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April 2020

# QIAseq<sup>®</sup> Human Exome Handbook

For hybridization-capture–based enrichment of human exonic sequences from human whole genome NGS libraries for Illumina<sup>®</sup> instruments

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# Kit Contents

<b>QIAseq Human Exome Kit</b>	<b>(24)</b>	<b>(96)</b>
<b>Catalog no.</b>	<b>333937</b>	<b>333939</b>
<b>No. of reactions</b>	<b>3</b>	<b>12</b>
<b>Box 1 of 2</b>		
Human Exome Probe Set	12 µl	48 µl
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 x 20 µl
RNase-free Water	1.5 ml	1.5 ml
<b>Box 2 of 2</b>		
Streptavidin Binding Beads	300 µl	1.2 ml
Post Capture Binding Buffer	2 x 1.2 ml	9.6 ml
Wash Buffer A	2 x 675 µl	5.4 ml
Wash Buffer B	2 x 1.05 ml	8.4 ml

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## Shipping and Storage

The QIAseq Human Exome Kit consists of 2 boxes. Box 1 is shipped on dry ice. Box 2 is shipped on cool pack (4°C). Store Box 1 immediately upon receipt at –30 to –15°C in a constant-temperature freezer. Store Box 2 immediately upon receipt at 2–8°C. Under these conditions, the components are stable until the expiry date indicated on the label.

## Intended Use

QIAseq Human Exome Kits are intended for molecular biology applications. This product is 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 a convenient and compact PDF format at [www.qiagen.com/safety](http://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 Human Exome Kit is tested against predetermined specifications to ensure consistent product quality.

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# Introduction

As of today, more than 6000 genes have been identified to cause inheritable monogenic disease when harboring specific variations. Next-generation sequencing (NGS) of DNA is the primary tool for the detection of genetic variations, including single nucleotide polymorphisms (SNPs), copy number variation, and small insertions and deletions. Target-enrichment technologies like gene panels, which focus on specific disease groups with clear etiology, have been proven highly effective in detecting disease-causing mutations. However, inherited diseases with unknown genetic origin or ambiguous etiology require more comprehensive analyses to identify disease-causing mutations or to associate yet unrelated genes with a disease. Whole exome sequencing is the most comprehensive approach of targeted DNA sequencing that covers all exons of the human genome. Despite targeting the coding regions of approximately 20,000 human genes, the targeted region comprises only about 1–2% of the human genome. In comparison to whole genome sequencing, whole exome sequencing enables users to target only well-interpretable regions, which effectively helps to increase sequencing depth and sample throughput while minimizing sequencing cost.

## QIAseq Human Exome Kit

The QIAseq Human Exome Kit uses a hybridization capture-based target enrichment approach to specifically enrich exonic sequences of the human genome from indexed whole genome libraries. The flexible workflow allows simultaneous hybridization capture from up to 8 samples with as little as 200 ng input per library. Libraries can be generated from enzymatically fragmented genomic DNA, physically sheared genomic DNA, as well as from circulating cell-free DNA. Depending on the DNA input type, we recommend using the library preparation kits listed in Table 1.

**Table 1. Recommended library preparation kits to be used for hybridization capture**

QIAseq Library Kit	Application
QIAseq FX DNA Library Kits*	Enzymatic fragmentation of genomic DNA Enzymatic fragmentation of formalin-compromised DNA
QIAseq Ultralow Input Library Kits†	Physical shearing of genomic DNA Physical shearing of formalin-compromised DNA
QIAseq cfDNA Library Kits‡	cfDNA from plasma

\* Follow the dedicated QIAseq FX protocol in this handbook.

† Follow the protocol for Ultralow Input Library Kits on [www.qiagen.com/UltraLowInputLibraryKit](http://www.qiagen.com/UltraLowInputLibraryKit).

‡ Follow the protocol for cfDNA Library Kits on [www.qiagen.com/cfDNALibraryKits](http://www.qiagen.com/cfDNALibraryKits).

**Note:** See ordering information at the end of this handbook.

Each of the listed QIAseq Library Kits includes QIAseq Unique Dual-Index (UDI) or Combinatorial Dual-Index (CDI) Adapters that are fully compatible for use with the QIAseq Human Whole Exome hybridization capture workflow and allow multiplexing of up to 384 samples per sequencing run. Please refer to the respective library kit handbook for more information on UDI and CDI Adapters.

Up to 8 indexed whole genome libraries can be combined in one hybrid capture reaction. During QIAGEN's hybridization workflow, library fragments containing exonic sequences are specifically captured by biotinylated probes in as little as 30 min. After hybridization, probes and hybridized library fragments are immobilized on beads via Biotin-Streptavidin interaction. Nonspecific targets are effectively removed by a series of washing steps. Finally, specific targets are amplified using the Post Hybrid-Capture PCR Mix that allows even amplification of DNA regions with vastly different GC contents, which minimizes sequencing bias caused by PCR.

## QIAseq Human Exome Probe Set

Hybridization capture is a powerful tool to capture DNA targets by sequence-specific interaction between probes and their target molecules. The Human Exome Probe Set targets Consensus Coding Sequence (CCDS)-annotated protein-coding regions of the human exome based on the hg38 genome build. A set of 5'-biotinylated double-stranded 120 nt DNA probes

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was designed to capture both strands of about 192,000 target regions. Noncoding transcripts, long UTRs, and low confidence exons are excluded, which ensures high capture specificity and reduces the total target region size to 33 Mb to facilitate increased sample multiplexing or sequencing depth per sequencing run. Target region definition files in BED format are available for download at [www.qiagen.com](http://www.qiagen.com).

## QIAseq One-4-All Blocking Oligos

QIAseq One-4-All Blocking Oligos included in the QIAseq Human Exome Kit are designed to effectively bind and block all Illumina TruSeq®-compatible NGS adapters irrespective of the index sequence length. 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.

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# Equipment and Reagents to be Supplied by User

- Agencourt® AMPure® XP Beads (cat. no. A63880, A63881) 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 Axxygen 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)

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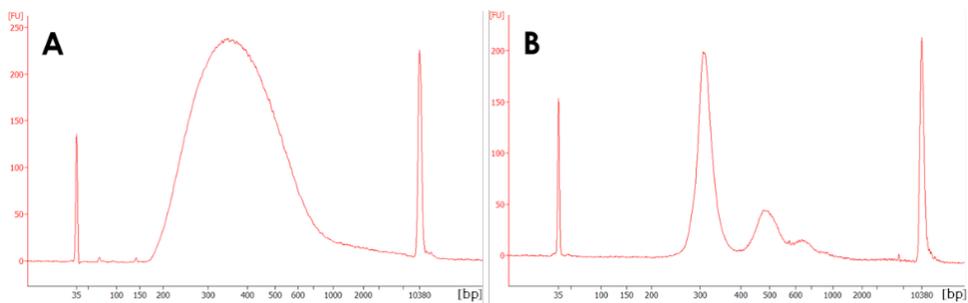
# Important Notes

## 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 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  $-15^{\circ}\text{C}$  and plan your workflow accordingly.

## Quality control of input libraries

- Control and quantify each indexed library using a capillary electrophoresis method such as the QIAxcel Advanced System or the Agilent 2100 Bioanalyzer using High Sensitivity DNA chips.
- A typical indexed whole genome library (QIAseq FX DNA Library Kit) has an average size distribution centered around 400 bp and is free of adapter dimers at a typical yield of  $>500$  ng (Figure 1A).
- A typical indexed cfDNA library (QIAseq cfDNA Library Kit) has 1 or 2 side peaks representing the N2 and N3 fraction at a typical yield of  $>200$  ng (Figure 1B). Libraries from physically sheared DNA (QIAseq Ultralow Input Kit) will reflect the size distribution of the sheared input DNA.



**Figure 1. Capillary electrophoresis device traces of different hybridization capture input libraries. A:** QIAseq FX DNA Library with a typical mean size distribution of 350–450 bp that is free of adapter dimers. **B:** A typical cfDNA library with the major library peak at 300 bp and additional peaks representing the N2 and N3 fractions. An example electrophoresis trace of the final postcapture library pool is shown at the end of the hybrid capture workflow protocol.

## Indexing recommendations

- Use QIAseq UDI Y-Adapters or QIAseq CDI Y-Adapters to generate whole genome libraries for hybridization capture.
- When using non-QIAseq Y-Adapters, make sure the adapters are Illumina TruSeq compatible and refer to the manufacturer’s instructions for adapter dilution and sample indexes.
- Use different sample indexes for all samples within a hybridization capture pool.
- Consider using different sample indexes also across hybridization capture pools to allow postcapture multiplexing depending on your sequencing instrument.

## Description of protocols

The QIAseq FX DNA Library Kit for Use with the QIAseq Human Exome Kit protocol (page 12) describes the generation of whole genome libraries using an optimized workflow for the QIAseq FX DNA Library Kit.

The QIAseq Human Exome Kit protocol (page 18) describes the use of the QIAseq Human Exome Kit and is suitable for input libraries generated using QIAseq FX DNA Library Kits, QIAseq Ultralow Input Library Kits, QIAseq cfDNA Library Kits, or alternative methods.

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# Protocol: QIAseq FX DNA Library Kit for Use with the QIAseq Human Exome Kit

This protocol describes the generation of indexed whole genome libraries using the QIAseq FX DNA Library Kit.

## Fragmentation, end repair, and A-addition

This protocol describes the FX reaction for single-tube fragmentation, end repair, and A-addition.

### Important points before starting

- Before setting up the reaction, it is critical to accurately determine the amount of input DNA.
- Ensure input DNA is in water, 10 mM Tris, QIAGEN's Buffer EB or low TE (0.1x TE, 0.1 mM EDTA). If input DNA is in 1x TE, please remove EDTA from the DNA by following the protocol in Appendix A, page 33.
- For the preparation of indexed whole genome libraries to be used for hybridization capture, we recommend using 50 ng of input DNA.

### Things to do before starting

- Prepare fresh 80% ethanol.
- Thaw reagents on ice. Once reagents are thawed, mix buffers thoroughly by quick vortexing to avoid any localized concentrations. Briefly spin down vortexed reagents before use.
- Program thermocyclers. For increased speed and convenience, all incubation steps of the protocol can be preprogrammed and saved in advance.

## Procedure

1. Program a thermocycler according to Table 2. Be sure to use the instrument's heated lid, and if possible, set the temperature of the heated lid to 70°C.

**Table 2. Fragmentation, end-repair, and A-addition cycling conditions.**

Step	Incubation temperature	Incubation time
1	4°C	1 min
2	32°C	22 min*
3	65°C	30 min
4	4°C	Hold

\* For 50 ng of input gDNA, a fragmentation time of 22 min typically results in an average library fragment size centered around 400 bp. To adjust the fragmentation to DNA, input amount, and quality, please refer to the *QIAseq FX DNA Library Handbook*, [www.qiagen.com/HB-2015](http://www.qiagen.com/HB-2015).

2. Start the program. When the thermocycler block reaches 4°C, pause the program.
3. Prepare the FX reaction mix in a PCR plate or tube on ice according to Table 3. We recommend using 50 ng of high quality input DNA. Mix well by gently pipetting (do not vortex to mix).

**Table 3. FX reaction mix setup (per sample)**

Component	Volume (µl)
FX buffer, 10x	5
Purified DNA (50 ng)*	Variable
Nuclease-free water	Variable
<b>Total without FX Enzyme Mix</b>	<b>40</b>

\* Input amounts of below 20 ng may result in reduced library complexity and are therefore not recommended.

4. Add 10 µl FX enzyme mix to each reaction and mix well by pipetting up and down 6–8 times. It is critical to keep the reactions on ice for the entire time during reaction setup.
5. Briefly spin down the PCR plate/tubes and immediately transfer to the prechilled thermocycler (4°C). Resume the cycling program.

6. When the thermocycler program is complete and the sample block has returned to 4°C, remove samples and place them on ice.
7. Immediately proceed with adapter ligation as described in the next protocol.

## Adapter ligation

This protocol describes adapter ligation. Make sure to use QIAseq UDI or CDI Y-Adapters, or other TruSeq-compatible adapters that are fully compatible with QIAGEN's One-4-All Blocking Oligos for hybridization capture. If adapters from another supplier are used, follow the manufacturer's instructions. QIAseq Universal Blockers are designed to work with TruSeq-compatible dual-indexed adapters with up to 12 bp index motives.

### Things to do before starting

- Equilibrate Agencourt AMPure XP beads to room temperature (15–25°C) for 20–30 min before use.
- Vortex and spin down the thawed adapter plate before use.

### Procedure

8. Remove the protective adapter plate lid, pierce the foil seal for each adapter well to be used and transfer 5 µl from one DNA adapter well to each 50 µl sample from the previous protocol. Track the barcodes from each adapter well used for each sample.
9. Replace the adapter plate lid and freeze unused adapters. The adapter plate is stable for a minimum of 10 freeze-thaw cycles.  
**Important:** Only 1 single adapter should be used per ligation reaction. Do not reuse adapter wells once the foil seal has been pierced.
10. Prepare the ligation Master Mix (per DNA sample) according to Table 4 in a separate PCR plate or tube on ice and mix well by pipetting.

**Table 4. Ligation master mix setup (per sample)**

Component	Volume (µl)
DNA Ligase Buffer, 5x	20
DNA ligase	10
Nuclease-free water	15
<b>Total</b>	<b>45</b>

11. Add 45 µl of the ligation Master Mix to each sample for a total of 100 µl and mix well by pipetting. Incubate the ligation reaction at 20°C for 15 min.  
**Important:** Do not use a thermocycler with a heated lid.
12. Proceed immediately to adapter ligation cleanup using 0.8x (80 µl) Agencourt AMPure XP beads.
13. Add 80 µl of resuspended Agencourt AMPure XP beads to each ligated sample and mix well by pipetting.
14. Incubate the mixture for 5 min at room temperature. Pellet the beads on a magnetic stand (e.g., DynaMag) for 2 min, then carefully discard the supernatant.
15. 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.
16. Incubate the beads 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.
17. Elute by resuspending in 52 µl Buffer EB or 10 mM Tris-Cl, pH 8.0. Pellet the beads and transfer 50 µl supernatant to a new tube.
18. Add 1.0x (50µl) of resuspended Agencourt AMPure XP beads and mix well by pipetting.
19. Perform steps 14–16, and then elute by resuspending in 26 µl Buffer EB or 10 mM Tris-Cl, pH 8.0.
20. Pellet the beads and carefully transfer 23.5 µl to a new PCR tube. If not proceeding immediately, the sample can be stored at –30 to –15°C.

## Amplification of library DNA

PCR-based library amplification is always required prior to hybridization capture. This protocol is for high-fidelity amplification of the DNA library using the amplification reagents provided in the QIAseq FX DNA Library Kit.

### Things to do before starting

- Thaw QIAseq HiFi PCR Master Mix and Primer Mix on ice. Once reagents are thawed, mix them thoroughly by quick vortexing to avoid any localized concentrations.
- Always start with the cycling conditions specified in this protocol. The cycling has been optimized for use with QIAseq HiFi PCR Master Mix for even and high-fidelity amplification of sequencing libraries.

### Procedure

21. Program a thermocycler with a heated lid according to Table 5.

**Table 5. Library amplification cycling conditions**

Time	Temperature	Number of cycles
2 min	98°C	1
20 s	98°C	6 (100 ng input DNA) 7 (50 ng input DNA) 8 (20 ng input DNA)
30 s	60°C	
30 s	72°C	
1 min	72°C	1
∞	4°C	Hold

**Note:** 6–8 amplification cycles are recommended based on the input DNA amount and quality.

22. Prepare a reaction mix on ice according to Table 6. Mix the components in a PCR tube or 96-well PCR plate.

**Table 6. Reaction mix for library enrichment**

Component	Volume (µl)
HiFi PCR Master Mix, 2x	25
Primer Mix Illumina Libr. Amp (10 µM each)	1.5
Library DNA	23.5
<b>Total reaction volume</b>	<b>50</b>

23. Transfer the PCR tube or plate to the thermocycler and start the program.
24. Once PCR is complete, add 50 µl of resuspended Agencourt AMPure XP Beads to each reaction (50 µl) and pipet up and down thoroughly to mix.
25. Incubate the mixture for 5 min at room temperature. Pellet the beads on a magnetic stand (e.g., DynaMag) and carefully discard the supernatant.
26. 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.
27. 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.
28. Elute by resuspending in 25 µl of Buffer EB or 10 mM Tris-Cl, pH 8.0. Pellet the beads on the magnetic stand. Carefully transfer 23 µl of the supernatant into a new tube.
29. Assess the quality and quantity of the library using a capillary electrophoresis device such as QIAGEN QIAxcel or Agilent Bioanalyzer. Check for the expected size distribution (see Figure 1A).  
**Note:** The library should show a distribution centered around approximately 350 bp.
30. The purified library can be safely stored at –30 to –15°C in a DNA LoBind tube until ready to use for sequencing or other applications.

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# Protocol: QIAseq Human Exome Kit

## 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

- Generate indexed whole genome libraries using the QIAseq FX DNA Library protocol on page 12 or any other compatible library preparation workflow (see guidelines in “Introduction” and Table 1).
- Quantify the amplified, indexed whole genome libraries (see guidelines in “Important Notes” and Figure 1).
- Thaw the Human Exome Probe Set, All-4-One Blocking Oligos, 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 3 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 7 or follow the recommendations in Table 8. Make sure to not exceed 3200 ng total input per capture pool.

**Table 7. 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 8. Recommended library pooling strategy for hybridization capture**

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

\* 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.

- Calculate the volume ( $\mu\text{l}$ ) of each amplified library needed for pooling, e.g., for a library concentration of 25 ng/ $\mu\text{l}$ , 16  $\mu\text{l}$  are needed to add 400 ng of indexed library to the capture pool.
- Transfer the calculated volumes for each library to a 1.5 ml LoBind tube. Use multiple tubes if performing multiple hybridization reactions at once.
- Add the Human Exome Probe Set and blocking reagents to each library pool according to Table 9.

**Table 9. Complement the library pool with probes and blocking reagents**

Component	Volume ( $\mu\text{l}$ )
Pool of indexed libraries (from step 3)	Variable
Human Exome Probe Set	4
One-4-All Blocking Oligos	8
One-4-All Blocking Solution	5

5. Pulse-vortex the complemented library pool to mix and pulse-spin the tube. Make sure there are minimal bubbles present.
6. Completely evaporate all liquid content of the complemented library pool by using a SpeedVac system (or a similar evaporator device). If needed, accelerate the evaporation of larger volumes by setting the temperature to 60°C.
7. When evaporation of the complemented library pool is complete, store the dried library pool on ice and proceed immediately to step 8 (Hybridization capture protocol).  
Alternatively, the dried library pool can be stored at -30 to -15°C for up to 3 days.

## Hybridization capture

During the hybridization step, QIAseq Exome 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

8. 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.

9. Program a thermocycler with a heated lid according to Table 10.

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

**Table 10. Conditions for hybridization capture**

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

\* Hybridization times of less than 30 min may result in reduced target region coverage.

† 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.

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

11. Vortex the 65°C Fast Hybridization Solution and immediately transfer 20 µl to the tube containing the dried complemented library pool from step 7.

**Important:** Fast Hybridization Solution is viscous. Pipet slowly to ensure accurate volume transfer.

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

13. Repeat step 12 for 2 more times, 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 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 to the thermocycler and continue the program at 95°C.

**Important:** Make sure the heated lid is set to 85°C, the tube is tightly sealed and the correct lid spacers for your thermocycler are being used.

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 Human Exome 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.
- Inspect the Post Capture Binding Buffer, Wash Buffer A, and Wash Buffer B for any precipitate that may have formed. Heat buffers to 48°C and mix thoroughly until all precipitate is dissolved.
- 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.

**Note:** Leave the wash buffers 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.

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22. Add 200  $\mu$ l Post Capture Binding Buffer and mix by pipetting.
  23. Incubate the beads on a magnetic stand for 1 min or until the solution is clear, 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  $\mu$ l 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, 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  $\mu$ l of preheated 70°C Wash Buffer A. Mix by pipetting.
31. Incubate for 5 min at 70°C.

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32. Place the tube on a magnetic stand for 1 min or until the solution is clear, then remove and discard the clear supernatant without disturbing the pellet.

Remove the tube from the magnetic stand, and add another 200  $\mu$ l 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, then remove and discard the clear supernatant without disturbing the pellet.

36. Remove the tube from the magnetic stand, and add 200  $\mu$ l of preheated 48°C Wash Buffer B. Mix by pipetting.

37. Pulse-spin the tube, 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, 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  $\mu$ l 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  $\mu$ l 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  $\mu$ l aliquots and store at –20°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  $-20^{\circ}\text{C}$ , thaw a 22.5  $\mu\text{l}$  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 11.

**Table 11. Postcapture amplification conditions**

Time	Temperature	Number of cycles
2 min	98°C	1
20 s	98°C	7 cycles*
30 s	60°C	
30 s	72°C	
1 min	72°C	1
$\infty$	4°C	Hold

\* Perform 8 cycles when using less than 1000 ng of total capture pool input.

44. Prepare the reaction mix on ice according to Table 12. Mix the components in a PCR tube or 96-well PCR plate.

**Table 12. Reaction mix for postcapture amplification**

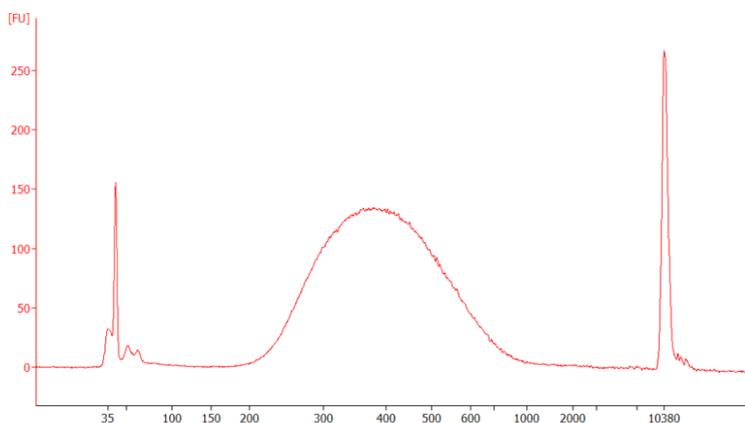
<b>Component</b>	<b>Volume (µl)</b>
Bead slurry with captured targets (from step 41)	22.5
Post Hybrid-Capture PCR Mix	25
Primer Mix Illumina Library Amplification	2.5
<b>Total reaction volume</b>	<b>50</b>

45. Transfer the PCR tube or plate to the thermocycler and start the program.
46. Once PCR is complete, add 75 µl of resuspended Agencourt AMPure XP 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  $-20^{\circ}\text{C}$  in a DNA LoBind tube until ready to use for sequencing.



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

# Sequencing Guidelines

## Pooling 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., 2 nM). To determine the total number of samples to be sequenced in a single sequencing run, consider specifications of the sequencing instrument, target region size (33 Mb) and the desired coverage per sample.

The QIAseq Human Exome Kit achieves a very high coverage uniformity throughout the target region with a typical Fold-80 base penalty in the range of 1.3–1.5. 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 the approximately 1.3- to 1.5-fold of the desired minimal coverage to reliably obtain that coverage throughout the target region.

To calculate the number of whole exome libraries per sequencing run, assume approximately 0.11 Gb of sequence output for every 1x of whole exome coverage.

### Example:

Instrument:	Illumina NextSeq® 550, High Output	
Specified output:	120 Gb	
Desired minimum coverage:	30x	
Fold-80-corrected coverage:	45x	[1.5 * 30x]
Maximum number of exomes:	24	[120 Gb/(0.11 * 45x)]

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## 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 CDI and UDI Y-Adapters are available for download at [QIAGEN.com](http://QIAGEN.com).
- A description of run setup for Illumina instruments and definition files for QIAseq CDI-Y and UDI-Y adapters to be used for Illumina Experiment Manager and Local Run Manager are available at [www.qiagen.com](http://www.qiagen.com).
- Sequencing on the NextSeq, HiSeq X<sup>®</sup>, HiSeq<sup>®</sup> 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 Illumina Experiment Manager, BaseSpace<sup>®</sup>, or Local Run Manager to plan the run, the software will automatically reverse complement index sequences when necessary.

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## 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” section for QIAseq Y-Adapters at [www.qiagen.com](http://www.qiagen.com).

- Read Type: **Paired End**
- Index Reads: **2**
- Enable Adapter Trimming
- Cycles for QIAseq CDI Y-Adapters
  - Read 1 and Read 2: **151**
  - Index 1 and Index 2: **8**
- Cycles for QIAseq UDI Y-Adapters
  - 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](http://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](http://www.qiagen.com)).

## Comments and suggestions

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### Low exome 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. Do not use PCR-free whole genome libraries. For input amounts smaller than 1000 ng, use 8 cycles of postcapture amplification.                                |
| 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 and use 8 cycles of postcapture amplification.  |
| 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 $\mu$ l 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.  |
| e) Incompatible adapters used                         | Using incompatible non-TruSeq-like adapters for the generation of whole genome libraries will result in failure to amplify captured library fragments. Only use QIAseq CDI or UDI Y-Adapters or other TruSeq-compatible adapters for the generation of indexed whole genome libraries.                                  |

### Decreased target region specificity

- |  |  |
|--|--|
| 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. |
|--|--|

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### Comments and suggestions

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- |    |  |  |
|----|--|--|
| 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. We recommend to only use QIAseq Unique or Combinatorial Dual-index Adapters included in QIAseq Library Kits.  |
| b) | Suboptimal hybridization and washing temperature | Check thermal cycler programs and make sure thermal cyclers and heating blocks are properly calibrated.  |
| c) | 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.<br><br>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. |

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## Appendix: Removal of Divalent Cations and EDTA from Input Nucleic Acid

Refer to manufacturer's protocol for details on methods of purification.

1. If DNA is in a volume of less than 50  $\mu$ l, adjust the volume to 50  $\mu$ l with nuclease-free water.
2. Add 90  $\mu$ l of resuspended Agencourt AMPure XP beads to the reaction for a ratio of 1.8x and mix well by pipetting. If DNA is in a volume greater than 50  $\mu$ l, scale the volume of Agencourt AMPure XP beads appropriately such that the ratio of beads to DNA is 1.8x.
3. Incubate the mixture for 5 min at room temperature. Pellet the beads on a magnetic stand for 2–4 min and carefully discard the supernatant without disturbing the beads.
4. Wash the beads with 200  $\mu$ l 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.
5. Incubate the beads on the magnetic stand for 5–10 min or until the beads are dry. Remove from the magnetic stand.
6. Elute by resuspending in 45  $\mu$ l of QIAGEN's Buffer EB or 10 mM Tris·Cl, pH 8.0. Pellet the beads on the magnetic stand for 2 min. Carefully transfer 42.5  $\mu$ l of supernatant into a new tube.
7. Determine the concentration of the purified DNA using Qubit<sup>®</sup>, PicoGreen<sup>®</sup>, or another fluorometric method.

# Ordering Information

Product	Contents	Cat. no.
QIAseq Human Exome Kit (24)	For 3 hybridization reactions: Buffers, blocking reagents, Human Exome Probe Set, Streptavidin beads, wash buffers, and postcapture enrichment reagents for hybrid capture of up to 24 whole genome libraries	333937
QIAseq Human Exome Kit (96)	For 12 hybridization reactions: Buffers, blocking reagents, Human Exome Probe Set, Streptavidin beads, wash buffers, and postcapture enrichment reagents for hybrid capture of up to 96 whole genome libraries	333939
<b>QIAseq library kits for the generation of indexed whole genome libraries for use in hybridization capture</b>		
QIAseq FX DNA Library Kits (96)	QIAseq FX DNA Library UDI-A Kit (96)	180479
	QIAseq FX DNA Library UDI-B Kit (96)	180480
	QIAseq FX DNA Library UDI-C Kit (96)	180481
	QIAseq FX DNA Library UDI-D Kit (96)	180482
	QIAseq FX DNA Library CDI Kit (96)	180484
	For 96 reactions: Buffers and reagents for DNA fragmentation, end repair, A-addition, ligation and library amplification; for use with Illumina instruments; includes a plate containing 96 adapters with different barcodes (pierceable foil seal allowing usage of defined parts of plate)	

<b>Product</b>	<b>Contents</b>	<b>Cat. no.</b>
QIAseq FX DNA Library Kits (24)	QIAseq FX DNA Library UDI Kit (24) QIAseq FX DNA Library CDI Kit (24) For 24 reactions: Buffers and reagents for DNA fragmentation, end repair, A-addition, ligation and library amplification; for use with Illumina instruments; includes a plate containing 24 adapters with different barcodes (pierceable foil seal allowing usage of defined parts of plate)	180477 180483
QIAseq cfDNA All-in-One Kits	QIAseq cfDNA All-in-One Kit (96) QIAseq cfDNA All-in-One Kit (24) Reagents and buffers for the isolation of cfDNA for NGS. Reagents for the generation of indexed whole genome libraries from cfDNA; for use with Illumina instruments; includes a plate containing 96 adapters with different barcodes (pierceable foil seal allowing usage of defined parts of plate)	180025 180023
QIAseq cfDNA Library Kit (96)	QIAseq cfDNA Library Kit (96) Reagents and buffers for the generation of indexed whole genome libraries from cfDNA; for use with Illumina instruments; includes a plate containing 96 adapters with different barcodes (pierceable foil seal allowing usage of defined parts of plate)	180015
<b>QIAseq Library Quantification Kits for use with Illumina instruments</b>		
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](http://www.qiagen.com) or can be requested from QIAGEN Technical Services or your local distributor.

# Document Revision History

Date	Changes
02/2020	Initial release
04/2020	Deleted instruction to "thaw the Human Exome Probe Set, All-4-One Blocking Oligos, and All-4-One Blocking Solution on ice, then pulse vortex and pulse-spin" in "Things to do before starting", page 20.

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## Notes

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## Notes

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