April 2025

QIAseq® xHYB CGP DNA/RNA Panel Handbook

For library preparation and hybridization capture–based enrichment for comprehensive genomic profiling from FFPE samples

Sample to Insight

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Kit Contents

QIAseq xHYB CGP DNA Panel	(24)	(96)
Catalog no.	333122	333125
No. of hybridization reactions	3	12
Typical no. of samples	24	96
xHYB Probe panel for DNA target enrichment for comprehensive genomic profiling	12 µL	48 µL

QIAseq xHYB CGP RNA Panel	(24)	(96)
Catalog no.	334122	334125
No. of hybridization reactions	3	12
Typical no. of samples	24	96
xHYB Probe panel for RNA target enrichment for comprehensive genomic profiling	12 µL	48 µL

QIAseq xHYB CGP DNA/RNA Panels	(24)	(96)
Catalog no.	335122	335125
No. of hybridization reactions	3	12
Typical no. of samples	24	96
xHYB Probe panel for DNA and RNA target enrichment for comprehensive genomic profiling	12 µL	48 µL

QIAseq xHYB Human Reagent Kit Catalog no. No. of hybridization reactions Typical no. of samples	(24) 333430 3 24	(96) 333195 12 96
Box 1 of 2		
One-4-All Blocking Oligos	24 µL	96 µL
One-4-All Blocking Solution	15 µL	60 µL
Fast Hybridization Solution	60 µL	240 µL
Vapor-Lock	500 µL	500 µL
Post Hybrid-Capture PCR Mix (2x)	660 µL	660 µL
Primer Mix Illumina Library Amplification 12 rxn	20 µL	3 × 20 µL
RNase-free Water	1.5 mL	1.5 mL
Box 2 of 2		
Streptavidin Binding Beads	300 µL	1.2 mL
Post Capture Binding Buffer	2 × 1.2 mL	9.6 mL
Wash Buffer A	2 × 675 µL	5.4 mL
Wash Buffer B	2 × 1.05 mL	8.4 mL

QIAseq Multimodal DNA/RNA Lib Kit Catalog no. Number of samples	(24) 334842 24	(96) 334845 96
10x FX Buffer	48 µL	225 µL
5x WGS FX Mix	96 µL	475 µL
QIAseq FastSelect™ –rRNA HMR (8)	12 µL	12 µL
Side Reaction Reducer	48 µL	192 µL
FG Solution	170 µL	170 µL
ATP Solution	36 µL	290 µL
PAP Enzyme	96 µL	96 µL
PAP Dilution Buffer	192 µL	192 µL
T4 Polynucleotide Kinase	125 µL	125 µL
DNA Ligase	120 µL x 2	1100 µL
UPH Ligation Buffer, 2.5x	1152 µL	1500 µL x 3
Buffer GE2 gDNA Elimination Buffer	72 µL	250 µL
US RT Buffer, 5X	150 µL	450 µL
100 mM DTT	20 µL	80 µL
10 mM dNTP	55 µL	235 µL
EZ Reverse Transcriptase	36 µL	96 µL x 2
RNase Inhibitor	12 µL	96 µL
Nuclease-free Water	10 mL	10 mL
QIAseq Beads	10 mL	38.4 mL

QIAseq Multimodal DNA/RNA UDI (24) (DNA and RNA indexing for 24 samples)	(24)
Catalog no.	**334852**
MDNA24X (QIAseq Multimodal MDNA Index Plate 24) Each plate allows indexing of 24 DNA samples. Each well in the plate is single-use and contains 9 µL of UDI index pairs for DNA libraries.	1
MRNA-24X (QIAseq Multimodal MRNA Index Plate 24) Each plate allows indexing of 24 RNA samples. Each well in the plate is single-use and contains 9 µL of UDI index pairs for RNA libraries.	1
non-UMI Adapter	60 µL
UMI Adapter	60 µL
MM RNA RT Primer	24 µL
MM RNA TSO Primer	24 µL
HiFi Ultra Buffer	384 µL
HiFi Ultra Polymerase	192 µL
MM F-R Primer Mix	77 µL

QIAseq Multimodal DNA/RNA UDI Set A (DNA and RNA indexing for 96 samples) Catalog nos.	(96) 334855
MDNA-96AX (QIAseq Multimodal MDNA Index 96 Set A) Each plate allows indexing of 96 DNA samples. Each well in the plate is single-use and contains 9 µL of UDI index pairs for DNA libraries.	1
MRNA-96AX (QIAseq Multimodal MRNA Index 96 Set A) Each plate allows indexing of 96 RNA samples. Each well in the plate is single-use and contains 9 µL of UDI index pairs for RNA libraries.	1
non-UMI Adapter	275 µL
UMI Adapter	275 µL
MM RNA RT Primer	115 µL
MM RNA TSO Primer	115 µL
HiFi Ultra Buffer	1536 µL
HiFi Ultra Polymerase	850 µL
MM F-R Primer Mix	340 µL

Product	Description	Cat. no.
QIAseq N Blocking Oligos (12)	Oligos designed to bind and block all Illumina Nextera-compatible NGS adapters for 12 reactions of hybridization capture	334871

Shipping and Storage

QIAseq xHYB CGP Panels are shipped on dry ice. Store spike-in panels immediately upon receipt at –30°C to –15°C in a constant-temperature freezer. The QIAseq xHYB Human Reagent Kit is shipped on a cool pack (4°C). Store the automation kit immediately upon receipt at 2–8°C.

QIAseq Multimodal DNA/RNA Library Kit (except QIAseq Beads and Nuclease-free Water) is shipped on dry ice and should be stored immediately upon receipt at –30°C to –15°C in a constant-temperature freezer. QIAseq Beads and Nuclease-free Water are shipped in a separate box on cold packs and should be stored immediately upon receipt at 2–8°C.

QIAseq Multimodal DNA/RNA Index Kits are shipped on dry ice and should be stored immediately upon receipt at –30°C to –15°C in a constant-temperature freezer.

Under these conditions, the components are stable until the expiry date indicated on the label.

Intended Use

QIAseq xHYB Panels, QIAseq xHYB Human Reagent Kits, QIAseq Multimodal DNA/RNA Library and Index Kits are intended for molecular biology applications. These product are not intended for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN® products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at **www.qiagen.com/safety** where you can find, view, and print the SDS for each QIAGEN kit and kit component.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of QIAseq xHYB CGP DNA/RNA Panels is tested against predetermined specifications to ensure consistent product quality.

Introduction

The QIAseq xHYB CGP Panel represents a significant advancement in comprehensive genomic profiling, tailored to enhance cancer research and diagnostics through its multiomic and multimodal capabilities.

This robust panel targets over 720 genes, ensuring full coverage of coding regions and microsatellite instability (MSI) markers, critical for precise tumor profiling and biomarker identification. Its compatibility with cell-free DNA (cfDNA) facilitates the accurate detection of gene fusions, essential for a deeper understanding of cancer biology. The panel's multiomic capability integrates both DNA and RNA analyses, empowering researchers to explore tumor heterogeneity and gene expression dynamics. Customization options further enhance its versatility, allowing users to add specific targets or utilize an optional RNA panel that focuses on over 230 genes.

The multimodal library preparation streamlines both DNA and RNA workflows, optimizing resource use and increasing efficiency, while flexible hybridization times and automation options improve workflow adaptability for diverse research applications.

In summary, the QIAseq xHYB CGP Panel serves as a powerful tool for advancing oncology through comprehensive multiomic genomic analysis, providing valuable insights that can lead to the discovery of new biomarkers and therapeutic targets in cancer, meeting the growing demand for accessible and effective genomic profiling solutions in the evolving landscape of cancer research.

Principle and procedure

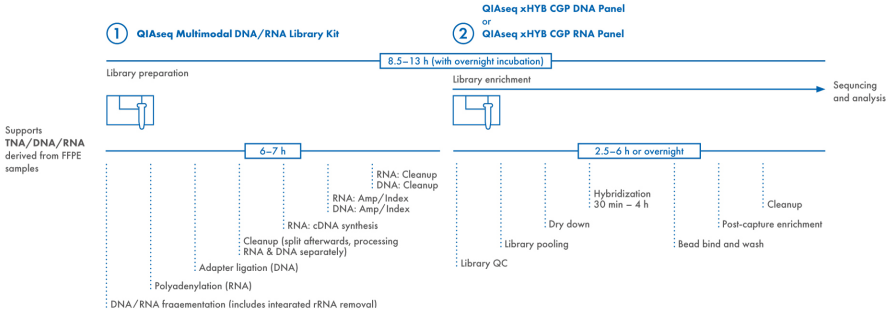

Figure 1. Workflow principle of the QIAseq xHYB CGP Panels. **Step 1**: Generation of indexed libraries using QIAseq Multimodal DNA/RNA Library Kit. **Step 2**: Enrichment of xHYB CGP Targets.

Step 1: Generation of indexed libraries

QIAseq Multimodal DNA/RNA Lib Kit enables construction of WGS and WTS libraries. We recommend using minimum of 40 ng DNA and 80 ng RNA in good quality for CGP DNA/RNA Panels. The following reactions occur in a streamlined, single-tube workflow.

Step 2: Enrichment of human xHYB targets

During xHYB enrichment, indexed library fragments are bound to biotinylated double-stranded DNA capture probes with highly flexible hybridization times ranging from 30 minutes to overnight incubation. Bound fragments are immobilized on streptavidin beads and non-targeted fragments are washed away. Enriched library fragments are amplified using a proprietary postcapture amplification mix that allows even amplification of DNA regions with vastly different GC contents, which minimizes sequencing bias caused by PCR.

QIAseq One-4-All Blocking Oligos

QIAseq xHYB Human Hybrid Capture Panels include One-4-All Blocking Oligos designed to effectively bind and block all Illumina TruSeq®-compatible NGS adapters with index sequence length of up to 12 nt. All QIAseq Dual-Index Y-Adapters (Unique and Combinatorial) are fully compatible with One-4-All Blocking Oligos for hybridization capture. Using these blocking oligos, the target capture specificity is largely enhanced by preventing capture of library fragments via their adapter sequences during the hybridization reaction.

QIAseq N Blocking Oligos

The RNA libraries generated using the QIAseq Multimodal DNA/RNA Library Kit contain the Nextera® adapter sequence. QIAseq N Blocking Oligos are available (cat. no. 334871, purchased separately) for effectively binding and blocking Nextera-compatible NGS adapters. Using these blocking oligos, the target capture specificity is largely enhanced by preventing capture of library fragments via their adapter sequences during the hybridization reaction.

QIAseq xHYB CGP Panel

QIAseq xHYB CGP DNA panel is a Comprehensive Genomic Profiling assay in with 729 genes enabling thorough assessment of all DNA variant types, including SNVs, CNVs, indels, as well as MSI and TMB. It can be combined with QIAseq xHYB CGP RNA panel targeting 274 genes for fusion detection. This broad and deep analysis supports advanced cancer research applications.

Description of protocols

The QIAseq Multimodal DNA/RNA Library Kit is for use with QIAseq xHYB CGP Panel protocols describes the generation of whole genome and whole transcriptome libraries using an optimized workflow. Alternatively, you may use the QIAseq FX DNA Library Kit, and refer to its respective handbook at **www.qiagen.com/QseqFX-DNA**

The QIAseq Multimodal DNA/RNA Library Kit can also be used for the generation of DNA-only or RNA-only libraries if needed.

The QIAseq xHYB CGP Panel protocol describes the hybridization capture workflow for any human xHYB panel and is suitable for input libraries generated using QIAseq Multimodal DNA/RNA Library Kit, QIAseq FX DNA Library Kits, QIAseq Ultralow Input Library Kits, or alternative methods.

Equipment and Reagents to be Supplied by User

- QIAseq Beads (cat. nos. 333923, 333903, and 333927) for bead-based library purification
- 100% ethanol (ACS grade)
- Nuclease-free water
- Buffer EB (cat. no. 19086)
- PCR tubes or plates
- Pipette tips and pipettes
- DNA LoBind tubes (from Axygen™ or Eppendorf®)
- Vortexer
- Microcentrifuge
- Magnetic racks for magnetic beads separation (e.g., Thermo Fisher Scientific DynaMag™ Magnet)
- Thermocycler with heated lid
- Capillary electrophoresis device, e.g., QIAGEN QIAxcel®, Agilent® Bioanalyzer®, or similar to evaluate the DNA fragmentation profile (optional)
- 2 heating blocks for 1.5–2.0 mL tubes
- An evaporation device (e.g., Eppendorf® Concentrator Plus, cat. no. 305000100) equipped with a rotor for 1.5 mL tube and/or a rotor for 0.2 mL PCR tubes/plates.

- The following controls can be used to verify the performance of the QIAseq xHYB CGP Panels:
    - SeraSeq® Lung & Brain CNV Mix, +6Copies (cat. no. 0710-0415)
    - Seraseq Myeloid Mutation DNA Mix (cat. no. 0710-0408)
    - Seraseq Tri-LvlTumor Mut DNA Mixv2 HC (cat. no. 0710-0089)
    - Seraseq gDNA TMBMix Score 20 WT (cat. no. 0710-1324)
    - Seraseq gDNA TMBMix Score 20 tumor (cat. no. 0710-1324)
    - Seraseq Myeloid Fusion RNA (cat. no. 0710-0407)
    - Seraseq FFPE Tumor Fusion RNA v4 Reference Material (cat. no. 0710-0496)
    - Seraseq Fusion RNA Mix v4 (cat. no. 0710-0497)
    - Seraseq FFPE NTRK Fusion RNA Reference Material (cat. no. 0710-1031)
    - Seraseq Whole Transcriptome RNA Seq Mix (cat. no. 0710-2129)
    - NA24385 (from Coriell Institute, control for copy number detection)

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- and RNase-free.
- Before starting, wipe down the work area and pipettes with an RNase and DNA cleaning product.
- For consistent hybridization capture and amplification, ensure the thermocycler used in this protocol is in good working condition and has been calibrated within the manufacturer's specifications.
- Read the entire protocol before beginning. Take note of stopping points where samples can be frozen at –30 to –15°C and plan your workflow accordingly.

Quality control of input libraries

- Control and quantify each indexed library using a capillary electrophoresis method such as using High Sensitivity DNA chip with the QIAxcel Connect System, Agilent 2100 Bioanalyzer, or TapeStation®.
- A typical indexed whole genome library has an average size distribution centered around 400 bp and is free of adapter dimers at a typical yield of >500 ng (refer to Figure 3 in "Recommendations: Library QC & Quantification").
- Libraries from physically sheared DNA will reflect the size distribution of the sheared input DNA.

Indexing recommendations

- Use QIAseq Multimodal DNA/RNA UDI to generate whole genome and/or whole transcriptome libraries for hybridization capture.
- Use different sample indexes for all samples within a hybridization capture pool.
- Consider using different sample indexes also across hybridization capture pools to allow post capture multiplexing depending on your sequencing instrument.

Protocol: Nucleic Acid Fragmentation

Important points before starting

- This protocol describes fragmentation of nucleic acids samples.
- Recommended starting amounts of nucleic acid:
  - For DNA, use a minimum of 40 ng in good quality with the QIAseq xHYB Kit CGP panel and reagent kits.
  - For RNA, use a minimum of 80 ng in good quality with the QIAseq xHYB Kit CGP panel and reagent kits.
- Set up reactions on ice.
- Unless specifically indicated, do not vortex any reagents or reactions.

Procedure

1. Thaw nucleic acid sample(s) 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 the fragmentation.
   a. Thaw 10x FX Buffer, QIAseq FastSelect –rRNA HMR (8) at room temperature (15–25°C).
   b. Mix by flicking the tube, and then centrifuge briefly.

      Note: 5x WGS FX Mix and Side Reaction Reducer should be removed from the freezer and placed on ice just before use. After use, immediately return the enzymes to the freezer.
3. Dilute an aliquot of QIAseq Fastselect –rRNA HMR 1:10 with Nuclease-free Water. Mix by flicking the tube, and then centrifuge briefly.

4. On ice, prepare the fragmentation mix according to Table 1. Briefly centrifuge, mix by pipetting up and down 20 times, and then 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.

Table 1. Reaction mix for FFPE samples

Component	Volume/reaction
DNA (see input recommendation in the "Important points before starting" section)*	Variable A
RNA (see input recommendation in the "Important points before starting" section)*	Variable B
10x FX Buffer	2 µL
5x WGS FX Mix	4 µL
QIAseq FastSelect –rRNA HMR (diluted 1:10)	1 µL
Side Reaction Reducer	1.6 µL
Nuclease-free Water	11.4 µL – variable A (DNA) – variable B (RNA)
Total	**20 µL**

*Instead of adding DNA and RNA separately, total nucleic acid containing both DNA and RNA can be added. If adding total nucleic acid, base the input amount on DNA.

5. Program the thermal cycler according to Table 2. Use the instrument's heated lid.

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

Table 2. Incubation conditions for nucleic acid fragmentation

Step	Incubation temperature (°C)	Incubation time for FFPE DNA (min)
1	4	1
2	32	12
3	65	30
4	60	2
5	55	2
6	37	5
7	25	5
8	4	Hold

6. Transfer the tubes/plate prepared in step 3 to the prechilled thermal cycler and resume the program.
7. Upon completion, allow the thermal cycler to return to 4°C.
8. Place the samples on ice and immediately proceed to "Protocol: RNA Polyadenylation".

Protocol: RNA Polyadenylation

Important points before starting

- Set up reactions on ice.
- Do not vortex any reagents or reactions.

Procedure

1. Prepare the reagents required for the polyadenylation.
    a. Thaw PAP Dilution Buffer, 10x, and ATP Solution on ice.
    b. Mix by flicking the tube, and then centrifuge briefly.

    Note: T4 Polynucleotide Kinase and PAP Enzyme should be removed from the freezer and placed on ice just before use. After use, immediately return the enzymes to the freezer.
2. Dilute PAP Enzyme from 5 U/µL to 1 U/µL as follows:
    a. Prepare 1x PAP Dilution Buffer by diluting 2 µL of the 10x PAP Dilution Buffer with 18 µL Nuclease-free Water.
    b. Use the 1x PAP Dilution Buffer to dilute an aliquot of the PAP Enzyme from 5 U/µL to 1 U/µL. Briefly centrifuge, mix by pipetting up and down 20 times, and then briefly centrifuge again.
3. Prepare the RNA polyadenylation mix according to Table 3. Briefly centrifuge, mix by pipetting up and down 20 times, and then 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.

Table 3. Reaction mix for RNA polyadenylation

Component	Volume/reaction (µL)
Fragmentation reaction (already in tube)	20
ATP Solution	1.25
T4 Polynucleotide Kinase	1
Diluted PAP Enzyme (1 U/µL)*	1
Nuclease-free Water	1.75
Total	**25**

* Ensure PAP Enzyme has been diluted from its stock 5 U/µL concentration to 1 U/µL using 1x PAP Dilution Buffer.

4. Incubate the reactions in a thermal cycler according to Table 4. Use the instrument's heated lid.

Table 4. Incubation conditions for RNA polyadenylation

Step	Temperature (°C)	Time (min)
1	4	1
2	30	10
3	4	Hold

5. Upon completion, place the reactions on ice and proceed to "Protocol: DNA Ligation", next section.

Protocol: DNA Ligation

Important points before starting

- The product from "Protocol: RNA Polyadenylation" is the starting material for this protocol.
- Set up reactions on ice.
- Do not vortex any reagents or reactions.
- Prepare fresh 80% ethanol daily.
- 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

1. Prepare the reagents required for the DNA ligation.
   a. Thaw UMI Adapter and UPH Ligation Buffer, 2.5x, at room temperature.

      Note: The UMI Adapter contains a UMI.

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

      Note: DNA Ligase should be removed from the freezer and placed on ice just before use. After use, immediately return the enzyme to the freezer.
2. Prepare the DNA ligation mix according to Table 5. Briefly centrifuge, mix by pipetting up and down 20 times, and then 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.

Table 5. Reaction mix for DNA ligation

Component	Volume/reaction (µL)
RNA polyadenylation reaction (already in tube)	25
UPH Ligation Buffer, 2.5x	40
UMI Adapter*	2.5
DNA Ligase	10
Nuclease-free Water	22.5
Total	**100**

* For FFPE samples with input <100 ng, use UMI Adapter with 1:10 dilution.

3. Incubate the reactions in a thermal cycler according to Table 6.

   Important: Do not use the heated lid.

Table 6. Incubation conditions for DNA ligation

Step	Temperature (°C)	Time (min)
1	4	1
2	20	15
3	4	Hold

4. Add 90 µL QIAseq Beads, and then mix by vortexing.
5. Incubate for 5 min at room temperature.
6. Place the tubes/plate on a magnetic rack for 2 min (for tubes) or 10 min (for plates).

   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 because they contain the DNA of interest.

Note: For plates, the following may improve performance. After 8 min, remove 90 µL supernatant. Leave it on the magnetic stand for 2 min and remove 90 µL supernatant. Then, briefly centrifuge the plate, return it to the magnetic stand for 1 min, and use a 10 µL pipette to remove the remaining supernatant.

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

   Important: Completely remove all traces of the ethanol after the second wash. Remove the ethanol with a 200 µL pipette tip first, spin down briefly, and then use a 10 µL pipette tip to remove any residual ethanol.
9. With the beads still on the magnetic stand, air-dry at room temperature for 5–10 min.

   Note: Visually inspect the pellet to confirm that it is completely dry. If the pellet is not completely dry, additional drying time may be required.
10. Remove the beads from the magnetic stand, and then elute the DNA from the beads by adding 52 µL Nuclease-free Water. Mix well by pipetting.
11. Return the tube/plate to the magnetic rack until the solution has cleared.
12. Transfer 50 µL of the supernatant to clean tubes/plate wells.
13. Add 55 µL of QIAseq Beads and mix by vortexing.
14. Incubate for 5min at room temperature.
15. Place the tubes/plate on a magnetic rack for 2 min (for tubes) or 10 min (for plates).

    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 because they contain the DNA of interest.

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

17. Repeat the ethanol wash.

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

18. With the beads still on the magnetic stand, air-dry at room temperature for 5–10 min.

    Note: Visually inspect the pellet to confirm that it is completely dry. If the pellet is not completely dry, additional drying time may be required.

19. Remove the beads from the magnetic stand, and then elute the DNA from the beads by adding 18 µL Nuclease-free Water. Mix well by pipetting.

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

21. For each sample, aliquot 7.5 µL of the eluate into two separate clean tubes/plate wells.

    Aliquot 1: Proceed to "Protocol: DNA Library Indexing" on the next page.

    Aliquot 2: Proceed to "Protocol: RNA Reverse Transcription" on page 32

    Alternatively, the samples can be stored at –30 to –15°C in a constant-temperature freezer.

Protocol: DNA Library Indexing

Important points before starting

- The starting material is a 7.5 µL sample aliquot from "Protocol: DNA Ligation".
- Set up reactions on ice.
- Do not vortex any reagents or reactions.
- Use MDNA index plates, either MDNA-24X or MDNA-96AX.
- Prepare fresh 80% ethanol daily.
- 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

1. Prepare the reagents required for DNA Library indexing.
   a. Thaw HiFi Ultra Buffer, 5x; MM F-R Primer Mix, and MDNA-24X or MDNA-96AX index plate at room temperature.
   b. Mix by either flicking the tube or vortexing the index plate, and then centrifuge briefly. Plate should be centrifuged at 1000 x *g* for 1 min.

      Note: HiFi Ultra Polymerase should be removed from the freezer and placed on ice just before use. After use, immediately return the enzyme to the freezer.
2. Prepare the reactions according to Table 7. Briefly centrifuge, mix by pipetting up and down 20 times, and then 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.

Table 7. Reaction mix for DNA indexing

Component	Volume/reaction (µL)
Sample aliquot (from "Protocol: DNA Ligation")	7.5
HiFi Ultra Buffer, 5x	8
MM F-R Primer Mix	1.6
Nuclease-free Water	16.4
Well from MDNA-24X or MDNA-96AX index plate*	2.5
HiFi Ultra Polymerase	4
Total	**40**

*Ensure proper technique to prevent cross-contamination. Additionally ensure that every sample has a unique index and that no well is used twice.

3. Program a thermal cycler as described in Table 8, using cycle numbers described in Table 9.

   Important: Set up the ramp rate of the thermal cycler at ≤2°C/s.

Table 8. Cycling conditions for DNA indexing

Step	Time	Temperature (°C)
Hold	2 min	98
2-step cycling		
Denaturation	20 s	98
Annealing/Extension	1 min	60
Cycle number	See Table 9.	
Hold	3 min	72
Hold	∞	4

Table 9. Cycle number recommendations for DNA indexing, based on original sample input

DNA Input	FFPE sample (cycles)
40 ng	14
100 ng	13
250 ng	12

4. After the reaction is complete, add 36 µL QIAseq Beads, and then mix by vortexing or pipetting up and down several times.
5. Incubate for 5 min at room temperature.
6. Place the tubes/plate on a magnetic rack for 2 min (for tubes) or 10 min (for plates) to separate the beads from the supernatant.

   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 because they contain the DNA of interest.
7. With the beads still on the magnetic stand, add 200 µL of 80% ethanol. Rotate the tube 2–3 times or move the plate side-to-side between the 2 column positions of the magnet to wash the beads. Carefully remove and discard the wash.
8. Repeat the ethanol wash twice, for a total of 3 washes.

   Important: Completely remove all traces of the ethanol after this second wash. Remove the ethanol with a 200 µL pipette tip first, spin down briefly, and then use a 10 µL pipette tip to remove any residual ethanol.
9. With the beads still on the magnetic stand, air-dry at room temperature for 5–10 min.

   Note: Visually inspect the pellet to confirm that it is completely dry. If the pellet is not completely dry, additional drying time may be required.

10. Remove the beads from the magnetic stand, and then elute the DNA from the beads by adding 24 µL Nuclease-free Water. Mix well by pipetting.
11. Return the tube/plate to the magnetic rack until the solution has cleared.
12. Transfer 22 µL of the supernatant to clean tubes/plate wells.
13. The library is now ready for sequencing or hybrid capture. Proceed to "Recommendations: Library QC & Quantification" on page 54. Alternatively, the samples can be stored at –30 to –15°C in a constant-temperature freezer.

Protocol: RNA Reverse Transcription

Important points before starting

- The starting material is a 7.5 µL sample aliquot from "Protocol: DNA Ligation".
- Set up reactions on ice.
- Do not vortex any reagents or reactions.
- Prepare fresh 80% ethanol daily.
- 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

1. Prepare the reagents required for sample pre-treatment.

   Note: Buffer GE2 gDNA elimination buffer should be removed from the freezer and placed on ice just before use. After use, immediately return the tube to the freezer.

2. Prepare the sample pretreatment mix according to Table 10. Briefly centrifuge, mix by pipetting up and down 20 times, and then 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.

Table 10. Reaction mix for sample pretreatment

Component	Volume/reaction (µL)
Sample aliquot (from "Protocol: DNA Ligation")	7.5
Buffer GE2 gDNA elimination buffer	2
Total	**9.5**

3. Incubate the reactions in a thermal cycler according to Table 11. Use the instrument's heated lid.

Table 11. Incubation conditions for sample pretreatment

Step	Incubation temperature (°C)	Incubation time (min)
1	42	5
2	75	10
3	4	Hold

4. Prepare the reagents required for reverse transcription.
   a. Thaw US RT Buffer, 5x; DTT (100 mM); dNTP (10 mM); MM RNA RT Primer; and MM RNA TSO at room temperature.
   b. Mix by flicking the tubes and then centrifuge briefly.

      Note: The RNase Inhibitor and EZ Reverse Transcriptase should be removed from the freezer and placed on ice just before use. After use, immediately return the enzymes to the freezer.

5. To the treated sample, prepare the reverse transcription mix according to Table 12, next page. Briefly centrifuge, mix by pipetting up and down 20 times, and then 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.

Table 12. Reaction mix for reverse transcription

Component	Volume/reaction (µL)
Treated Sample	9.5
US RT Buffer, 5x	4
DTT (100 mM)	0.5
dNTP (10 mM)	2
MM RNA RT Primer	1
MM RNA TSO	1
RNase Inhibitor	0.5
EZ Reverse Transcriptase	1.5
Total	**20**

6. Incubate the reactions in a thermal cycler according to Table 13. Use the instrument's heated lid.

Table 13. Incubation conditions for reverse transcription

Step	Incubation temperature (°C)	Incubation time (min)
1	4	1
2	42	90
3	70	10
4	4	1
5	4	Hold

7. Add 20 µL Nuclease-Free Water and 44 µL QIAseq Beads and mix by vortexing or by pipetting up and down several times.
8. Incubate for 5 min at room temperature.
9. Place the tubes/plate on a magnetic rack for 2 min (for tubes) or 10 min (for plates).

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 because they contain the DNA of interest.

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

    Important: Completely remove all traces of the ethanol after this second wash. Remove the ethanol with a 200 µL pipette tip first, spin down briefly, and then use a 10 µL pipette tip to remove any residual ethanol.
12. With the beads still on the magnetic stand, air-dry at room temperature for 5–10 min.

    Note: Visually inspect the pellet to confirm that it is completely dry. If the pellet is not completely dry, additional drying time may be required. Ethanol carryover to the next universal PCR step will affect PCR efficiency.
13. Remove the beads from the magnetic stand, and then elute the DNA from the beads by adding 42 µL Nuclease-free Water.
14. Return the tube/plate wells to the magnetic rack until solution the solution has cleared.
15. Transfer 40 µL of the eluate to clean tubes/plate wells.
16. Add 44 µL QIAseq Beads and mix by vortexing or by pipetting up and down several times.
17. Incubate for 5 min at room temperature.
18. Place the tubes/plate wells on a magnetic rack for 2 min (for tubes) or 10 min (for plates). 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 because they contain the DNA of interest.

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

20. Repeat the ethanol wash.

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

21. With the beads still on the magnetic stand, air-dry at room temperature for 5–10 min.

    Note: Visually inspect the pellet to confirm that it is completely dry. If the pellet is not completely dry, additional drying time may be required. Ethanol carryover to the next universal PCR step will affect PCR efficiency.

22. Remove the beads from the magnetic stand, and then elute the DNA from the beads by adding 22 µL Nuclease-free Water.

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

24. Transfer 20 µL of the eluate to clean tubes/plate wells.

25. Proceed to "Protocol: RNA Library Indexing" on the facing page. Alternatively, the samples can be stored at –30 to –15°C in a constant-temperature freezer.

Protocol: RNA Library Indexing

Important points before starting

- The starting material is the 20 µL sample from "Protocol: RNA Reverse Transcription".
- Set up reactions on ice.
- Do not vortex any reagents or reactions.
- Use MRNA index plates, either MRNA-24X or MRNA-96AX.
- Prepare fresh 80% ethanol daily.
- 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

1. Prepare the reagents required for RNA Library indexing.
   a. Thaw HiFi Ultra Buffer, 5x; MM F-R Primer Mix, and MRNA-24X or mRNA-96AX index plate at room temperature.
   b. Mix by either flicking the tube or vortexing the index plate, and then centrifuge briefly. Plate should be centrifuged at 1000 x *g* for 1 min.

   Note: HiFi Ultra Polymerase should be removed from the freezer and placed on ice just before use. After use, immediately return the enzyme to the freezer.
2. Prepare the reactions according to Table 14. Briefly centrifuge, mix by pipetting up and down 20 times, and then 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.

Table 14. Reaction mix for RNA indexing

Component	Volume/reaction (µL)
Sample aliquot (from "Protocol: RNA Reverse Transcription")	20
HiFi Ultra Buffer, 5x	8
MM F-R Primer Mix	1.6
Nuclease-free Water	3.9
Well from MRNA-24X or MRNA-96AX index plate*	2.5
HiFi Ultra Polymerase	4
Total	**40**

*Ensure proper technique to prevent cross-contamination. Additionally ensure that every sample has a unique index and that no well is used twice.

3. Program a thermal cycler as described in Table 15, using cycle numbers described in .

   Important: Set up the ramp rate of the thermal cycler at ≤2°C/s.

Table 15. Cycling conditions for RNA indexing

Step	Time	Temperature (°C)
Hold	2 min	98
2-step cycling		
Denaturation	20 s	98
Annealing/Extension	1 min	60
Cycle number	See Table 16.	
Hold	3 min	72
Hold	∞	4

Table 16. Cycle number recommendations for RNA indexing, based on original sample input

RNA Input (ng)	FFPE sample (cycle)
80	24
200	23
500	22

4. After the reaction is complete, add 36 µL QIAseq Beads, and then mix by vortexing or pipetting up and down several times.
5. Incubate for 5 min at room temperature.
6. Place the tubes/plate on a magnetic rack for 2 min (for tubes) or 10 min (for plates) to separate the beads from the supernatant.

   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 because they contain the DNA of interest.
7. With the beads still on the magnetic stand, add 200 µL of 80% ethanol. Rotate the tube 2–3 times or move the plate side-to-side between the 2 column positions of the magnet to wash the beads. Carefully remove and discard the wash.
8. Repeat the ethanol wash twice, for a total of 3 washes.

   Important: Completely remove all traces of the ethanol after this second wash. Remove the ethanol with a 200 µL pipette tip first, spin down briefly, and then use a 10 µL pipette tip to remove any residual ethanol.
9. With the beads still on the magnetic stand, air-dry at room temperature for 5–10 min.

   Note: Visually inspect the pellet to confirm that it is completely dry. If the pellet is not completely dry, additional drying time may be required.

10. Remove the beads from the magnetic stand, and then elute the DNA from the beads by adding 24 µL Nuclease-free Water. Mix well by pipetting.
11. Return the tube/plate to the magnetic rack until the solution has cleared.
12. Transfer 22 µL of the supernatant to clean tubes/plate.
13. The library is now ready for sequencing or hybrid capture. Proceed to "Recommendations: Library QC & Quantification" on page 54. Alternatively, the samples can be stored at –30 to –15°C in a constant-temperature freezer.

Protocol: Target Enrichment from Indexed Whole-genome Libraries Using QIAseq xHYB Human Hybrid Capture Panels

Pool libraries and dry down the indexed library pool

Pooling of amplified, indexed whole genome libraries enables processing multiple libraries in a single hybridization-capture reaction. We recommend pooling of up to 8 indexed libraries for one hybridization capture reaction. Alternatively, hybrid-capture may be performed for a single library. The amount of indexed libraries to be pooled depends on the number of samples per pool. Make sure to only use PCR-amplified libraries for hybridization capture.

Things to do before starting

- Quantify the amplified, indexed whole genome libraries (see guidelines in "Important Notes").
- Thaw the xHYB Probe Set, All-4-One Blocking Oligos for DNA libraries, or QIAseq N Blocking oligos for RNA libraries, and All-4-One Blocking Solution on ice. Then, pulse vortex and pulse-spin.
- To immediately proceed to hybridization capture after library pool dry-down, equilibrate the Vapor-Lock reagent to room temperature and heat the Fast Hybridization Solution to 65°C before pool evaporation is complete. Alternatively, the dried library pool can be stored at –30 to –15°C for up to 7 days.

Procedure

1. For each capture pool, determine the per-library amount (ng) according to the desired pool size referring to the guidelines in Table 17 or follow the recommendations in Table 18. Make sure to not exceed 3200 ng total input per capture pool.

Table 17. Guidelines for pooling indexed libraries for hybridization capture

Library pooling guidelines	
Recommended number of libraries per capture pool	8
Maximum total input per capture pool	3200 ng
Minimal input per library	200 ng

Table 18. Recommended library pooling strategy for hybridization capture

Number of indexed samples per pool	Amount of each indexed library per pool (ng)
1	200–500
2	200–500
3	200–500
4	200–500
6	200–500
8	200–400

* Do not exceed a total DNA input of 3200 ng per pool.

Note: To maintain equal representation of libraries during sequencing, use the same input amount for all libraries. Using less than 200 ng input per library may result in reduced complexity.

2. Calculate the volume (µL) of each amplified library needed for pooling, e.g., for a library concentration of 25 ng/µL, 16 µL are needed to add 400 ng of indexed library to the capture pool.

3. Transfer the calculated volumes for each library to a 0.2 mL PCR tube/strip/plate. If the total library pool volume exceeds 180 µL, use a 1.5 mL LoBind tube. Use multiple tubes/wells if performing multiple hybridization reactions at once.

   Recommendation: If the volume allows, pool libraries in a 0.2 mL PCR tube/strip or plate.

4. Add the xHYB Probe Set and blocking reagents to each library pool according to Table 19.

Table 19. Complement the library pool with probes and blocking reagents

Component	Volume (µL)
Pool of indexed libraries (from step 3)	Variable
xHYB Probe Set (CGP DNA or RNA panel)*	4
One-4-All Blocking Oligos for DNA libraries or QIAseq N Blocking oligos for RNA libraries	8
One-4-All Blocking Solution	5

*Use the CGP DNA probe set for DNA library pools and the CGP RNA panel for RNA library pools.

5. Completely evaporate all liquid content of the complemented library pool by using a SpeedVac system (or a similar evaporator device) equipped with an appropriate rotor for tube and/or 96-well plates. If needed, accelerate the evaporation of larger volumes by setting the temperature to 60°C.

   Alternative: Evaporate all liquid content by placing the opened tube/plate in a heating block or thermal cycler with the lid opened. Evaporate at 60°C over night or until all liquid has evaporated.

6. When evaporation of the complemented library pool is complete, store the dried library pool on ice and proceed immediately to step 7 (Hybridization capture protocol). Alternatively, the dried library pool can be stored at –30 to –15°C for up to 7 days.

Hybridization capture

During the hybridization step, QIAseq xHYB Probes bind specifically to targeted library fragments present in the pool. The flexible hybridization protocol allows adjusting the duration of hybridization from as little as 30 min to 4 h. Incubating for 1 h or longer can improve the capture performance. Incubating for longer than 4 h or overnight will not enhance performance significantly, but may be considered for highest flexibility in experiment planning.

Things to do before starting

- Thaw the Vapor-Lock reagent and equilibrate to room temperature.
- Heat the Fast Hybridization Solution to 65°C in a heating block for at least 10 min and make sure all precipitate is dissolved.
- When performing the hybridization capture reaction for 1 h or less, start preparing reagents needed for binding the hybridized targets to Streptavidin Binding Beads as outlined at the beginning of the next chapter.

Procedure

7. Thaw and equilibrate the Vapor-Lock reagent to room temperature. Heat the Fast Hybridization Solution to 65°C for 10 min in a heating block. Vortex and make sure all precipitate is dissolved. Keep the Fast Hybridization Solution at 65°C until used.
8. Program a thermocycler with a heated lid according to Table 20.

   Important: The heated lid must be set to 85°C to prevent evaporation.

Table 20. Conditions for hybridization capture

Step	Incubation temperature (°C)	Incubation time
1	95	1 min
2	95	5 min
3	60	30 min–4 h*
4	60	Hold†

* Hybridization times of less than 30 min may result in reduced target region coverage. Hybridization time may be extended to overnight incubation without negative effects on specificity or uniformity.

† Program the thermocycler to maintain 60°C after hybridization is complete. Do not allow the hybridization reaction to cool down as this negatively affects off-target rate.

9. Start the program. When the thermocycler block reaches 95°C, pause the program in step 1.

10. Vortex the 65°C Fast Hybridization Solution and immediately transfer 20 µL to the tube/well containing the dried complemented library pool from step 6.

    Important: Fast Hybridization Solution is viscous. Pipet slowly to ensure accurate volume transfer. For optimal volume transfer, set the pipette to 20 µL; then, slowly aspirate and dispense back into the reagent tube twice, and with the same pipette tip, slowly aspirate and transfer the entire volume to the reaction tube.

11. If the dried library pool was in a 0.2 mL PCR tube/plate, proceed to step 15.

    If the dried library pool was in a 1.5 mL LoBind tube, continue with step 12.

12. Vortex the tube for 5 s, and then incubate for 2 min at 65°C.

13. Repeat step 12 for 2 more times, and then pulse-spin the tube.

    Note: A white precipitate may form. This does not affect the hybridization procedure.

14. Transfer the complete solution to a 0.2 mL PCR tube/plate and pulse-spin. Ensure that there are no bubbles present.

15. Add 30 µL of Vapor-Lock reagent. Pulse-spin the tube to ensure all liquid is at the bottom of the tube.

    Note: Vapor-Lock will form a phase on top of the hybridization reaction after pulse-spin. This does not affect the hybridization procedure.

16. Transfer the tube/plate to the thermocycler and continue the program at 95°C.

    Important: Make sure the heated lid is set to 85°C and the tube is tightly sealed with fresh lids/foil. Make sure to use the correct lid spacers for your thermocycler.

17. Leave the hybridization reaction in the thermocycler until ready for binding of captured library fragments to Streptavidin beads. Continue with the protocol "Bind hybridized targets to Streptavidin beads" at least 30 min before hybridization is complete.

    Alternatively, the hybridization reaction may remain in the thermocycler over night to continue with binding the hybridized targets to Streptavidin beads the next day.

    Important: Do not allow the hybridization reaction to cool to less than 60°C after the program is complete.

Bind hybridized targets to Streptavidin beads

In this step, the biotin-coupled xHYB Probes and their hybridized targets will be captured on Streptavidin beads. Nonspecific targets will be washed away during a series of washing steps. Start preparing the Streptavidin Binding Beads and wash buffers at least 30 min before hybridization is complete.

Things to do before starting

- Preheat a heating block for 1.5 mL tubes to 70°C.
- Preheat a heating block for 1.5 mL tubes to 48°C.
- Heat the whole reagent bottles of the Wash Buffer A and Wash Buffer B to 48°C and mix thoroughly. Leave both reagent bottles at 48°C until needed.
- Heat the whole reagent bottle of the Post Capture Binding Buffer to 48°C and mix thoroughly. Then leave the reagent bottle at room temperature until needed.
- Equilibrate Streptavidin Binding Beads to room temperature for at least 30 min.

Procedure

18. Per reaction, aliquot 450 µL Wash Buffer A and preheat to 70°C.
19. Per reaction, aliquot 700 µL Wash Buffer B and preheat to 48°C.

    Important: For steps 18 and 19, do not aliquot chilled buffers. Heat the entire reagent bottles of Wash Buffer A and Wash Buffer B to 48°C and mix thoroughly. Then, prepare aliquots.

    Note: Leave the wash buffer aliquots in the heating blocks until needed.
20. Homogenize the pre-equilibrated Streptavidin Binding Beads by vortexing.
21. Transfer 100 µL homogenized Streptavidin Binding Beads to a new 1.5 mL LoBind tube. Prepare 1 tube for each hybridization reaction.
22. Add 200 µL Post Capture Binding Buffer and mix by pipetting.

    Important: Do not use chilled Post Capture Binding Buffer. Make sure to heat the buffer to 48°C before use and mix well. Then allow the buffer to cool to room temperature.

23. Incubate the beads on a magnetic stand for 1 min or until the solution is clear, and then remove and discard the clear supernatant without disturbing the pellet. Remove from the magnetic stand.

24. Repeat steps 22 and 23 for 2 more times for a total of 3 washing steps.

25. After removing the supernatant from the third wash, add a final 200 µL Post Capture Binding Buffer and homogenize the beads by vortexing. Keep the homogenized bead slurry at room temperature until needed.

26. After the hybridization reaction is complete, open the lid of the thermocycler, open the tube, and swiftly transfer the complete volume including Vapor-Lock into the tube containing the bead slurry from step 25. Mix well by pipetting.

    Important: Do not allow the hybridization reaction to cool to less than 60°C before transferring to the Streptavidin Binding Beads. Rapid transfer directly from the thermocycler is essential for minimizing the off-target rate.

27. Incubate the mixture at room temperature for 30 min on a shaker, rotator, or similar device to prevent the beads from settling.

    Alternatively, incubate the mixture at room temperature for 30 min, while agitating in 5-min intervals to prevent beads from settling.

    Note: Aggressive mixing is not required. Do not vortex.

28. Pulse-spin the tube to ensure all liquid is at the bottom of the tube.

29. Incubate the beads on a magnetic stand for 1 min or until the solution is clear, and then remove and discard the clear supernatant including Vapor-Lock without disturbing the pellet.

    Note: Small amounts of Vapor-Lock may remain after removal of supernatant and throughout each wash step. This does not affect the final capture product.

30. Remove the tube from the magnetic stand, and add 200 µL of preheated 70°C Wash Buffer A. Mix by pipetting.
31. Incubate for 5 min at 70°C.
32. Place the tube on a magnetic stand for 1 min or until the solution is clear, and then remove and discard the clear supernatant without disturbing the pellet.

    Remove the tube from the magnetic stand, and add another 200 µL of preheated 70°C Wash Buffer A. Mix by pipetting.
33. Incubate for 5 min at 70°C.
34. Pulse-spin the tube and transfer the entire volume, including the beads to a new 1.5 mL LoBind tube.
35. Place the tube on a magnetic stand for 1 min or until the solution is clear, and then remove and discard the clear supernatant without disturbing the pellet.
36. Remove the tube from the magnetic stand, and add 200 µL of preheated 48°C Wash Buffer B. Mix by pipetting.
37. Pulse-spin the tube, and then incubate for 5 min at 48°C.
38. Place the tube on a magnetic stand for 1 min or until the solution is clear, and then remove and discard the clear supernatant without disturbing the pellet.
39. Repeat steps 36 to 38 for 2 more times for a total of 3 washes.
40. After the final wash, use a 10 µL pipette to remove all residual supernatant. Proceed immediately to the next step.

    Important: Do not allow the beads to dry.
41. Remove the tube from the magnetic stand, and add 45 µL RNase-free water. Homogenize by pipetting, and store the bead slurry on ice.
42. Proceed directly to “Postcapture amplification”.

Alternatively, aliquot the bead slurry into two 22.5 µL aliquots and store at –30 to –15°C for later use.

Important: Do not discard the beads. The capture product is still bound to the Streptavidin beads.

Postcapture amplification

Captured targets bound to Streptavidin Binding Beads will be amplified using QIAGENs Post Hybrid-Capture PCR Mix. The amplified hybrid capture library will be purified and is ready for QC and sequencing on Illumina instruments.

Things to do before starting

- Prepare fresh 80% ethanol.
- If stored at –30 to –15°C, thaw a 22.5 µL aliquot of the Streptavidin Binding Bead slurry on ice (from step 42).
- Equilibrate Agencourt AMPure XP beads to room temperature for 20–30 min before use.
- Thaw Post Hybrid-Capture PCR Mix and Primer Mix Illumina Library Amplification on ice. Once reagents are thawed, mix by pulse-vortexing to avoid any localized concentrations. Briefly spin down vortexed reagents before use.

Procedure

43. Program a thermocycler with a heated lid according to Table 21 and Table 22.

Table 21. Postcapture amplification conditions

Time	Temperature (°C)	Number of cycles
2 min	98	1
20 s 30 s 30 s	98 60 72	7–13 cycles*
1 min	72	1
∞	4	Hold

* See Table 22 for the optimal cycle numbers depending on input amount and xHYB panel used.

Table 22. Number of postcapture amplification cycles

xHYB Panel	<500 ng capture input	500 ng – 2.0 µg capture input	>2.0 µg capture input
CGP DNA Panel	9 cycles	8 cycles	7 cycles
CGP RNA Panel	13 cycles	12 cycles	11 cycles

44. Prepare the reaction mix on ice according to Table 23. Mix the components in a PCR tube or 96-well PCR plate.

Table 23. Reaction mix for postcapture amplification

Component	Volume (µL)
Bead slurry with captured targets (from step 41)	22.5
Post Hybrid-Capture PCR Mix	25
Primer Mix Illumina Library Amplification	2.5
Total reaction volume	**50**

45. Transfer the PCR tube or plate to the thermocycler and start the program.

46. Once PCR is complete, add 75 µL of resuspended QIAseq beads to each reaction (50 µL) and pipet up and down thoroughly to mix.

47. Incubate the mixture for 5 min at room temperature. Pellet the beads on a magnetic stand, and carefully discard the supernatant.

48. Wash the beads by adding 200 µL of 80% ethanol. Pellet the beads on the magnetic stand and discard the supernatant. Repeat the wash once for a total of 2 ethanol washes. Remove as much excess ethanol as possible.

49. Incubate on the magnetic stand for 5–10 min or until the beads are dry. Overdrying of beads may result in lower DNA recovery. Remove from the magnetic stand.

50. Elute by resuspending in 32 µL of Buffer EB, 10 mM Tris·Cl, pH 8.0, or RNase-free water. Incubate at room temperature for 2 min.

51. Pellet the beads on the magnetic stand. Carefully transfer 30 µL of the clear supernatant into a new tube.

52. Assess the quality and size distribution of the library using a capillary electrophoresis device such as QIAGEN QIAxcel Advanced or Agilent Bioanalyzer 2100. Check for the expected size distribution (see Figure 2). For best sequencing results, quantitate the captured library using a qPCR assay such as the QIAseq Library Quant Assay Kit or compatible method.

    Note: The library should show a distribution similar to the whole genome libraries used for the respective capture pool.

53. The purified library can be safely stored at –30 to –15°C in a DNA LoBind tube until ready to use for sequencing.

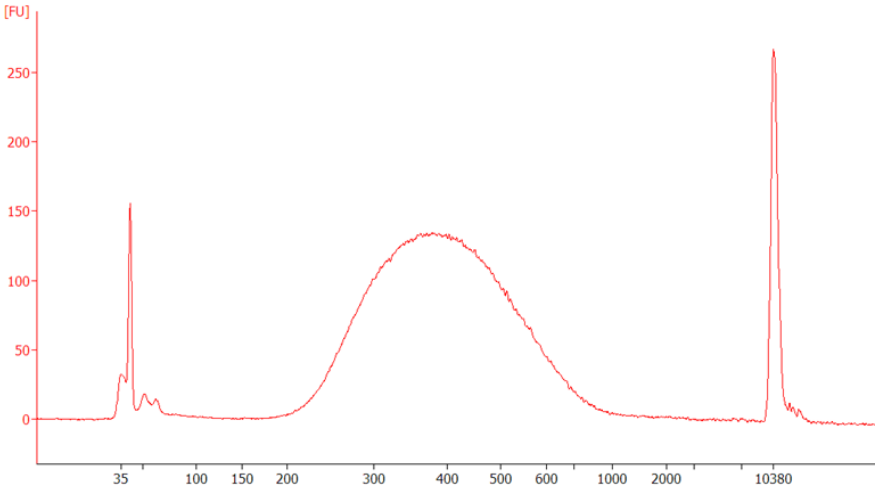

Figure 2. Electrophoresis trace data of a final hybrid capture library pool.

Recommendations: Library QC & Quantification

NGS Library QC

Perform QC with the QIAxcel Connect or similar instrument. Check for the correct size distribution of library fragments (~400–500 bp median size) and for the absence of adapters or adapter–dimers (~<200 bp) (Figure 3).

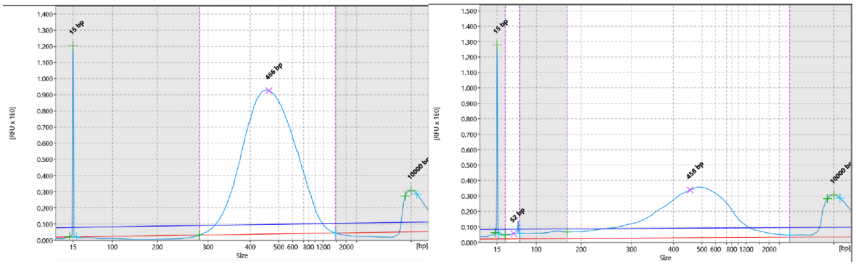

Figure 3. QIAseq Multimodal WGS DNA (left) and WTS RNA (right) Libraries.

Preferred library quantification method

Quantify the library using Nanodrop® or QIAexpert if proceeding to "Hybrid Capture", or qPCR-based method if proceeding to sequencing.

Sequencing Guidelines

Pooling and multiplexing guidelines

Depending on the sequencing instrument used, combining multiple hybridization capture pools may be advisable for cost efficient sequencing. Before combining equal volumes of hybrid capture pools, quantify each pool and dilute to a defined concentration (e.g., 5 nM). To determine the total number of samples to be sequenced in a single sequencing run, consider specifications of the sequencing instrument, the safe target cluster density, target region size of the used xHYB panel and the desired coverage per sample. Please refer to Table 24a and Table 24b for initial multiplexing guidelines per sequencing run to achieve a minimal average target region coverage of 1200x for DNA Library. Multiplexing and loading may need to be adjusted based on your local conditions like the quantification method used and your preferences for target cluster density.

xHYB panels achieve a very high coverage uniformity throughout the target region with a typical Fold-80 base penalty in the range of 1.3–1.5 or better. This means that the average target region coverage needs to be increased by approximately 1.4 to raise 80% of non-zero-coverage target bases to the mean coverage level. When planning the sequencing pool, consider approximately 1.3- to 1.5-fold of the desired minimal coverage to reliably obtain that coverage throughout the target region.

Table 24a. Multiplexing guidelines for Illumina instruments using 2 x 149 bp paired-end sequencing to achieve a minimal average target coverage of 1200x for DNA Library. Actual coverage may vary depending on input DNA quality and the sequencing instrument used

	# (DNA library+RNA library)	# (DNA library)	# (RNA library)*
DNA library reads (M)	75	75	–
Reads for RNA library (M)	50	–	50

*When sequencing an RNA library alone (without DNA), we recommend adding 20% pHiX.

Table 24b. Multiplexing guidelines for Illumina instruments using 2 x 149 bp paired-end sequencing to achieve a minimal average target coverage of 1200x for DNA Library. Actual coverage may vary depending on input DNA quality and the sequencing instrument used

Platform and Sequencing Chemistry

Instrument	Version	Read length	Capacity (paired-ends reads)	Capacity (M)	# (DNA library+RNA library)	# (DNA library)	# (RNA library)*
NextSeq® 500/550	Mid output	2 x 149 bp	260 M	260	2	3	5
NextSeq 500/550	High output	2 x 149 bp	800 M	800	6	10	16
NextSeq 1000/2000	P1	2 x 149 bp	200 M	200	1	2	4
NextSeq 1000/2000	P2	2 x 149 bp	800 M	800	6	10	16
NextSeq 1000/2000	P3	2 x 149 bp	2400 M	2400	19	32	48
NovaSeq™ 6000	SP (2 lanes per flow cell)	2 x 149 bp	1.6 B	1600	12	21	32
NovaSeq 6000	S1 (2 lanes per flow cell)	2 x 149 bp	3.2 B	3200	25	42	64
NovaSeq 6000	S2 (2 lanes per flow cell)	2 x 149 bp	8.2 B	8200	65	109	164
NovaSeq 6000	S4 (4 lanes per flow cell)	2 x 149 bp	20 B	20,000	160	266	400
NovaSeq X Series	1.5 B	2 x 149 bp	3.2 B	3200	25	42	64
NovaSeq X Series	10 B	2 x 149 bp	20 B	20,000	160	266	400
NovaSeq X Series	25 B	2 x 149 bp	52 B	52,000	416	693	1040

Table 24b. Multiplexing guidelines for Illumina instruments using 2 x 149 bp paired-end sequencing to achieve a minimal average target coverage of 1200x for DNA Library. Actual coverage may vary depending on input DNA quality and the sequencing instrument used (continued)

Platform and Sequencing Chemistry

Instrument	Version	Read length	Capacity (paired-ends reads)	Capacity (M)	# (DNA library+RNA library)	# (DNA library)	# (RNA library)*
AVITI/AVITI LT	Low Output (per flow cell)	2 x 149 bp	500 M	500	4	6	10
AVITI/AVITI LT	Midium Output (per flow cell)	2 x 149 bp	1 B	1000	8	13	20
AVITI	High Output (per flow cell)	2 x 149 bp	2 B	2000	16	26	40

*When sequencing an RNA library alone (without DNA), we recommend adding 20% pHiX.

Sequencing

- Always ensure that libraries have been quantified using QIAseq Library Quant Assay or a compatible method to enable equal library representation within the sequencing pool and exact pool concentrations for optimal flow cell loading and best sequencing performance.
- For complete instructions on how to denature sequencing libraries and set up a sequencing run, please refer to the system-specific Illumina documents.
- Depending on the adapter type used for library preparation, please refer to the respective QIAseq Library handbook for detailed information on QIAseq Combinatorial and Unique Dual-Index Adapters.
- Editable and ready to use sample sheets, including all sample indexes of QIAseq Multimodal DNA/RNA UDI are available for download at **www.qiagen.com**

- A description of run setup for Illumina instruments and definition files for QIAseq Multimodal DNA/RNA UDI to be used for Illumina Local Run Manager are available at **www.qiagen.com**
- Sequencing on the NextSeq, HiSeq X®, HiSeq® 3000/4000 systems follow a different dual-indexing workflow than other Illumina systems. If manually creating sample sheets on these instruments, enter the reverse complement of the i5 index adapter sequence. If using BaseSpace®, or Local Run Manager to plan the run, the software will automatically reverse complement index sequences when necessary.

Sequencing setup on Illumina instruments

The following guidelines outline the most important settings for Illumina instruments. More detailed instructions on how to configure a run and how to create a sample sheet can be found in the "Product Resources" at **www.qiagen.com**

- Read Type: **Paired End**
- Index Reads: **2**
- Enable Adapter Trimming
- Cycles:
  - Read 1 and Read 2: **149**
  - Index 1 and Index 2: **10**

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 CGP hybrid capture library yields	
a. Suboptimal hybrid capture input quality or amount	Make sure to use high-quality indexed whole genome libraries as input that have been quantified using a fluorometric method or capillary electrophoresis.
b. Hybridization time too short	Hybrid capture input amounts smaller than 1000 ng may result in decreased library yields when using hybridization times of 30 min or less. Increase hybrid capture input, hybridize for 1 h or longer.
c. Inappropriate amount of Streptavidin Binding Beads	Insufficient Exome library yield may be a result of deficient postcapture amplification. Streptavidin Binding beads may inhibit postcapture amplification. Ensure to use no more than 100 µL of Streptavidin Binding beads per hybridization capture reaction. Only amplify half of the final bead slurry per PCR.
d. Inappropriate hybridization or washing temperature	Hybridization capture performs optimally at a very narrow temperature range. Ensure your thermal cycler and heating blocks are calibrated to accurately reach the intended temperature for the hybridization reaction and washing steps.
Decreased target region specificity or uniformity of CGP DNA libraries	
a. Incorrect use of Blocking Oligos or Blocking Solution	Only use QIAseq One-4-All Blocking Oligos and One-4-All Blocking Solution as outlined in the protocol.

		Comments and suggestions
b.	Wrong library adapter type used	One-4-All Blockers delivered with the kit are designed for Illumina TruSeq compatible adapters, but not for other adapter types. N-Blockers are designed for Illumina Nextera compatible adapters.
c.	Suboptimal hybridization and washing temperature	Check thermal cycler programs and make sure thermal cyclers and heating blocks are properly calibrated. For some heating blocks, it may be necessary to adjust the washing temperatures by 1°C or 2°C to match the optimal washing conditions.
d.	Suboptimal hybridization and washing conditions	Follow the protocol for hybridization and washing steps. Take care not to confuse the usage order of Wash Buffers A and B. Ascertain that Fast Hybridization Solution is heated to 65°C before use and neither the Fast Hybridization Solution nor the Wash Buffers show precipitates when aliquoting.
Unequal read representation of libraries within a hybrid capture pool		
a.	Unequal hybrid capture input	Use equal amounts of indexed whole genome libraries to be pooled for a hybrid capture reaction. Quantify indexed whole genome libraries before hybrid capture by using a capillary electrophoresis method such as the QIAxcel Advanced System or the Agilent 2100 Bioanalyzer using High Sensitivity DNA chips.
b.	Suboptimal library enrichment conditions	Do not use PCR-free whole genome libraries. Always follow the library enrichment protocol outlined in the respective library preparation protocol using QIAGENs HiFi PCR Master Mix for library enrichment. Make sure input libraries are not overamplified. Quantify indexed whole genome libraries using the QIAGEN Library Quant Assay and assess library quality via capillary electrophoresis.

Ordering Information

Product	Contents	Cat. no.
QIAseq xHYB CGP DNA Panel (24)	xHYB probe panel for DNA target enrichment for comprehensive genomic profiling, fixed panel for 24 samples	333122
QIAseq xHYB CGP DNA Panel (96)	xHYB probe panel for DNA target enrichment for comprehensive genomic profiling, fixed panel for 96 samples	333125
QIAseq xHYB CGP RNA Panel (24)	xHYB probe panel for RNA target enrichment for comprehensive genomic profiling, fixed panel for 24 samples	334122
QIAseq xHYB CGP RNA Panel (96)	xHYB probe panel for RNA target enrichment for comprehensive genomic profiling, fixed panel for 96 samples	334125
QIAseq xHYB CGP DNA/RNA Panels (24)	xHYB probe panels for DNA and RNA target enrichments for comprehensive genomic profiling, fixed panel for 24 samples	335122
QIAseq xHYB CGP DNA/RNA Panels (96)	xHYB probe panels for DNA and RNA target enrichments for comprehensive genomic profiling, fixed panel for 96 samples	335125
QIAseq xHYB Reagent Kit (24)	Reagents required for hybrid capture-based target enrichment. The kit contains 3 hybrid capture reactions, which are typically sufficient for 24 samples.	333430
QIAseq xHYB Reagent Kit (96)	Reagents required for hybrid capture-based target enrichment. The kit contains 12 hybrid capture reactions, which are typically sufficient for 96 samples.	333195
QIAseq library kits for the generation of indexed whole genome and whole transcriptome libraries for use in hybridization capture		
QIAseq Multimodal DNA/RNA Lib Kit (24)	Reagents (except indexes) for multimodal (DNA and RNA) library preparation of 24 samples: 24 DNA and 24 RNA libraries	334842
QIAseq Multimodal DNA/RNA Lib Kit (96)	Reagents (except indexes) for multimodal (DNA and RNA) library preparation of 96 samples: 96 DNA and 96 RNA libraries	334845
QIAseq Multimodal DNA/RNA UDI (24)	DNA and RNA indexes for 24 samples for a total of 48 reactions	334852
QIAseq Multimodal DNA/RNA UDI (96)	DNA and RNA indexes for 96 samples for a total of 192 reactions	334855

Product	Contents	Cat. no.
QIAseq N Blocking Oligos (12)	Oligos designed to bind and block all Illumina Nextera-compatible NGS adapters for 12 reactions of hybridization capture	334871
QIAseq Library Quantification Kits for use with Illumina instruments		
QIAseq Library Quant Assay Kit	Laboratory-verified forward and reverse primers for 500 x 25 µL reactions (500 µL); DNA Standard (100 µL); Dilution Buffer (30 mL); (1.35 mL x 5) GeneRead® qPCR SYBR® Green Mastermix	333314

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 Service or your local distributor.

Document Revision History

Date	Description
04/2025	Initial release.

Limited License Agreement for QIAseq xHYB CGP DNA/RNA Panel

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