June 2025

QIAseq® xHYB Long Read Panel Handbook

Sample to Insight® solution for long-read targeted next-generation sequencing of human samples

Sample to Insight

Table of Contents

Kit Contents 4
Shipping and Storage 8
Intended Use 9
Safety Information 10
Quality Control 11
Introduction 12
    Principle and procedure 14
Equipment and Reagents to be Supplied by User 18
Important Notes 20
Protocol: Long- Read Enzymatic Fragmentation and Indexed Human DNA Library Construction 21
    Long-read fragmentation, end polishing, and A-addition 22
    Adapter ligation 24
    Size-selection after ligation 26
    Size-selection after library amplification 28
Protocol: Long - Read Mechanical Fragmentation and Indexed Human DNA Library Construction 30
    Adapter ligation 33
    Size-selection after ligation 34
    Size-selection after library amplification 37
Protocol: Hybrid Capture and Wash 38
    Pool libraries and overnight HYB capture reaction 38
    Preparing streptavidin beads 41
    Binding and washing of hybridized targets to streptavidin beads 42
    Post-capture library amplification 43
    Size-selection of secondary amplification 46

Protocol: Library QC and Quantification 48
Protocol: Data Analysis 49
Troubleshooting Guide 50
Appendix A: Cleanup of DNA Samples Containing EDTA/EGTA 51
Appendix B: Preparing Long-Read Libraries for Sequencing on Oxford Nanopore Platform 53
Optimal protocol (sample input greater than 400 ng captured DNA) 54
Non-optimal protocol for end-repair and Nanopore adapter ligation (samples less than 400 ng input) 57
Appendix C: Overamplification of Libraries 60
Long-read reconditioning of overamplified libraries 61
Size-selection after post-capture recondition 62
Appendix D: Unique Dual-Index Primers for PCR-Based Indexing 63
Ordering Information 70
Document Revision History 71

Kit Contents

Component part no.	Component name	QIAseq xHYB Long Read HYB Kit E (24)	QIAseq xHYB Long Read HYB Kit E (96)	QIAseq xHYB Long Read HYB Kit S (24)	QIAseq xHYB Long Read HYB Kit S (96)
334332	QIAseq xHYB Long Read Reagent Kit E (24), Box 1	1 pc	–	–	–
334332	QIAseq xHYB Long Read Reagent Kit (24), Box 2	1 pc	–	–	–
334332	QIAseq xHYB Long Read Reagent Kit (24), Box 3	1 pc	–	–	–
334335	QIAseq xHYB Long Read Reagent Kit E (96), Box 1	–	1 pc	–	–
334335	QIAseq xHYB Long Read Reagent Kit (96), Box 2	–	1 pc	–	–
334335	QIAseq xHYB Long Read Reagent Kit (96), Box 3	–	1 pc	–	–
334342	QIAseq xHYB Long Read Reagent Kit S (24), Box 1	-	–	1 pc	–
334342	QIAseq xHYB Long Read Reagent Kit (24), Box 2	-	–	1 pc	–
334342	QIAseq xHYB Long Read Reagent Kit (24), Box 3	-	–	1 pc	–
334345	QIAseq xHYB Long Read Reagent Kit S (96), Box 1	-	–	–	1 pc
334345	QIAseq xHYB Long Read Reagent Kit (96), Box 2	-	-	-	1 pc
334345	QIAseq xHYB Long Read Reagent Kit (96), Box 3	-	-	-	1 pc

QIAseq xHYB Long Read Reagent Kit E Box 1 Cat. no. No. of Hybridization Reactions Typical no. of samples	(24) 334332 3 24	(96) 334335 12 96
Fragmentation Buffer, 10x	108 µL × 1	432 µL × 1
Fragmentation Enzyme Mix	192 µL × 1	768 µL × 1
FX Enhancer	54 µL × 1	216 µL × 1
FA Buffer	60 µL × 1	240 µL × 1
FERA Solution	60 µL × 1	110 µL × 2
FG solution	170 µL × 1	170 µL × 2
UPH Ligation Buffer	1152 µL × 1	3840 µL × 1
DNA Ligase	360 µL × 1	1100 µL × 1
LR Adapter	120 µL × 1	480 µL × 1
Size Selection Reagent	3.4 mL × 1	13.6 mL × 1
MUDI plate	9 µL x 24	9 µL x 96
Buffer EB	1.2 mL × 1	4.8 mL × 1
HMW Wash Buffer	2.2 mL × 1	8.8 mL × 1
2X HiFi LR Master Mix	1 mL x 2	8 mL x 1
LR Buffer XL	600 µL x 1	600 µL x 2
LR Primer Mix	1300 µL x 1	1300 µL x 2

QIAseq xHYB Long Read Reagent Kit S Box 1 Cat. no. No. of Hybridization Reactions Typical no. of samples	(24) 334342 3 24	(96) 334345 12 96
ERA Buffer, 10x	60 µL × 1	480 µL × 1
ERA Enzyme	120 µL × 1	960 µL × 1
UPH Ligation Buffer	1152 µL × 1	3840 µL × 1
DNA Ligase	360 µL × 1	1100 µL × 1
LR Adapter	120 µL × 1	480 µL × 1
Size Selection Reagent	3.4 mL × 1	13.6 mL × 1
MUDI plate	9 µL × 24	9 µL × 96
Buffer EB	1.2 mL × 1	4.8 mL × 1
HMW Wash Buffer	2.2 mL × 1	8.8 mL × 1
2x HiFi LR Master Mix	1 mL × 2	8 mL × 1
LR Buffer XL	600 µL × 1	600 µL × 2
LR Primer Mix	1300 µL × 1	1300 µL × 2

QIAseq xHYB Long Read Reagent Box 2 Cat. no. No. of samples	(24) 334332/334342 24	(96) 334335/334345 96
LR Amp SB	270 µL × 1	270 µL × 2
One-4-All Blocking Oligos	24 µL ×1	96 µL × 1
One-4-All Blocking Solution	15 µL × 1	60 µL × 1
HYB Mix	150 µL × 1	150 µL × 2
Enhanced blocking buffer	18 µL × 1	85 µL × 1
Vapor-Lock	500 µL × 1	500 µL × 1
LR Amp Enhancer	84 µL × 1	84 µL × 1
HN Buffer	30 µL × 1	30 µL × 1

QIAseq xHYB Long Read Reagent Kit Box 3 Cat. no. No. of samples	(24) 334332/3343422 24	(96) 334335/334345 96
Bead Booster	48 µL × 1	144 µL × 1
Nuclease-free Water	10 mL ×1	50 mL × 1
QIAseq Beads	10 mL × 1	38.4 mL × 1
Streptavidin Binding Beads	300 µL × 1	1200 µL × 1
Binding Buffer	6.25 mL × 1	6.25 mL × 2
Wash Buffer 1	3.25 mL × 1	3.25 mL × 2
Wash Buffer 2	5 mL × 1	5 mL × 2

QIAseq xHYB Long Read Panel Cat. no. No. of hybridization reactions No. of samples	(96) 334322 3 24	(96) 334325 12 96
HLA Panel (LXHS-200Z), probes	12	48 µL x 1
Hereditary Cancer Panel (LXHS-3200Z), probes	12	48 mL x 1

QIAseq xHYB Long Read Custom Panel Cat. no. No. of hybridization reactions No. of samples		(96) 334355 12 96
QIAseq xHYB Long Read Custom Panel, probes		48 µL x 1

Shipping and Storage

The QIAseq xHYB Long Read Panels are shipped in three boxes. Boxes 1 and 2 are shipped on dry ice, and Box 3 is shipped on blue ice. Upon receipt, all the components in Boxes 1 and 2 should be stored immediately at –30°C to –15°C in a constant-temperature freezer. All the components in Box 3, the beads, and wash buffers should be stored immediately at 2–8°C.

When stored correctly, QIAseq xHYB Long Read Panels are good until the expiration date printed on the kit box lid, outer package.

Intended Use

The QIAseq xHYB Long Read Panels 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 xHYB Long Read Panels is tested against predetermined specifications to ensure consistent product quality.

Introduction

While widely used, short-read sequencing has inherent limitations that can hinder comprehensive genomic analyses. Short reads often fail to span long repetitive regions, leading to incomplete or ambiguous assemblies. They also struggle to resolve haplotypes over extended genomic distances, making it difficult to unambiguously differentiate between alleles. Additionally, short-read lengths often miss or inaccurately characterize large and complex structural variations.

Long-read sequencing offers several advantages over traditional short-read methods, particularly in addressing challenges associated with complex genomic regions. Long-read sequencing accurately reports on repetitive sequences and complex structural variations, providing a clearer and more complete view of the genome. Furthermore, the ability to phase long stretches of DNA enables precise haplotyping of the highly polymorphic HLA region, for example. Long-read sequencing can also identify large insertions, deletions, inversions, and translocations with greater accuracy than short-read methods.

Targeted capture and detection of large structural variations (SVs) associated with hereditary cancer using long-read sequencing can provide crucial insights. SVs in DNA, such as large insertions, deletions, inversions, duplications, and translocations, play a significant role in the development and progression of cancer. These genomic alterations can disrupt genes, regulatory elements, and chromosomal architecture, leading to changes in gene expression, protein function, and genome stability. The detection and characterization of large SVs are crucial for understanding cancer biology, identifying biomarkers, and developing targeted therapies.

Long-read sequencing offers a transformative approach to understanding the role of large SVs in cancer. By overcoming the limitations of traditional techniques, it provides a more complete and accurate picture of the cancer genome. This capability not only enhances our understanding of cancer biology but also supports the development of precision medicine approaches that target specific genomic alterations.

Approaches using amplicon-based targeting with long-read sequencing technologies suffer from limitations. Designing primers for highly polymorphic or repetitive regions can be difficult, potentially leading to inefficient amplification or biases; one allele may fail to amplify due to primer mismatches, leading to incomplete or biased representation of heterozygous regions. Another limitation of amplicon-based targeting for long-read sequencing is that multiplexing many large amplicons becomes problematic as larger genomic regions are interrogated. Also, primer placement with respect to large structural variations that have unknown or poorly defined breakpoints is difficult and prone to bias.

Although amplicon-based methods are useful for targeted sequencing, their limitations make alternative approaches, such as hybrid capture-based long-read sequencing, more suitable for comprehensive and unbiased analyses in complex genomic regions. The QIAseq xHYB Long Read Panel has been optimized for the production of long human DNA libraries with high fidelity and uniform coverage over high-GC and other challenging regions of the human genome with minimal bias. The targeted human long-read hybrid capture probe pool designs are also optimized specifically for excellent uniformity and coverage over these challenging genomic regions.

Principle and procedure

The QIAseq xHYB Long Read Panel uses a workflow that consists of library construction with PCR-based indexing, followed by hybrid capture for targeted enrichment.

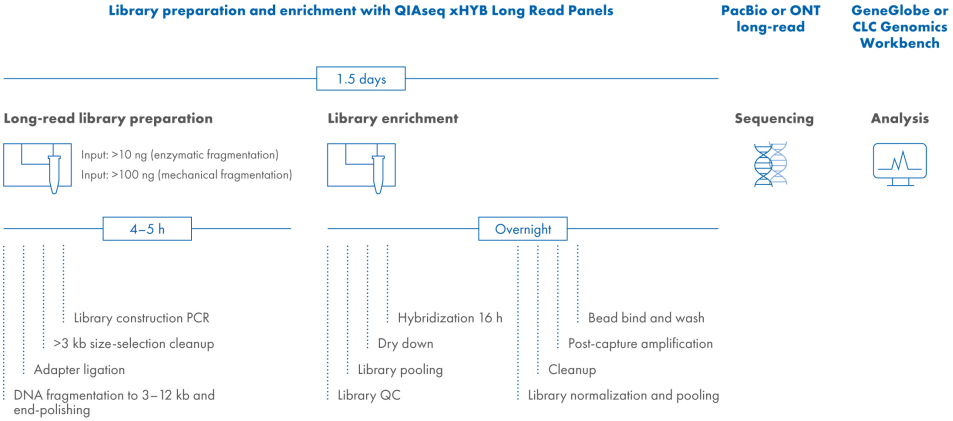

Figure 1. Scheme of optimized QIAseq xHYB long-read library construction and target enrichment. The QIAseq xHYB long-read workflow illustrating the production of 3–10 kb human DNA libraries using either enzymatic or mechanical fragmentation. After DNA fragmentation and adapter ligation, size selection is performed to remove DNA fragments smaller than 3 kb, libraries are then amplified with PCR-based indexing, and another round of size selection is performed before library QC. The purified, size-selected libraries are then pooled and concentrated by drying down. The dried-down libraries are resuspended and hybridized with biotinylated probes overnight. After washing, pre-amplification is performed to produce sufficient library material to allow efficient post-capture secondary amplification. Size selection is then performed, followed by library QC, with the resulting captured material ready for Pacific Biosystems or Oxford Nanopore long-read library processing.

QIAseq xHYB long-read DNA library construction

The QIAseq xHYB long-read workflow begins with enzymatic or mechanical fragmentation that is optimized to produce DNA fragments between 3 and 10 kb in length. Mechanical fragmentation is performed with Covaris® g-TUBEs using physical DNA shearing based on centrifuge speed. Mechanical shearing is not optimized for high-throughput fragmentation, as tube handling and number are restricted by centrifuge size and physical manipulation of g-TUBEs. In contrast, enzymatic fragmentation facilitates high-throughput fragmentation due to a simple, multichannel pipette–based workflow. For mechanical fragmentation, the DNA is size selected after fragmentation to concentrate the fragmented DNA from the g-TUBE fragmentation volume (50 µL) and to remove DNA that was fragmented smaller than 3 kb. The mechanical and enzymatic fragmentation workflows utilize different end-polishing systems: enzymatic fragmentation is performed with FX-based fragmentation and end-polishing, and end-polishing for mechanical fragmentation uses ERA-based polishing and A-addition. After the fragmented DNA is end polished and an "A" is added to the 3' end, this product is ready for long-read adapter ligation where the adapter is added to both ends of the DNA fragments. Following adapter ligation, bead-based size selection is performed to remove DNA fragments smaller than 3 kb, which will preferentially amplify over longer DNA fragments if not removed. The libraries are then amplified using PCR-based indexing to allow sample pooling and produce sufficient mass for subsequent hybrid capture.

Hybrid capture targeted enrichment

The size-selected, PCR-indexed long fragment human libraries are pooled with equal mass from each library, and enhanced blocking buffer is added to decrease non-specific hybridization. The pooled libraries are then dried down using a SpeedVac® system. The dried-down pooled libraries are resuspended and denatured. In a separate tube, the QIAseq xHYB long-read panel is mixed with hybridization mix and is denatured. After both pooled libraries and the QIAseq xHYB long-read panel cool down, the QIAseq xHYB long-read panel is added to the pooled libraries and this is placed overnight in a thermal cycler where the probes will hybridize to their targets. After overnight incubation, the biotinylated probes, along with any capture products, are bound to streptavidin-coated beads. The bound probes and streptavidin-coated beads are washed to remove any non-specific library fragments, and the streptavidin-bound library is resuspended with LR-amp enhancer; elution is performed with sodium hydroxide and neutralized with HN buffer. A pre-amplification PCR is then performed to convert the single-stranded captured DNA into double-stranded libraries and produce sufficient mass to undergo efficient secondary amplification in a larger volume to yield enough material for long-read sequencing library processing for either Pacific Biosystems or Oxford Nanopore long-read sequencing platforms. A final size selection is performed after amplification, quality control on the resulting libraries with Qubit® BR quantitation, and size-distribution assessment using one of the following approaches: 0.7% agarose gel electrophoresis, QIAxcel®, or TapeStation® genomic DNA assay, which are important to determine double-stranded DNA concentration and size-distribution of the capture libraries.

Long-read sequencing

For Pacific Biosystems long-read sequencing: After performing QC, libraries are ready for SMRTbell® adapter ligation. Refer to the Pacific Biosystems SMRTbell adapter ligation and loading protocols.

For Oxford Nanopore long-read sequencing: Refer to Appendix B: Preparing Long-Read Libraries for Sequencing on Oxford Nanopore Platform.

Data analysis

A simplified downstream data analysis is available through QIAGEN's GeneGlobe® Data Analysis portal for HLA haplotyping. Hereditary cancer large structural variant detection is performed with CLC Genomics Workbench.

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.

Required products

- **Preferred Library Quantification Method**: Qubit HS assay (Thermo Fisher Scientific, cat. no. Q32854) and Qubit BR assay (Thermo Fisher Scientific, cat. no. Q32853)
- **Size-distribution assessment**: 0.7% agarose gel electrophoresis, or QX DNA Size Marker Large-Fragment Kit, QX DNA Size Marker Large-Fragment Kit (cat. no. 929710), QX Nitrogen Cylinder (cat. no. 929705), and QIAxcel DNA High Resolution Kit (cat. no. 929002).
- **Alternative fragmentation**: g-TUBE (Covaris, Inc., cat. no. 520079)

Consumables and reagents

- Nuclease-free pipette tips and tubes
- 1.5 mL LoBind® tubes (Eppendorf, cat. no. 022431021)
- PCR tubes (0.2 mL individual tubes or tubes strips) (VWR, cat. no. 20170-012 or 93001-118)
- Ice
- Nuclease-Free Water

Laboratory equipment

- Single-channel pipettor
- Multichannel pipettor
- Microcentrifuge
- Thermal cycler
- Vortexer
- Magnetic rack/plate for 1.5 or 2 mL tubes (DynaMag™-2 Thermo Fisher Scientific 12321D or equivalent) or magnetic rack/plate for 96-well plates (DynaMag-96 Side Magnet, Thermo Fisher Scientific, cat. no. 12331D or equivalent)

  Note: If using a magnetic rack/plate for 96-well plates, then DynaMag-2 (or equivalent) is also required for the streptavidin bead capture and subsequent wash steps.
- Vacuum Concentrator (Thermo Fisher Scientific, SpeedVac Vacuum Concentrator System)
- Agarose gel electrophoresis chamber and power supply, QIAxcel, or TapeStation capillary electrophoresis instrument with Genomic DNA assay reagents
- Heat block that holds 1.5 mL tubes

Table 1. Optional positive DNA controls from Coriell

Target	Cat. no.
CEPH/UTAH PEDIGREE 1463	NA12878

Important Notes

General precautions

- Use good laboratory practices to minimize cross-contamination of nucleic acid products.
- Always use PCR tubes, microcentrifuge tubes, and pipette tips that are certified, sterile, and DNase free.
- Before starting, wipe down work area and pipettes with DNA cleaning product such as LookOut® DNA Erase (Sigma-Aldrich).
- For consistent library construction and hybridization reactions, ensure that the thermal cycler used in this protocol is in good working order and has been calibrated according to the manufacturer's specifications.
- Read the entire protocol before beginning. Take note of the required product, notes, recommendations, and stopping points.
- **Recommended library quantification method**: For post-ligation quantification, use Qubit HS assay (Thermo Fisher Scientific, cat. no. Q32854). After library construction and post-capture secondary amplification, use Qubit BR assay (Thermo Fisher Scientific, cat. no. Q32853).
- **Indexing recommendations**: 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. This is facilitated by the integration of index sequences into long-read library amplification primers using different sample indexes for each sample. This is achieved using the QIAseq xHYB long-read PCR-indexing primers. The QIAseq xHYB long-read PCR-indexing primers are described in Appendix D: Unique Dual-Index Primers for PCR-Based Indexing.

Protocol: Long-Read Enzymatic Fragmentation and Indexed Human DNA Library Construction

Important points before starting

- Use high quality and intact DNA samples.
- If DNA samples contain TE buffer or EDTA in general, follow the protocol in Appendix A: Cleanup of DNA Samples Containing EDTA/EGTA.
- A total of 200 ng DNA is recommended as input into the enzymatic fragmentation reaction.
- Ensure reactions are thoroughly mixed, as well as prepared and incubated at the recommended temperatures.
- Before cleanup, ensure that the QIAseq Beads have been equilibrated to room temperature for 30 minutes.
- Thaw 5x concentration size-selection buffer and HMW wash buffer to room temperature, vortex thoroughly, and spin down before diluting 1:5 for required size-selection volumes.

  Note: The QIAseq Beads need to be homogenous. Thoroughly resuspend the beads immediately before use and process the beads quickly. If delay in the protocol occurs, simply vortex the beads again.
- Size-selection and HMW wash buffers must be diluted 1:5 before use. Dilute only the volume that is needed (+10%). One library construction requires 660 µL size-selection buffer and 440 µL HMW wash buffer; 24 library constructions would require 15.9 mL size-selection buffer and 10.6 mL of HMW wash buffer.
- After dilution to 1x, size-selection and HMW wash buffers are stable at 4°C for 2 weeks.

- Pre-program the thermal cyclers. For increased speed and convenience, all incubation steps of the protocol can be pre-programmed and saved in advance. Additionally, the program can be started and paused while setting up the reaction.

Long-read fragmentation, end polishing, and A-addition

Procedure

1. Thaw reagents and samples on ice. Once reagents are thawed, mix buffers thoroughly by inverting tubes several times to avoid any localized concentrations. Mix the enzyme mix by inverting the tube several times. Briefly spin down vortexed reagents.
2. Prepare the long-fragmentation reaction following Table 2 on ice. Mix well by pipetting up and down 12 times, then spin down.

Table 2. Long-fragmentation setup

Component	Per sample
Fragmentation Buffer, 10x	1 µL
FA buffer*	2.5 µL
Purified DNA	x µL
Fragmentation enzyme mix	1 µL
Nuclease-free water	5.5–x µL
Total volume	**10 µL**

* Follow the important points regarding FA buffer. Dilution is made with water, that is, for 2x dilution use 2 µL of FA buffer and 2 µL of nuclease-free water. Both 1x and 2x dilution should be tested.

3. Incubate the reactions in a thermal cycler by following Table 3.

   Note: Transfer directly to the thermal cycler from ice.

Table 3. Fragmentation reaction

Temperature (°C)	Time
4	1 min
32	10 min
4	Hold

4. Immediately place reaction on ice.
5. Prepare the following FX reaction from Table 4 on ice. Mix well by pipetting up and down 12 times, then spin down.

Table 4. FX reaction setup

Component	Per sample
Fragmentation Buffer, 10x	3.5 µL
Fera solution	1.35 µL
Previous product	10 µL
FX enhancer	2.25 µL
FG	2.25 µL
Fragmentation enzyme mix	7 µL
Nuclease-free water	18.65 µL
Total volume	**45 µL**

6. Incubate the reactions in a thermal cycler following Table 5.

Table 5. FX reaction

Temperature (°C)	Time
4	1 min
70	60 min
4	Hold

Adapter ligation

Important points before starting

- Before cleanup, thaw size-selection buffer and HMW wash buffer. Ensure that the QIAseq Beads, size-selection buffer, and HMW wash buffer have been equilibrated to room temperature for 30 minutes.

  Note: The QIAseq Beads need to be homogenous. Thoroughly resuspend the beads immediately before use and process the beads quickly. If delay in the protocol occurs, simply vortex the beads again.

- Pre-program the thermal cyclers. For increased speed and convenience, all incubation steps of the protocol can be pre-programmed and saved in advance. Additionally, the program can be started and paused while setting-up the reaction.

Procedure

1. Prepare the following ligation reaction from Table 6 on ice. Mix well by pipetting up and down 12 times, then spin down. Add 55 µL of ligation master mix to the reaction from Table 4.

Table 6. Ligation setup

Component	Per sample (µL)
Fragmentation reaction	45
DNA adapter	5
UPH Ligation Buffer	40
DNA ligase	10
Total volume	**100**

2. Incubate the reactions in a thermal cycler following Table 7 with no heat on the thermal cycler lid.

Table 7. Ligation reaction

Temperature (°C)	Time
4	1 min
20	15 min
4	Hold

3. Perform the following size selection with QIAseq Beads, size-selection buffer, and HMW wash equilibrated to room temperature for 30 min.

Size-selection after ligation

Procedure

1. Add 90 µL (0.9x) QIAseq Beads to ligation reaction.
2. Mix by gently pipetting up and down. Spin down. Incubate at room temperature for 5 min.
3. Place on the magnetic rack/plate until clear. Discard the supernatant.
4. Add 200 µL size-selection buffer and mix by pipetting. Spin down.
5. Incubate at room temperature for 5 min.
6. Place on the magnetic rack/plate until clear. Discard the supernatant.
7. Add 200 µL size-selection buffer and mix by pipetting. Spin down.
8. Incubate at room temperature for 5 min.
9. Place on the magnetic rack/plate until clear. Discard the supernatant.
10. Add 200 µL HMW bead wash. Do not disturb beads. Remove the wash buffer.
11. Briefly spin down, place on the magnetic rack/plate, and remove the remaining wash buffer with 10 µL pipettor.
12. Add 20 µL buffer EB and pipet into mix. Incubate at 37°C for 10 min.
13. Put on the magnetic rack/plate until clear. Leave the tube on the magnetic rack/plate.
14. Transfer 17 µL of purified DNA sample to PCR tube. Save the remainder for Qubit HS assay (1 µL).
15. Determine the yield after ligation with Qubit High-sensitivity assay (HS).

16. Based on the Qubit HS DNA yield from the ligation, follow the library construction cycle numbers in Table 8.

Table 8. Cycle numbers based on yield from ligation

ng/µL	Cycle number
0.2–1	14
<1–4.5	12
<4.5–8.5	11
<8.5–12	10

17. Prepare the library amplification reaction from Table 9 on ice.

Table 9. Library amplification setup

Component	Per sample (µL)
2X PCR master mix	50
LR amp buffer XL	7.5
Long read primers	18
Indexing primers	3
Nuclease-free water	4.5
Fragmented DNA	17
Total volume	**100**

18. Mix well by pipetting up and down 12 times, then spin down and follow the cycling protocol in Table 10, using the cycle number indicated for ligation yields from Table 8 above.

Table 10. Library amplification cycling conditions

Step	Time	Temperature (°C)
1 cycle	30 s	98
X cycles	10 s	98
	3 min	68
Hold	∞	4

19. Perform the following purification with the QIAseq Beads, size-selection buffer, and HMW wash equilibrated to room temperature for 30 min. Ensure that the 5x concentrated size-selection buffer and HMW wash buffer have been diluted 1:5 with NFH20 with the appropriate volumes for the number of reactions.

Size-selection after library amplification

Procedure

1. Add 1 µL of bead booster, mix and incubate 1 min at room temperature.
2. Add 90 µL (0.9x) QIAseq Beads to each sample.
3. Mix by gently pipetting up and down. Spin down. Incubate at room temperature for 5 min.
4. Place on the magnetic rack/plate until clear. Discard the supernatant.
5. Add 200 µL size-selection buffer and mix by pipetting and spin down.
6. Incubate at room temperature for 5 min.

7. Place on the magnetic rack/plate until clear. Discard supernatant.
8. Add 200 µL HMW bead wash. Do not disturb beads. Remove wash buffer.
9. Briefly spin down, place on the magnetic rack/plate and remove remaining wash buffer with 10 µL pipettor.
10. Elute with 20 µL Buffer EB, pipet into mix. Incubate at 37°C for 10 min.
11. Place on the magnetic rack/plate for 1 min or until clear. Leave the tube on the magnetic rack/plate, and transfer 17.5 µL to a new tube.
12. QC library construction amplification with Qubit BR assay and 0.7% agarose gel, QIAxcel, or Genomic DNA TapeStation.

Stopping point. Samples can be stored at –20°C.

Proceed to Protocol: Hybrid Capture and Wash.

Protocol: Long-Read Mechanical Fragmentation and Indexed Human DNA Library Construction

Important points before starting

- Use high quality and intact DNA samples.
- A total of 200 ng of DNA is recommended as input into the mechanical fragmentation reaction.
- Ensure reactions are thoroughly mixed, as well as prepared and incubated at the recommended temperatures.
- Before clean up, ensure that the QIAseq Beads have been equilibrated to room temperature for 30 minutes.
- Thaw 5x concentration size-selection buffer and HMW wash buffer to room temperature, vortex thoroughly, and spin down before diluting 1:5 for required size-selection volumes.

  Note: QIAseq Beads need to be homogenous. Thoroughly resuspend the beads immediately before use and process the beads quickly. If a delay in the protocol occurs, simply vortex the beads again.
- Size-selection and HMW wash buffers must be diluted 1:5 before use. Dilute only the volume that is needed (+10%). One library construction, including size selection after g-TUBE fragmentation requires 880 µL size-selection buffer and 660 µL HMW wash buffer: 24 library constructions would require 21.1 mL size-selection reagent and 15.85 mL of HMW wash buffer.
- After dilution to 1x, size-selection and HMW wash buffers are stable at 4°C for 2 weeks.
- Pre-program the thermal cyclers. For increased speed and convenience, all incubation steps of the protocol can be pre-programmed and saved in advance. Additionally, the

program can be started and paused while setting up the reaction.

- Eppendorf 5424 and 5415 R centrifuge models are suggested for mechanical fragmentation.
- Use RPM values; do not use RCF.
- Producing DNA fragments larger than 8 kb may reduce yield and compromise post-capture amplification.
- Centrifugation speed for 6–8 kb fragmentation may need to be empirically determined depending on centrifuge age, calibration status, or centrifuge model.

Long-read mechanical fragmentation, end-polishing, and A-addition

Procedure

1. Mix 200 ng of DNA with lab-grade water to make 50 µL total.
2. Pipet diluted DNA into Covaris g-TUBE and centrifuge sample 1 min at 7200 RPM.
3. Remove g-TUBEs, and invert and place inverted tubes back in centrifuge for 1 min at 7200 RPM.
4. Recover 50 µL from inverted g-TUBE cap and mix with 45 µL room temperature QIAseq Beads.
5. Incubate 5 min.
6. Capture beads on the magnetic rack/plate, add 200 µL of size-selection buffer, and mix by pipetting. Spin down and incubate at room temperature for 5 min.
7. Remove size-selection buffer, and apply 200 µL HMW wash without disturbing the beads.
8. Remove HMW wash, pulse-spin tubes, capture beads on the magnetic rack/plate, and remove any residual wash buffer with a 10 µL pipette.
9. Add 20 µL 10mM Tris-Cl pH 8.0, and incubate for 10 min at 37°C.

10. Perform Qubit HS DNA quantitation.
    - A total of 200 ng input is optimal for library construction; however, 10–1000 ng may be used with modifications to library construction cycling.
11. Prepare the end-repair reaction outlined in Table 11, following the thermal cycler program in Table 12. Mix reactions by pipetting up and down 12 times.

Table 11. ERA end-polishing setup

Component	Per sample
ERA buffer, 10x	2.25 µL
Nuclease-free water	17.75–x µL
DNA input	x µL
ERA enzyme	2.5 µL
Total volume	**22.5 µL**

Table 12. ERA end-polishing reaction

Temperature (°C)	Time
4	1 min
20	30 min
65	30 min
4	Hold

Adapter ligation

Important points before starting

- Before cleanup, thaw size-selection buffer and HMW wash buffer. Ensure that the QIAseq Beads, size-selection buffer, and HMW wash buffer have been equilibrated to room temperature for 30 minutes.

  Note: QIAseq Beads need to be homogenous. Thoroughly resuspend the beads immediately before use and process the beads quickly. If a delay in the protocol occurs, simply vortex the beads again.

- Pre-program the thermal cyclers. For increased speed and convenience, all incubation steps of the protocol can be pre-programmed and saved in advance. Additionally, the program can be started and paused while setting up the reaction.

Procedure

12. Add the ligation master mix in Table 13 to the end-repair mix. Follow the cycling protocol in Table 14. Mix ligation by gentle pipetting up and down 12 times.

Table 13. Adapter ligation setup

Component	Per sample (µL)
Product from ERA reaction	22.5
DNA adapter	2.5
UPH Ligation Buffer	20
DNA ligase	5
Total volume	**50**

Table 14. Adapter ligation reaction

Temperature (°C)	Time
4	1 min
20	15 min
4	Hold

Size-selection after ligation

Procedure

Perform the following purification with the QIAseq Beads, size-selection buffer, and HMW wash equilibrated to room temperature for 30 minutes.

13. Add 45 µL (0.9x) QIAseq Beads to ligation reaction.
14. Mix by gently pipetting up and down. Spin down. Incubate at room temperature for 5 min.
15. Place on the magnetic rack/plate until clear. Discard the supernatant.
16. Add 200 µL of size-selection buffer and mix by pipetting. Spin down.
17. Incubate at room temperature for 5 min.
18. Place on the magnetic rack/plate until clear. Discard the supernatant.
19. Add 200 µL of size-selection buffer and mix by pipetting. Spin down.
20. Incubate at room temperature for 5 min.
21. Place on the magnetic rack/plate until clear. Discard the supernatant.
22. Add 200 µL HMW bead wash. Do not disturb beads. Remove the wash buffer.

23. Briefly spin down, place on the magnetic rack/plate, and remove the remaining wash buffer with 10 µL pipettor.
24. Add 19.5 µL buffer EB and pipet into mix. Incubate at 37°C for 10 min.
25. Place on the magnetic rack/plate for 1 min or until clear. Leave the tube on the magnetic rack/plate.
26. Transfer 17 µL of purified DNA sample to PCR tube. Save the remainder for Qubit HS assay (1 µL).
27. Determine the yield after ligation with Qubit High-sensitivity assay (HS).
28. Based on the Qubit HS DNA yield from the ligation, follow the library construction cycle numbers in Table 15.

Table 15. Cycle number based on Qubit HS yield after ligation size selection

ng/µL	Cycle number
0.2–1	14
<1–4.5	13
<4.5–8.5	12

29. Prepare the library amplification reaction from Table 16 on ice.

Table 16. Library amplification setup

Component	Per sample (µL)
2x PCR master mix	50
LR amp buffer XL	7.5
Long read primers	18
Indexing primers	3
Nuclease-free water	4.5
Fragmented DNA	17
Total volume	**100**

30. Mix well by pipetting up and down 12 times, then spin down and follow the cycling protocol in Table 17 using the cycle number indicated for ligation yields from Table 15 above.

Table 17. Library amplification cycling conditions

Step	Time	Temperature
1 cycle	30 s	98°C
x cycles	10 s	98°C
	3 min	68°C
Hold	∞	4°C

Size-selection after library amplification

Procedure

Perform the following purification with QIAseq Beads, size-selection buffer, and HMW wash equilibrated to room temperature for 30 minutes.

31. Add 1 µL of bead booster, mix, and incubate 1 min at room temperature.
32. Add 90 µL (0.9x) QIAseq Beads to each sample.
33. Mix by gently pipetting up and down. Spin down. Incubate at room temperature for 5 min.
34. Place on the magnetic rack/plate until clear. Discard the supernatant.
35. Add 200 µL of size-selection buffer and mix by pipetting and spinning down.
36. Incubate at room temperature for 5 min.
37. Place on the magnetic rack/plate until clear. Discard the supernatant.
38. Add 200 µL HMW bead wash. Do not disturb beads. Remove the wash buffer.
39. Briefly spin down, place on the magnetic rack/plate, and remove remaining wash buffer with 10 µL pipettor.
40. Elute with 20 µL Buffer EB, and pipet into mix. Incubate at 37°C for 10 min. Spin down.
41. Place on the magnetic rack/plate for 1 min or until clear. Leave the tube on the magnetic rack/plate, and transfer 17.5 µL to a new tube.
42. QC library construction amplification with Qubit BR assay and 0.7% agarose gel, Genomic DNA TapeStation or QIAxcel.

Stopping point. Samples can be stored at –20°C.

Proceed to Protocol: Hybrid Capture and Wash.

Protocol: Hybrid Capture and Wash

Pool libraries and overnight HYB capture reaction

Important points before starting

- Ensure reactions are thoroughly mixed, as well as prepared and incubated at the recommended temperatures.
- Pre-program thermal cyclers. For increased speed and convenience, all incubation steps of the protocol can be pre-programmed and saved in advance. Additionally, the program can be started and paused while setting up the reaction.
- Thaw the QIAseq xHYB Probe Set, Enhanced Blocking Buffer, One-4-All Blocking Oligos, and One-4-All Blocking Solution on ice, then pulse vortex and pulse-spin.
- To immediately proceed to hybridization after library pool dry-down, equilibrate the Vapor-Lock reagent to room temperature, and heat the Hybridization Solution to 65°C for 10 minutes or until all precipitate is dissolved before pool evaporation is complete. Alternatively, the dried library pool can be stored at –30°C to –15°C for up to 3 days.
- For each xHYB capture pool, a QIAseq probe panel/hybridization mix are prepared in a 1.5 mL LoBind tube. For each HYB capture pool, the dried-down libraries/One-4-All Blocking Solution/One-4-All Blocking Oligos are prepared in a separate tube. After both tubes are finished incubating at room temperature or 23°C, they are combined.

Procedure

Table 18. Capture library pooling guidelines

Panel	Panel size targeted (bp)	µg input	ng/library (8 libraries)
HLA or custom	<200,000	5	625
Hereditary cancer or custom	>200,000	1.5	188

1. Pool the libraries according to Table 18.

   Note: Smaller capture pools like the HLA panel require 5 µg pooled human DNA library material, whereas larger panels like the hereditary cancer panel require 1.5 µg of pooled human DNA library material.

2. Add 3.5 µL enhanced blocking buffer to pooled libraries and dry-down completely with a SpeedVac set at 45°C.

3. Heat the hybridization mix at 65°C for 5 min or until the solution is clear and there is no precipitate.

4. Prepare the probes as in Table 19 and heat in a thermal cycler as outlined in Table 20.

Table 19. QIAseq probe prep

Component	Per sample (µL)
Hybridization mix	20
Nuclease-free water	4
Probe	4
Total volume	**28**

Table 20. QIAseq probe prep reaction

Temperature	Time (min)
95°C	2
On ice	5
RT (23°C)	5

Important: Do not use PCR plates for the hybridization preparation step as excessive evaporation may occur during overnight incubation.

5. Resuspend the dried library as in Table 21, following the thermal cycler guide in Table 22.

Table 21. Resuspension of dried-down library

Component	Per sample
Dried library	–
Blocker solution	5 µL
Blocking oligos	8 µL
Total volume	**13 µL**

Table 22. Blocking of dried-down library reaction

Temperature (°C)	Time (min)
95	5
23 (room temperature)	<5

6. Carefully mix probe solution by pipetting then add 28 µL (all) to resuspended library.
7. Add 30 µL Vapor Lock.

8. Carefully mix capture reaction thoroughly by gentle pipetting, making sure not to generate bubbles. Spin down.
9. Incubate Hybridization reaction at 70°C for 16 hours with heated lid at 85°C.

Preparing streptavidin beads

Important points before starting

- Inspect Binding Buffer, Wash Buffer 1, and Wash Buffer 2 for any precipitate.
- If precipitate is observed, heat buffer at 48°C until all precipitate is dissolved into solution.
- Prepare 450 µL of Wash Buffer 1 for each Hybridization Reaction, and preheat to 60°C.
- Prepare 650 µL of Wash Buffer 2 for each Hybridization Reaction, and preheat to 48°C.
- Equilibrate Streptavidin Binding Beads and DNA Purification Beads to room temperature for at least 30 minutes.
- Thaw the HYB Elute Buffer on ice. Once buffer is thawed, mix by pulse-vortexing to avoid any localized concentrations. Briefly spin down vortexed reagents before use.

Procedure

1. For each hybridization reaction, add 100 µL of streptavidin beads to a clean 1.5 mL LoBind tube.
2. Add 200 µL of binding buffer to the tube and mix by pipetting.
3. Place tube on magnetic rack/plate for 1 min. Discard the supernatant.
4. Repeat wash (steps 1 to 2) two additional times.
5. Add 200 µL of Binding Buffer to the tube and resuspend by vortexing.
6. Warm the streptavidin beads 5 min at 60°C.

Note: Ensure accurate heating block temperature with thermometer. Perform post-capture washes in heat block in temperature-controlled room above 21.1°C. It is important to minimize temperature fluctuations during the post-capture wash procedure.

Binding and washing of hybridized targets to streptavidin beads

Procedure

1. After hybridization and while still on thermal cycler at 70°C, transfer the full volume of hybridization reaction (71 µL) the to corresponding tube of streptavidin beads.
2. Mix tube of hybridization buffer with streptavidin beads and transfer to 60°C heat block for 5 min. After 5 min, pulse spin down the tubes.
3. Place on the magnetic rack/plate 1 min. Discard supernatant. Remove tube from magnetic rack/plate.
4. Add 200 µL of wash buffer 1. Mix by pipetting. Pulse spin down.
5. Transfer to a new 1.5 mL LoBind tube.
6. Incubate at 60°C for 5 min.
7. Place on the magnetic rack/plate for 1 min. Discard the supernatant.
8. Repeat steps 4–7 one time.
9. Remove tube from magnetic rack/plate and add 200 µL of wash buffer 2 (48°C). Mix by pipetting. Pulse spin down.
10. Incubate at 48°C for 5 min.
11. Place on the magnetic rack/plate for 1 min. Discard the supernatant.
12. Repeat wash (steps 9–11) two additional times for total of three washes.
13. After removing the last wash, pulse spin down and remove any residual wash buffer.

14. Pellet the beads to the bottom of the tube to improve bead resuspension/elution.
15. Add 7 µL LR amp enhancer, flick the tube several times, and pulse spin down.
16. Add 5 µL of prepared 0.2N NaOH (reagents supplied by user), and flick tube to mix. Incubate at room temperature (24°C) for 5 min.
17. Add 2 µL of HN buffer, flick tube to mix then pulse spin down.
18. Transfer the tubes to the magnetic rack/plate.
19. Proceed directly to "Post-capture library amplification".

Post-capture library amplification

Important points before starting

- The post-capture amplification must be set up on the same day as streptavidin library capture and washing. The un-amplified, captured DNA is not stable for storage.
- Prepare one pre-amplification per capture pool. A total of 20 µL of pre-amplification is used in one secondary amplification; the remainder of pre-amplification is stored at –20°C.
- Before cleanup, ensure that the QIAseq Beads, size-selection buffer, and HMW wash buffer have been equilibrated to room temperature for 30 minutes.

  Note: The QIAseq Beads need to be homogenous. Thoroughly resuspend the beads immediately before use and process the beads quickly. If a delay in the protocol occurs, simply vortex the beads again.
- Size-selection and HMW wash buffers must be diluted 1:5 before use. Dilute only the volume that is needed (+10%). Two post-capture amplifications would require 440 µL of size-selection buffer and 220 µL HMW wash buffer.

- Pre-program the thermal cyclers. For increased speed and convenience, all incubation steps of the protocol can be pre-programmed and saved in advance. Additionally, the program can be started and paused while setting up the reaction.
- Thaw 2x HiFi LR Master Mix, LR Primer Mix, LR buffer XL, and LR-amp SB on ice. Once reagents are thawed, mix by pulse-vortexing to avoid any localized concentrations. Briefly spin down vortexed reagents before use.

Procedure

1. For each capture, use 11.75 µL of the capture eluate in the pre-amplification reaction in Table 23. Mix amplification reaction by pipetting up and down 12 times.

Table 23. Pre-amplification reaction setup

Component	Per HYB capture reaction (µL)
2X PCR master mix	25
LR amp buffer XL	3.75
Long read primers	4.5
LR amp buffer SB	5
Captured DNA	11.75
Total volume	**50**

Use the PCR conditions from Table 24, and the indicated pre-amplification cycle number for individual probe pool capture from Table 25 below.

Table 24. Pre-amplification cycling conditions

Step	Time	Temperature (°C)
1 cycle	30 s	98
x cycles	10 s	98
	3 min	68
Hold	∞	4

Table 25. Pre-amplification cycle number for individual panels based on size of capture region

Panel	Panel size targeted	Pre-amplification cycle number
HLA	170 kb	20 cycles
Hereditary cancer	13 MB	19 cycles

2. Set up a secondary amplification using the following master mix in Table 26. Mix by pipetting up and down 12 times.

Table 26. Secondary amplification setup

Component	Per pre-amplification reaction (µL)
2X PCR master mix	100
LR amp buffer XL	15
Long read primers	18
LR amp buffer SB	20
Nuclease-free water	27
Pre-amp DNA*	20
Total volume	**200**

* Store remaining pre-amplification reaction at –20°C for a backup.

3. Split the 200 µL master mix from above into two PCR strip tubes for each secondary amplification reaction and follow the cycling protocol in Table 27 below.

Table 27. Secondary amplification cycling conditions

Step	Time	Temperature (°C)
1 cycle	30 s	98
2 cycles	10 s	98
	3 min	68
Hold	∞	4

Size-selection of secondary amplification

Procedure

1. Add 1 µL bead booster mix to each 100 µL secondary amplification reaction and incubate for 1 min at room temperature.
2. Pool both 100 µL reactions for each sample pool.
3. Add 180 µL (0.9x) QIAseq Beads to each sample.
4. Mix by gently pipetting up and down. Spin down. Incubate at room temperature for 5 min.
5. Place on the magnetic rack/plate until clear. Discard the supernatant.
6. Add 200 µL of size-selection buffer and mix by pipetting and spinning down. Incubate at room temperature for 5 min.
7. Place on the magnetic rack/plate until clear. Discard the supernatant.
8. Add 200 µL HMW wash. Do not disturb beads. Remove the wash buffer.

9. Briefly spin down, place on the magnetic rack/plate, and remove the remaining buffer with 10 µL pipettor.
10. Elute with 20 µL Buffer EB, pipet into the mix. Incubate at 37°C for 10 min.
11. Place on the magnetic rack/plate for 1 min or until clear. Leave the tube on the magnetic rack/plate, and transfer 17.5 µL to a new tube.
12. Quantify the post-capture amplification products with Qubit BR.
13. Proceed to Protocol: Library QC and Quantification.

Protocol: Library QC and Quantification

This protocol determines the quality and quantity of each xHYB long-read library.

Important points before starting

- A portion of the xHYB long-read post-capture library is the starting material for the library QC and quantification. When not in use, the xHYB post-capture library should be stored on ice.
- Library QC involves use of 0.7% agarose gel electrophoresis, QIAxcel, or TapeStation Genomic DNA assay.
- Library quantification involves use of Qubit BR dsDNA assay (cat. no. Q32853).

Library QC (Qubit BR and TapeStation or agarose gel)

1. Analyze 1 µL of the xHYB post-capture library Qubit dsDNA BR assay.
2. Analyze 1 µL of the xHYB post-capture library on TapeStation Genomic DNA assay; alternatively, assess the size distribution of the post-capture libraries on a QIAxcel instrument, or with 0.7% agarose gel electrophoresis, using 40 ng of material for visualization.

Protocol: Data Analysis

Important points before starting

- Register and sign-in to GeneGlobe data analysis center: **www.geneglobe.qiagen.com/us/analyze**
- GeneGlobe data analysis provides library demultiplexing and HLA haplotyping.
- CLC workbench provides large structural variant detection for the hereditary cancer panel.
- For PacBio reads, HiFi .BAM files must be converted to fastq format for HLA haplotyping on GeneGlobe. BAM HiFi reads can be imported to CLC workbench through import PacBio function.
- Oxford Nanopore reads must be basecalled before uploading to GeneGlobe or CLC workbench. For base calling, perform base calling after the run has completed using the super-accuracy base calling method: **Data analysis | Oxford Nanopore Technologies**

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 in 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 or protocols in this handbook (for contact information, visit **support.qiagen.com**).

	Comments and suggestions
Low library yield	
Library fragmented to larger than recommended median size	Ensure accurate pipetting for small volumes in the enzymatic fragmentation reaction. For g-TUBE fragmented libraries, fragmentation to larger than 8 kb median size will result in inefficient amplification. It may be necessary to empirically determine centrifuge speed that produces 6–10 kb fragmentation pattern, with 8 kb median. Titration of FA buffer in the enzymatic fragmentation reaction can be performed to decrease the fragment size and improve yield with enzymatic fragmentation. If yields from enzymatic fragmentation are low, it is recommended to test 2x and 4x dilutions of FA buffer to improve library yield.
Library amplification	
Presence of smeared library smaller than 3 kb and larger than 10 kb	If library amplification smears smaller than 3 kb and larger than 10 kb in size, then this indicates overamplification. Use Qubit HS DNA assay to determine yield after ligation. Use the correct library amplification cycle number based on ligation yield (Table 7 and Table 15). Overamplification during library construction may lead to decreased median length for post-capture libraries and may result in unbalanced pooling. Refer to Appendix C: Overamplification of Libraries.
Post-capture amplification	
Presence of smeared library smaller than 3 kb and larger than 10 kb	If the post-capture library smears smaller than 3 kb and larger than 10 kb in size, then this indicates over-amplification of the library. These libraries will need to be reconditioned using Appendix C: Overamplification of Libraries, for efficient PacBio and Oxford Nanopore adapter ligation.

Appendix A: Cleanup of DNA Samples Containing EDTA/EGTA

Important points before starting

- Before cleanup, ensure that the QIAseq Beads have been equilibrated to room temperature for 30 minutes.
- **Note**: The QIAseq Beads need to be homogenous. Thoroughly resuspend the beads immediately before use and process the beads quickly. If a delay in the protocol occurs, simply vortex the beads again.

Procedure

1. Add nuclease-free water to DNA sample so that final volume equals 50 µL.
2. Add 90 µL (1.8x) QIAseq Beads to each sample. Mix by pipetting up and down 10 times and then briefly centrifuge.
3. Incubate for 5 min at room temperature.
4. Place the tubes/plate on a magnetic rack/plate for 5 min. After the solution has cleared, carefully remove and discard the supernatant without disturbing the beads.

   Note: Keep the beads on the magnetic rack/plate while handling the supernatant and during the washing steps.

   Important: Do not discard the beads as they contain the DNA of interest.
5. Add 200 µL 80% ethanol while it is on the magnetic rack/plate. Let sit for 1 min. Carefully remove and discard the wash.

6. Repeat the ethanol wash in step 5.

   Important: Completely remove all traces of ethanol after the second wash. To do this, briefly centrifuge (1 s) and return the tubes or plate to the magnetic rack/plate. Remove the ethanol first with a 200 µL pipette, and then use a 10 µL pipette to remove any residual ethanol.

7. With the tubes/plate are still on the magnetic rack/plate, air-dry at room temperature for 2–5 min (up to 15 min when using a plate).

   Note: Visually inspect that the pellet is dry. When dry, the beads should lose its shiny appearance.

8. While still on the magnetic rack/plate, elute the DNA from the beads by adding 18.25 µL nuclease-free water. Remove tubes from the magnetic rack/plate and mix well by pipetting. Incubate for 2–5 min at room temperature.

9. Transfer 15.75 µL of the supernatant to a new PCR tube or plate.

Appendix B: Preparing Long-Read Libraries for Sequencing on Oxford Nanopore Platform

Reagents supplied by user

- NEBNext® Ultra™ II End Repair/dA-Tailing Module (NEB, E7546S)
- Salt-T4® DNA Ligase (NEB, M0467S)
- Ligation Sequencing Kit V14 (Oxford Nanopore, SQK-LSK114)
- Flongle Flow Cell (R10.4.1) (Oxford Nanopore, FLO-FLG114)
- Minion Flow cell (R10.4.1) (Oxford Nanopore, FLO-MIN114)

Important points before starting

- The optimal input for Oxford Nanopore library processing is 400–600 ng of captured, amplified 3–10 kb libraries.
- If available library is less than 400 ng of captured DNA, samples can be processed using the included non-optimal protocol that does not utilize size-selection. This will result in increased Nanopore adapter concatemer formation that will consume sequencing reads and result in a spike of reads below 1 kb.
- Before cleanup, ensure that the QIAseq Beads, size-selection buffer, and HMW wash buffer have been equilibrated to room temperature for 30 minutes.

  Note: The QIAseq Beads need to be homogenous. Thoroughly resuspend the beads immediately before use and process the beads quickly. If a delay in the protocol occurs, simply vortex the beads again.
- Size-selection and HMW wash buffers must be diluted 1:5 before use. Prepare the 1:5 dilutions in the final 1x volume sufficient for the number of post-capture amplifications with

additional 10% (1 library for Oxford Nanopore sequencing adapter processing requires 440 µL of 1x size-selection reagent and 220 µL of 1x HMW wash buffer).

- Allow Flongle or Minion flow cell to equilibrate to room temperature for at least 30 minutes.
- Thaw Oxford Nanopore loading reagents on ice, vortex thoroughly, and spin down.

Optimal protocol (sample input greater than 400 ng captured DNA)

Procedure

1. Prepare the following end-polishing reaction of captured DNA.

Table 28. End-polishing setup

Component	Per sample
Ultra II end-prep buffer	3.5 µL
Ultra II enzyme mix	1.5 µL
NFH20	x µL
Captured library (>400 ng)	x µL
Total volume	**30 µL**

Table 29. End-polishing reaction

Temperature (°C)	Time
4	1 min
20	5 min
65	5 min
4	Hold

2. Add 30 µL (1x) QIAseq Beads.

3. Mix by pipetting up and down 10 times, then vortex for 10 s. Spin down. Incubate at room temperature for 5 min.
4. Place on the magnetic rack/plate until solution is clear. Discard the supernatant.
5. Add 200 µL HMW wash buffer. Do not disturb beads. Remove the wash.
6. Spin down, and remove the rest of PEG.
7. Elute with 32 µL 10 mM Tris-Cl pH8.0. Incubate at 37°C for 10 min.
8. Transfer 30 µL of eluate to a new PCR strip tube for ligation setup.

Table 30. Oxford Nanopore adapter ligation setup

Component	Per sample (µL)
ONT LA adapter	2.5
ONT LNB ligation buffer	12.5
SALT-T4 DNA ligase	5
Product from end-polishing reaction	30
Total volume	**50**

Table 31. Oxford Nanopore adapter ligation reaction

Temperature (°C)	Time
4	1 min
20	10 min
4	Hold

9. Add 45 µL (0.9x) QIAseq Beads.
10. Mix by pipetting up and down 10 times. Spin down. Incubate at room temperature for 5 min.

11. Place on the -magnetic rack/plate until solution is clear. Discard the supernatant.
12. Add 200 µL size-selection buffer, pipet into the mix, then incubate at room temperature for 5 min.
13. Spin down, then place on the magnetic rack/plate until solution is clear. Discard the supernatant.
14. Add 200 µL size-selection buffer, pipet into mix, then incubate at room temperature for 5 min.
15. Spin down, then place on the magnetic rack/plate until the solution is clear. Discard the supernatant.
16. Add 250 µL ONT LFW buffer without disturbing the beads, and remove the LFW wash.
17. Spin down, and remove the residual wash.
18. Air-dry for 30 s.
19. Follow the Oxford Nanopore loading guidelines for Minion flow cells. For flongle flow cells, load 30 fmol of Nanopore-adapted capture libraries.

Non-optimal protocol for end-repair and Nanopore adapter ligation (samples less than 400 ng input)

Procedure

1. Prepare the following end-polishing reaction of captured DNA.

Table 32. End-polishing setup

Component	Per sample
Ultra II end-prep buffer	3.5 µL
Ultra II enzyme mix	1.5 µL
NFH20	x µL
Captured library (400 ng)	x µL
Total volume	**30 µL**

Table 33. End-polishing reaction

Temperature (°C)	Time
4	1 min
20	5 min
65	5 min
4	Hold

2. Add 30 µL (1x) QIAseq Beads.
3. Mix by pipetting up and down 10 times, then vortex for 10 s. Spin down. Incubate at room temperature for 5 min.
4. Place on the magnetic rack/plate until solution is clear. Discard the supernatant.
5. Add 200 µL HMW wash buffer. Do not disturb beads, then remove the wash.

6. Spin down, then remove the rest of PEG.
7. Elute with 32 µL 10 mM Tris-Cl pH8.0. Incubate at 37°C for 10 min.
8. Transfer 30 µL of eluate to new PCR strip tube for ligation setup.

Table 34. Oxford Nanopore adapter ligation setup

Component	Per sample (µL)
ONT LA adapter	2.5
ONT LNB ligation buffer	12.5
SALT-T4 DNA ligase	5
Product from end-polishing reaction	30
Total volume	**50**

Table 35. Oxford Nanopore adapter ligation reaction

Temperature (°C)	Time
4	1 min
20	15 min
4	Hold

9. Add 45 µL (0.9x) QIAseq Beads.
10. Mix by pipetting up and down 10 times. Spin down. Incubate at room temperature for 5 min.
11. Place on the magnetic rack/plate until solution is clear. Discard the supernatant.
12. Add 250 µL ONT LFW buffer without disturbing the beads, and remove the LFW wash.
13. Spin down, then remove the residual wash.
14. Air-dry for 30 s.

15. Elute with 7 µL 10 mM Tris-Cl pH8.0. Incubate at 37°C for 10 min.
16. Follow Oxford Nanopore loading guidelines for Minion flow cells. For flongle flow cells, load 30 fmol of Nanopore-adapted capture libraries.

Appendix C: Overamplification of Libraries

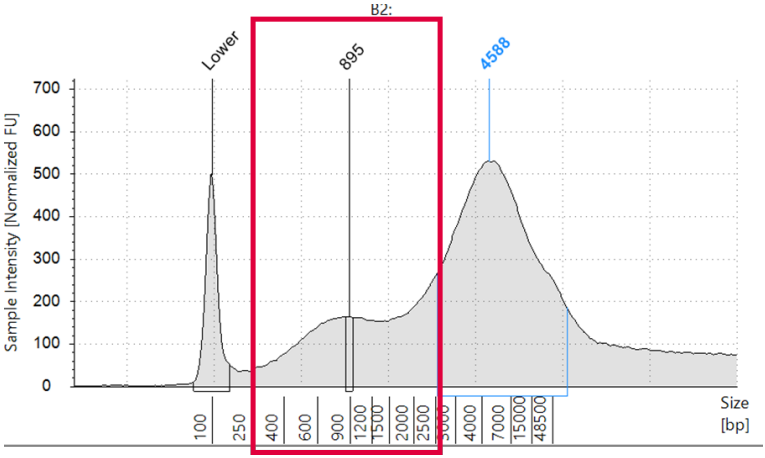

Figure 2. Example of library overamplification.

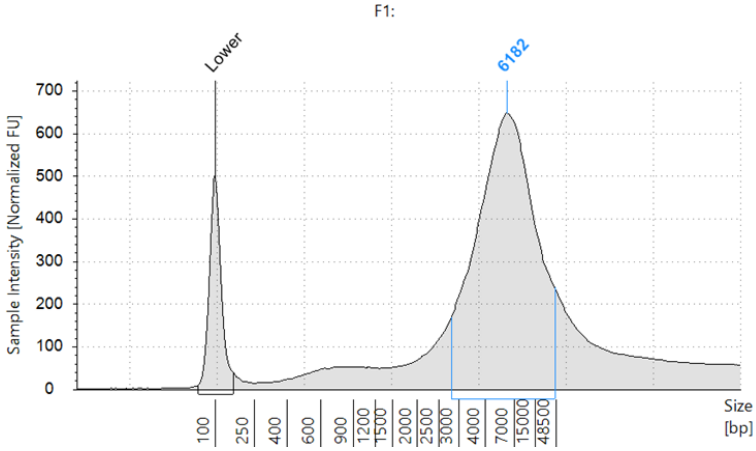

Figure 3. Example of clean library.

Overamplification of library shows a noticeable shoulder around 900 bp, well below the 3–10 kb long-read library size. The increase in the lower shoulder is due to overamplification and presence of single-stranded DNA that affects the migration. Overamplified libraries will not ligate efficiently to Pacific Biosystems or Oxford Nanopore sequencing adapters. Libraries that have been overamplified can be efficiently and quickly "reconditioned" using the following two-cycle long-read recondition protocol.

Long-read reconditioning of overamplified libraries

Table 36. Overamplified library recondition setup

Component	Per sample (µL)
2x PCR master mix	50
LR amp buffer XL	7.5
Long read primers	9
LR amp buffer SB	10
Nuclease-free water	7.5
Overamplified DNA	16
Total volume	**100**

Table 37. Library recondition reaction

Step	Time	Temperature (°C)
1 cycle	30 s	98
2 cycles	10 s	98
	3 min	68
Hold	∞	4

Size-selection after post-capture recondition

Procedure

1. Add 1 µL of bead booster, mix, and incubate for 1 min.
2. Add 90 µL (0.9x) QIAseq Beads to each sample.
3. Mix by gently pipetting up and down. Spin down. Incubate at room temperature for 5 min.
4. Place on the magnetic rack/plate until clear. Discard the supernatant.
5. Add 200 µL of size-selection buffer and mix by pipetting and spin down.
6. Incubate at room temperature for 5 min.
7. Place on the magnetic rack/plate until clear. Discard the supernatant.
8. Add 200 µL HMW bead wash. Do not disturb the beads. Remove the wash buffer.
9. Briefly spin down, place on the magnetic rack/plate, and remove the remaining wash buffer with 10 µL pipettor.
10. Elute with 20 µL Buffer EB, then pipet into mix. Incubate at 37°C for 10 min.
11. QC library construction amplification with Qubit BR assay and 0.7% agarose gel or Genomic DNA TapeStation.

Stopping point. Samples can be stored at –20°C.

Appendix D: Unique Dual-Index Primers for PCR-Based Indexing

The layout of the 24-plex and 96-plex single-use UDI LR primer plate is shown in Table 38 and Table 39. The index sequences used in the QIAseq Unique Dual-Index Kits are listed in Table 40. Index sequences are available for download at: **www.qiagen.com**

Table 38. QIAseq UDI Y-Adapter Plate (24) layout (UDI 1–24)

	1	2	3	4	5	6	7	8	9	10	11	12
A	UDI 001	UDI 009	UDI 017	–	–	–	–	–	–	–	–	–
B	UDI 002	UDI 010	UDI 018	–	–	–	–	–	–	–	–	–
C	UDI 003	UDI 011	UDI 019	–	–	–	–	–	–	–	–	–
D	UDI 004	UDI 012	UDI 020	–	–	–	–	–	–	–	–	–
E	UDI 005	UDI 013	UDI 021	–	–	–	–	–	–	–	–	–
F	UDI 006	UDI 014	UDI 769	–	–	–	–	–	–	–	–	–
G	UDI 007	UDI 015	UDI 023	–	–	–	–	–	–	–	–	–
H	UDI 008	UDI 016	UDI 024	–	–	–	–	–	–	–	–	–

Table 39. QIAseq UDI Y-Adapter Plate A (96) layout (UDI 1–96)

	1	2	3	4	5	6	7	8	9	10	11	12
A	UDI 001	UDI 009	UDI 017	UDI 025	UDI 033	UDI 041	UDI 049	UDI 057	UDI 065	UDI 073	UDI 081	UDI 089
B	UDI 002	UDI 010	UDI 018	UDI 026	UDI 034	UDI 042	UDI 050	UDI 058	UDI 066	UDI 074	UDI 082	UDI 090
C	UDI 003	UDI 011	UDI 019	UDI 027	UDI 035	UDI 043	UDI 051	UDI 059	UDI 067	UDI 075	UDI 083	UDI 091
D	UDI 004	UDI 012	UDI 020	UDI 028	UDI 036	UDI 044	UDI 052	UDI 060	UDI 068	UDI 076	UDI 084	UDI 092
E	UDI 005	UDI 013	UDI 021	UDI 029	UDI 037	UDI 045	UDI 053	UDI 061	UDI 069	UDI 077	UDI 085	UDI 093
F	UDI 006	UDI 014	UDI 769	UDI 030	UDI 038	UDI 046	UDI 054	UDI 062	UDI 070	UDI 078	UDI 086	UDI 094
G	UDI 007	UDI 015	UDI 023	UDI 031	UDI 039	UDI 047	UDI 055	UDI 063	UDI 071	UDI 079	UDI 087	UDI 095
H	UDI 008	UDI 016	UDI 024	UDI 032	UDI 040	UDI 048	UDI 056	UDI 064	UDI 072	UDI 080	UDI 088	UDI 096

Table 40. UDI index sequences used in the QIAseq UDI Y-Adapter Kits (24 and 96 A/B/C/D)

Indices for demultiplexing

Unique dual-index number	i7 bases for entry on sample sheet for demultiplexing	i5 bases for demultiplexing
UDI 001	TGAACGTTGT	ATGGCCGACT
UDI 002	ACCAGACTTG	CGATGAGCAC
UDI 003	ACTGGCGAAC	GATAAGTCGA
UDI 004	GCGTTAGGCA	TCACGCCTTG
UDI 005	TTATCGGCCT	AGGAACACAA
UDI 006	GAGGTATAAG	CTCAGTAGGC

Table 40. UDI index sequences used in the QIAseq UDI Y-Adapter Kits (24 and 96 A/B/C/D) (continued)

Indices for demultiplexing		
Unique dual-index number	**i7 bases for entry on sample sheet for demultiplexing**	**i5 bases for demultiplexing**
UDI 007	TCAAGGATTC	GAAGTGCCTG
UDI 008	CGAACCGAGA	TCTCTCGCCT
UDI 009	GAGCCAAGTT	AGGCACCTTC
UDI 010	AAGGCCGTAG	CTGTTGGTAA
UDI 011	TTAGAGAAGC	GCTGGTACCT
UDI 012	TCTAAGACCA	TAAGGAGCGG
UDI 013	TGTAACCACT	AATCGCTCCA
UDI 014	CCGACACAAG	CTCCTAATTG
UDI 015	CTCTGATGGC	GCCTCATAAT
UDI 016	CGGCCTGTTA	TGTATTGAGC
UDI 017	TGCATAGCTT	AGCCATAACA
UDI 018	AACCTTCTCG	CCACAAGTGG
UDI 019	AAGAGATCAC	GTTATCACAC
UDI 020	GCCTGAAGGA	TACCGTTCTT
UDI 021	ATTGTGCCTT	AGGCGTTAGG
UDI 769	CTCCTTGGCA	TGGTTGGCGA
UDI 023	TACCATGAAC	GTAATAGCCA
UDI 024	CATTGGCAGA	TAGCGCCGAT
UDI 025	CACTGCTATT	CATTCTTGGA
UDI 026	AATGGTAGGT	ATGCAAGGTT

Table 40. UDI index sequences used in the QIAseq UDI Y-Adapter Kits (24 and 96 A/B/C/D) (continued)

Indices for demultiplexing		
Unique dual-index number	**i7 bases for entry on sample sheet for demultiplexing**	**i5 bases for demultiplexing**
UDI 027	GATACCTATG	CGCCAGACAA
UDI 028	CACTAGGTAC	GAAGGTTGGC
UDI 029	AGCTCGTTCA	TCGCATCACG
UDI 030	TGTCAGTCTT	CCGGTCATGA
UDI 031	GATGAACAGT	ATTCACAAGC
UDI 032	ACAATCGGCG	CAACCTGTAA
UDI 033	GATTGAGTTC	GCCAGTCGTT
UDI 034	GTAATGCCAA	TGCCTTGTCG
UDI 035	TCGTTGCGCT	CTATCCGCTG
UDI 036	AGGTGAGTAT	AATGCCGGAA
UDI 037	TCGATAATGG	CGGTTATCCG
UDI 038	GCGTCTCTTC	GCGGAAGAGT
UDI 039	GTCTCCTGCA	TTGGTTAGTC
UDI 040	GAGCTTCATT	TTCAGTGTGA
UDI 041	AGGCCTACAT	AGAATTCTGG
UDI 042	TGTGGAACCG	CATTGACTCT
UDI 043	CGTATTAAGC	GCGGCTTCAA
UDI 044	CCAGTGGTTA	TTATGGTCTC
UDI 045	GCGTTCGAGT	CGTAACCAGG
UDI 046	CCTTCCGGTT	AGCTCAGATA

Table 40. UDI index sequences used in the QIAseq UDI Y-Adapter Kits (24 and 96 A/B/C/D) (continued)

Indices for demultiplexing		
Unique dual-index number	**i7 bases for entry on sample sheet for demultiplexing**	**i5 bases for demultiplexing**
UDI 047	CACAAGACGG	CCGGTGTTAC
UDI 048	GCTTACACAC	GACCTAACCT
UDI 049	AGGATGTCCA	TTGTAGAAGG
UDI 050	CACCTTATGT	CCTAGCACTA
UDI 051	AAGCGGCTGT	ATCGTGTTCT
UDI 052	TTCCTGTGAG	CCAACTTATC
UDI 053	AGTACAGTTC	GAAGCCAAGG
UDI 054	TACAGCCTCA	TGGAGTTCAA
UDI 055	GTTCTATTGG	CTTCAATCCT
UDI 056	ATATACCGGT	ATCTTGCGTG
UDI 057	CCTCGGAATG	CGTCTAAGGT
UDI 058	GTTCTGGAAC	GAGGTGAACA
UDI 059	AGATTCACCA	TCAGAACTAC
UDI 060	TCGGTCAGAT	CGGATATTGA
UDI 061	CACTCTCGCT	AGGAGTAGAT
UDI 062	GTTGGTCCAG	CCGCCGAATA
UDI 063	AGCTCGAAGC	GAGTCTATAC
UDI 064	AGAGGTTCTA	TTATTACCGG
UDI 065	ATGACTCGAA	CGCTCGTTAG
UDI 066	GAACAATCCT	AACAACGCTG

Table 40. UDI index sequences used in the QIAseq UDI Y-Adapter Kits (24 and 96 A/B/C/D) (continued)

Indices for demultiplexing		
Unique dual-index number	**i7 bases for entry on sample sheet for demultiplexing**	**i5 bases for demultiplexing**
UDI 067	TGGCAAGGAG	CGCGGCTATT
UDI 068	GAATATTGGC	GCTCGACACA
UDI 069	CCGGAACCTA	TTCTTCCAAC
UDI 070	ACTTGTTCGG	TTGGCGGTTG
UDI 071	CAAGTCCAAT	AACAGGCAAT
UDI 072	AACCGCAAGG	CAGAATGGCG
UDI 073	ACGTTGACTC	GTTGAGATTC
UDI 074	CCACTTAACA	TGTGTGCGGA
UDI 075	AGCAGTTCCT	GTTCGGCGAA
UDI 076	TCGCCTTCGT	AGCTGTATTG
UDI 077	TAGGACTGCG	CAGCGGATGA
UDI 078	TCCGAGCGAA	GTCCTTGGAT
UDI 079	TTCGGTTGTT	TCTAGATGCT
UDI 080	ACAGGAGGAA	CGAGCCACAT
UDI 081	CCTCCATTAA	ATGGAATGGA
UDI 082	AGTCGCGGTT	CATTCCTCAC
UDI 083	CTCATCCAGG	GCATAGGAAG
UDI 084	TGTGGTTGAA	TGTTCGTGTT
UDI 085	TTATGCGTGG	TAAGACCGTT
UDI 086	GCGAATGTAT	ATGGTACCAG

Table 40. UDI index sequences used in the QIAseq UDI Y-Adapter Kits (24 and 96 A/B/C/D) (continued)

Indices for demultiplexing		
Unique dual-index number	**i7 bases for entry on sample sheet for demultiplexing**	**i5 bases for demultiplexing**
UDI 087	GTCAAGCTCG	CCGACAGCTT
UDI 088	TAGAGTTGGA	GACGATATGA
UDI 089	CTGATGATCT	TTGTACTCCA
UDI 090	ACTAGGTGTT	GTGCACATAA
UDI 091	CTGTTAGCGG	AGGACAAGTA
UDI 092	ATCGCACCAA	CCGATTCGAG
UDI 093	CTTACTTGGT	GTAGGAACTT
UDI 094	CCTTAATGCG	TACACTACGA
UDI 095	TCTCGCCTAG	ATGACCTTGA
UDI 096	TCTTCAGAGA	CTACGTGACG

Unique Dual-Index adapters 1–24 are identical on the adapter plates of the QIAseq UDI Y-Adapter Kit (24) and QIAseq UDI Y-Adapter Kit A (96).

Note: Sequencing on the MiniSeq®, NextSeq®, HiSeqX®, and HiSeq® 3000/4000 systems follows a dual-indexing workflow different from other Illumina® systems, which requires the reverse complement of the i5 index adapter sequence.

Ordering Information

Product	Contents	Cat. no.
QIAseq xHYB Long-Read Panel (24)	xHYB probe panel for target enrichment for long read sequencing, fixed panel for 24 samples	334322
QIAseq xHYB Long-Read Reagent Kit E (24)	Kit containing reagents for library prep with enzymatic fragmentation and target enrichment for long read sequencing, fixed reagents for 24 samples	334332
QIAseq xHYB Long-Read Reagent Kit S (24)	Kit containing reagents for library prep with mechanical shearing and target enrichment for long read sequencing, fixed reagents for 24 samples	334342
QIAseq xHYB Long-Read Panel (96)	xHYB probe panel for target enrichment for long read sequencing, fixed panel for 96 samples	334325
QIAseq xHYB Long-Read Reagent Kit E (96)	Kit containing reagents for library prep with enzymatic fragmentation and target enrichment for long read sequencing, fixed reagents for 96 samples	334335
QIAseq xHYB Long-Read Reagent Kit S (96)	Kit containing reagents for library prep with mechanical shearing and target enrichment for long read sequencing, fixed reagents for 96 samples	334345
QIAseq xHYB Long-Read Custom Panel (96)	Custom xHYB probe panel for target enrichment for long read sequencing, fixed panel for 96 samples	334355
Related products		
MagAttract® HMW Kit	For 48 DNA preps: MagAttract Suspension G, Buffer ATL, Buffer AL, Buffer MB, Buffer MW1, Buffer PE, Proteinase K, RNase A, Buffer AE, Nuclease-Free Water	67563
PAXgene® Blood DNA Kit	The PAXgene Blood DNA system consists of PAXgene Blood DNA Tubes for blood collection and stabilization, and the PAXgene Blood DNA Kit for DNA purification in a single-tube procedure.	761133

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
June 2025	Initial revision

Limited License Agreement for QIAseq xHYB Long Read Panels

Use of this product signifies the agreement of any purchaser or user of the product to the following terms:

1. The product may be used solely in accordance with the protocols provided with the product and this Instructions for Use and for use with components contained in the panel only. QIAGEN grants no license under any of its intellectual property to use or incorporate the enclosed components of this panel with any components not included within this panel except as described in the protocols provided with the product, this Instructions for Use, and additional protocols available at www.qiagen.com. Some of these additional protocols have been provided by QIAGEN users for QIAGEN users. These protocols have not been thoroughly tested or optimized by QIAGEN. QIAGEN neither guarantees them nor warrants that they do not infringe the rights of third-parties.
2. Other than expressly stated licenses, QIAGEN makes no warranty that this panel and/or its use(s) do not infringe the rights of third-parties.
3. This panel and its components are licensed for one-time use and may not be reused, refurbished, or resold.
4. QIAGEN specifically disclaims any other licenses, expressed or implied other than those expressly stated.
5. The purchaser and user of the panel agree not to take or permit anyone else to take any steps that could lead to or facilitate any acts prohibited above. QIAGEN may enforce the prohibitions of this Limited License Agreement in any Court, and shall recover all its investigative and Court costs, including attorney fees, in any action to enforce this Limited License Agreement or any of its intellectual property rights relating to the panel and/or its components.

For updated license terms, see **www.qiagen.com**.

This page intentionally left blank

This page intentionally left blank

This page intentionally left blank