Quant System.
4. Upon completion of the sequencing run, proceed to ""Appendix B: Data Analysis Using QIAGEN's CLC Genomic Workbench" on page 51".

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 cfDNA input	Make sure cfDNA is free of cellular genomic DNA. If there is cellular genomic DNA, make sure input is based on cfDNA peak, not all the DNA in the sample.
b) Inefficient targeted enrichment or universal PCR	Check to see if correct thermocycling condition is used during target enrichment or universal PCR. Ensure no ethanol or bead carryover to the PCR.
Unexpected signal peaks	
a) Short peaks less than 200 bp	These are primer–dimers from targeted enrichment PCR or universal PCR. The presence of primer dimers indicates either not enough DNA input or inefficient PCR.
b) Larger library fragments after universal PCR	After the universal PCR, library fragments are larger than the intended peak and can be a PCR artifact due to overamplification of the DNA library. Overamplification of the library will not affect the QIAseq Targeted cfDNA Ultra sequencing performance. Decreasing the number of universal PCR 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 that the library is accurately quantified and that the correct amount is loaded onto the sequencing instrument.

	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 to see if the required input DNA 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 16.

Table 16. Combining an existing panel (at 50 µL) with a booster panel

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

Appendix B: Data Analysis Using QIAGEN's CLC Genomic Workbench

After sequencing, data from the QIAseq Targeted cfDNA Ultra can be analyzed using the Biomedical Genomics Analysis plugin for CLC 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.

Ordering Information

Product	Contents	Cat. no.
QIAseq Targeted cfDNA Ultra (12)	All reagents (except indexes) for targeted DNA sequencing; fixed panel for 12 samples; less than 200 genes	334051
QIAseq Targeted cfDNA Ultra (96)	All reagents (except indexes) for targeted DNA sequencing; fixed panel for 96 samples; less than 200 genes	334055
QIAseq Targeted cfDNA Ultra Custom (96)	All reagents (except indexes) for targeted DNA sequencing; custom panel for 96 samples	Inquire
QIAseq Targeted cfDNA Ultra Booster (96)	Pool of primers used in combination with either cataloged or custom panels	334085
QIAseq cfDNA Ultra Reagent Kit (96)	Kit containing library preparation and target enrichment reagents (except indexes and primers) for targeted DNA sequencing; fixed reagents for 96 samples	334455
QIAseq Targeted cfDNA Ultra Unique Dual Indices		
QIAseq Targeted cfDNA Ultra UDI Set A (96)	Box containing unique molecularly indexed adapters and primers, enough for a total of 96 samples, for indexing up to 96 samples for targeted panel sequencing on Illumina platforms; Set A (of A, B, C, and D) required for multiplexing 384 samples in one run	334165
QIAseq Targeted cfDNA Ultra UDI Set B (96)	Box containing unique molecularly indexed adapters and primers, enough for a total of 96 samples, for indexing up to 96 samples for targeted panel sequencing on Illumina platforms; Set B (of A, B, C, and D) required for multiplexing 384 samples in one run	334175
QIAseq Targeted cfDNA Ultra UDI Set C (96)	Box containing unique molecularly indexed adapters and primers, enough for a total of 96 samples, for indexing up to 96 samples for targeted panel sequencing on Illumina platforms; Set C (of A, B, C, and D) required for multiplexing 384 samples in one run	334185

Product	Contents	Cat. no.
QIAseq Targeted cfDNA Ultra UDI Set D (96)	Box containing unique molecularly indexed adapters and primers, enough for a total of 96 samples, for indexing up to 96 samples for targeted panel sequencing on Illumina platforms; Set D (of A, B, C, and D) required for multiplexing 384 samples in one run	334195
QIAseq Targeted cfDNA Ultra UDI (12)	Box containing unique molecularly-indexed adapters and primers, enough for a total of 12 samples, for indexing up to 12 samples for targeted panel sequencing on Illumina platforms	334151
Related products		
QIAseq Library Quant Assay Kit	Reagents for quantification of libraries prepared for Illumina or Ion Torrent platforms; assay format	333314
QIAamp Circulating Nucleic Acid Kit (50)	For 50 preps: QIAamp Mini Columns, Tube Extenders (20 mL), QIAGEN Proteinase K, Carrier RNA, Buffers, VacConnectors, and Collection Tubes	55114
QIAamp MinElute ccfDNA Mini Kit (50)	For 50 DNA preps: QIAamp UCP MinElute Columns, QIAGEN Proteinase K, Magnetic Bead Suspension, Buffers, Bead Elution Tubes, Collection Tubes	55204
QIAamp MinElute ccfDNA Midi Kit (50)	For 50 preps: QIAamp UCP MinElute Columns, QIAGEN Proteinase K, Magnetic Bead Suspension, Buffers, Bead Elution Tubes, and Collection Tubes	55284
EZ1&2 ccfDNA Kit (48)	For 48 preps: 48 reagent cartridges (EZ1&2 ccfDNA), Magnetic Bead Suspension, Elution Buffer, Large-Volume Tubes, Disposable Tip Holders, Disposable Filter-Tips, Elution Tubes	984854

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.

Document Revision History

Date	Changes
12/2022	Initial revision
04/2023	With reduced HiFi polymerase during TEPCR for panel with 1100 primers or less; Added two panels, Actionable Solid Tumor and Myeloid Neoplasms; Included NextSeq 1000/2000 in sequencing set up; Corrected UDI index plate name in index kit component.
07/2025	Change in list of Illumina NGS systems: removed HiSeq with NovaSeq; Update in Important Notes: Addition of column "10,000 primers" for number of multiplexed samples; Update in Protocols; addition of cycling conditions in Protocol: Target Enrichment.

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