May 2023

QIAseq® Stranded mRNA Library Kit Handbook

For enrichment of polyadenylated RNA and stranded RNAseq libraries for next-generation sequencing using dual indexing

Sample to Insight

Contents

Kit Contents

QIAseq Stranded mRNA Library Kit Catalog no. Number of reactions		UDI (24) 180440 24	UDI-A(96) 180441 96	UDI-B(96) 180442 96	UDI-C(96) 180443 96	UDI-D(96) 180445 96
Component	**Tube cap color**			**Volume**		
QIAseq Beads						
QIAseq Beads	N/A	10 mL	40 mL	40 mL	40 mL	40 mL
QIAseq Stranded mRNA Enrichment Kit						
Pure mRNA Beads*	Violet	2 x 600 µL	5 x 600 µL	5 x 600 µL	5 x 600 µL	5 x 600 µL
Buffer mRBB (binding buffer)	Clear	2 x 8 mL	4 x 8 mL	4 x 8 mL	4 x 8 mL	4 x 8 mL
Buffer OW2 (wash buffer)	Clear	2 x 19 mL	7 x 19 mL	7 x 19 mL	7 x 19 mL	7 x 19 mL
RNase-Free Water	Clear	1 x 10 mL	3 x 10 mL	3 x 10 mL	3 x 10 mL	3 x 10 mL
Buffer OEB (elution buffer)	Clear	2 x 1.5 mL	5 x 1.5 mL	5 x 1.5 mL	5 x 1.5 mL	5 x 1.5 mL
Quick-Start Protocol	N/A	1	1	1	1	1
QIAseq Stranded RNA Lib Enzyme Kit						
RT Buffer, 5x	Blue	1 x 215 µL	1 x 860 µL	1 x 860 µL	1 x 860 µL	1 x 860 µL
RT Enzyme	Violet	1 x 28 µL	1 x 112 µL	1 x 112 µL	1 x 112 µL	1 x 112 µL
RNase Inhibitor	Green	1 x 55 µL	1 x 212 µL	1 x 212 µL	1 x 212 µL	1 x 212 µL
Second Strand Buffer, 10x	Blue	1 x 275 µL	1 x 1.1 mL	1 x 1.1 mL	1 x 1.1 mL	1 x 1.1 mL
Second Strand Enzyme Mix	Violet	1 x 175 µL	1 x 700 µL	1 x 700 µL	1 x 700 µL	1 x 700 µL
DTT, 1 M	Clear	1 x 1 mL	1 x 1 mL	1 x 1 mL	1 x 1 mL	1 x 1 mL
Ultralow Input Ligase	Orange	2 x 65 µL	1 x 500 µL	1 x 500 µL	1 x 500 µL	1 x 500 µL
Primer Mix, 10uM	Clear	1 x 150uL	1 x 150uL	1 x 150uL	1 x 150uL	1 x 150uL
HiFI PCR Master Mix, 2x	Green	1 x 1.25mL	2 x 1.25mL	2 x 1.25mL	2 x 1.25mL	2 x 1.25mL
Ultralow Input Ligation Buffer, 4x	Yellow	1 x 900 µL	3 x 900 µL	3 x 900 µL	3 x 900 µL	3 x 900 µL
CleanStart PCR Mix, 2x	Red	1 x 660 µL	2 x 1.4 mL	2 x 1.4 mL	2 x 1.4 mL	2 x 1.4 mL
CleanStart PCR Primer Mix	Clear	1 x 36 µL	1 x 150 µL	1 x 150 µL	1 x 150 µL	1 x 150 µL
Ligation Initiator	Black	1 x 210 µL	1 x 840 µL	1 x 840 µL	1 x 840 µL	1 x 840 µL
QIAseq UDI Adapter Kit						
QIAseq UDI Y-Adapter Plate (24)	N/A	1	N/A	N/A	N/A	N/A
QIAseq UDI Y-Adapter Kit A (96)	N/A	N/A	1	N/A	N/A	N/A
QIAseq UDI Y-Adapter Kit B (96)	N/A	N/A	N/A	1	N/A	N/A

QIAseq Stranded mRNA Library Kit Catalog no. Number of reactions		UDI (24) 180440 24	UDI-A(96) 180441 96	UDI-B(96) 180442 96	UDI-C(96) 180443 96	UDI-D(96) 180445 96
QIAseq UDI Y-Adapter Kit C (96)	N/A	N/A	N/A	N/A	1	N/A
QIAseq UDI Y-Adapter Kit D (96)	N/A	N/A	N/A	N/A	N/A	1
QIAseq Y-Adapter Reference Card	N/A	1	1	1	1	1
Quick-Start Protocol	N/A	3	3	3	3	3

* **Caution**: Pure mRNA Beads contain 0.1% sodium azide (NaN3) as a preservative. Dispose of azide-containing solutions according to your institution's waste-disposal guidelines. See "Safety Information", page 9.

QIAseq Stranded mRNA Select Kit Catalog no. Number of reactions		(24) 180773 24	(96) 180775 96
Component	**Tube cap color**		**Volume**
QIAseq Beads			
QIAseq Beads	Clear	10 mL	40 mL
QIAseq Stranded mRNA Enrichment Kit			
Pure mRNA Beads*	Violet	2 x 600 µL	5 x 600 µL
Buffer mRBB (binding buffer)	Clear	2 x 8 mL	4 x 8 mL
Buffer OW2 (wash buffer)	Clear	2 x 19 mL	7 x 19 mL
RNase-Free Water	Clear	1 x 10 mL	3 x 10 mL
Buffer OEB (elution buffer)	Clear	2 x 1.5 mL	5 x 1.5 mL
Quick-Start Protocol	N/A	1	1
QIAseq Stranded RNA Lib Enzyme Kit			
RT Buffer, 5x	Blue	1 x 215 µL	1 x 860 µL
RT Enzyme	Violet	1 x 28 µL	1 x 112 µL
RNase Inhibitor	Green	1 x 55 µL	1 x 212 µL
Second Strand Buffer, 10x	Blue	1 x 275 µL	1 x 1.1 mL
Second Strand Enzyme Mix	Violet	1 x 175 µL	1 x 700 µL
DTT, 1 M	Clear	1 x 1 mL	1 x 1 mL
Ultralow Input Ligase	Orange	2 x 65 µL	1 x 500 µL
Ultralow Input Ligation Buffer, 4x	Yellow	1 x 900 µL	3 x 900 µL
CleanStart PCR Mix, 2x	Red	1 x 660 µL	2 x 1.4 mL
CleanStart PCR Primer Mix	Clear	1 x 36 µL	1 x 150 µL
Ligation Initiator	Black	1 x 210 µL	1 x 840 µL
QIAseq CDI Y-Adapter Kit			
QIAseq CDI Y-Adapter Plate (24)	N/A	1	N/A
QIAseq CD I Y-Adapter Plate (96)	N/A	N/A	1
QIAseq Y-Adapter Reference Card	N/A	1	1
Quick-Start Protocol	N/A	3	3

* **Caution**: Pure mRNA Beads contain 0.1% sodium azide (NaN3) as a preservative. Dispose of azide-containing solutions according to your institution's waste-disposal guidelines. See "Safety Information", page 9.

The QIAseq Stranded Library Kits ship with a QIAseq Y-Adapter plate with either unique dual-index (UDI) adapters or combinatorial dual-index (CDI) adapters. To multiplex more than 96 libraries in a single sequencing run, simply combine the kits with different UDI Y-adapter plates.

For example, to multiplex 384 samples in a single flow cell line, prepare and combine libraries from the QIAseq Stranded mRNA Library Kit UDI-A (96) with the QIAseq Stranded mRNA Library Kit UDI-B (96), QIAseq Stranded mRNA Library Kit UDI-C (96), and QIAseq Stranded mRNA Library Kit UDI-D (96). For more information on QIAseq Y-Adapter plates, please refer to Appendix A: QIAseq Dual-Index Y-Adapters, page 41.

Shipping and Storage

The QIAseq Stranded mRNA Select Kit (cat. nos. 180773, 180775), QIAseq Stranded mRNA Library Kit UDI (24) (cat. no. 180440), QIAseq Stranded mRNA Library Kit UDI-A (96) (cat. no. 180441), QIAseq Stranded mRNA Library Kit UDI-B (96) (cat. no. 180442), QIAseq Stranded mRNA Library Kit UDI-C (96) (cat. no. 180443), and QIAseq Stranded mRNA Library Kit UDI-D (96) (cat. no. 180445) are shipped in 4 boxes.

- In the QIAseq Stranded mRNA Enrichment Kit (cat. nos. 1105688, 1105689), the Buffers mRBB and Pure mRNA Beads should be stored at 2–8°C (do not freeze). Buffer OW2 (wash buffer), RNase-Free Water, and Buffer OEB (elution buffer) should be stored at room temperature (15–25°C).
- Store the QIAseq Stranded RNA Lib Enzyme Kit (cat. nos. 1122418, 1122419) at –30 to –15°C.
- Store the QIAseq UDI Y-Adapter Kit (cat. nos. 180312, 180314, 180316, 180318, 180310) and QIAseq CDI Y-Adapter Kit (cat. nos. 180301, 180303) at –30 to –15°C.
- The QIAseq Beads (cat. nos. 1107149, 1107460) should be stored at 2–8°C (do not freeze).

  Important: Do not use expired beads as this will significantly reduce library yield.

  If stored under these conditions, the kit contents are stable until the date indicated on the box labels.

Intended Use

The QIAseq Stranded mRNA Library Kit is 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 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 Stranded mRNA Library Kit is tested against predetermined specifications to ensure consistent product quality.

Introduction

A typical mammalian cell contains 10–30 pg of total RNA. The majority of RNA within cells are rRNAs, with mRNA accounting for only 1–5% of the total cellular RNA. Approximately 360,000 mRNA molecules are present in a single mammalian cell, with approximately 12,000 different mRNA transcripts per cell. Some mRNAs comprise as much as 3% of the mRNA pool, whereas others account for less than 0.01%. These "rare" or "low-abundance" mRNAs may have a copy number of only 5–15 molecules per cell. However, these rare species may account for as many as 11,000 different mRNA species, comprising 45% of the mRNA population. For more information, see Alberts, B., et al. (1994) *Molecular Biology of the Cell*. 3rd ed. New York: Garland Publishing, Inc.

In whole transcriptome next-generation sequencing (NGS) applications, it is of great interest to maximize the amount of information generated from a single sequencing run. Enriching for mRNA from the total cellular RNA pool allows scientists to reduce the amount of rRNA and other non-mRNAs in their RNAseq data and increases the sequencing results from mRNA. The QIAseq Stranded mRNA Enrichment Kit includes all the necessary reagents and buffers for the isolation of poly(A)+ mRNA.

NGS library preparation of RNA samples

The QIAseq Stranded mRNA Library Kits enable one-day, accurate stranded NGS library construction from a broad range of RNA inputs. This kit includes magnetized QIAseq Beads for fast and efficient reaction cleanups between protocol steps and Y-shaped sample index adapter plates, which enable sample multiplexing. A total of 384 samples can be sequenced together per lane on an Illumina NGS instrument by combining different sets of UDI sample index plates.

Compared to other protocols, many novel advancements are included in the kit. During reverse transcription, the optimized RT enzyme and buffers do not require the usage of toxic reagents

such as Actinomycin D to enhance strand specificity. In the second-strand synthesis reaction, a specialized combination of enzymes and optimized buffering not only enables degradation of the RNA strand, generation of a second cDNA strand, and generation of blunt DNA ends, but it also guarantees the A-base addition required for the efficient ligation of Illumina-compatible adapters. The novel strand-specific ligation step establishes the strand specificity of the QIAseq Stranded RNA Kit protocol without additional reagents or laborious and time-consuming protocol steps. Finally, the HiFi PCR Mix utilizes a high-fidelity, hot-start, DNA polymerase to efficiently amplify the library irrespective of GC content

Globin mRNA removal using QIAseq FastSelect RNA Removal Kits

When working with whole blood samples, globin mRNA represents a substantial library contaminant. While mRNA enrichment effectively eliminates rRNA, globin mRNA remains a concern. QIAseq FastSelect RNA Removal Kit (sold separately) is a breakthrough technology that rapidly and efficiently removes up to 99% of globin mRNA during RNAseq library preparation. Simply add the QIAseq FastSelect reagent during the NGS library preparation, and unwanted RNAs are eliminated from the library.

Principle and procedure

The QIAseq Stranded mRNA Library Kit comprises the QIAseq Stranded mRNA Enrichment Kit, the QIAseq Stranded RNA Lib Enzyme Kit, QIAseq Beads, and QIAseq Y-Adapter Kits. The QIAseq FastSelect RNA Removal Kit (sold separately) is designed for fast and efficient removal of globin mRNA during library preparation of whole blood samples. Together, these kits enable preparation of mRNA-enriched, globin-mRNA–depleted, and strand-specific NGS libraries from total RNA in less than 5.5 hours (Figure 1)

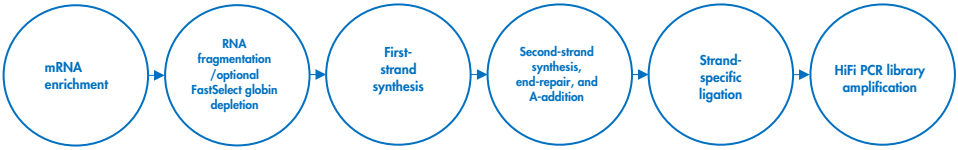

Figure 1.QIAseq Stranded mRNA Library Kit workflow. The QIAseq Stranded mRNA Library Kit provides all necessary reagents for mRNA enrichment and the preparation of strand-specific NGS libraries.
Optional: If you are working with total RNA from whole blood, the QIAseq FastSelect RNA Removal Kit provides all necessary reagents for the removal of globin mRNA. The HiFi PCR library amplification step utilizes a high-fidelity DNA polymerase to amplify the NGS libraries.

The following reactions are part of the workflow (Figure 2):

- **mRNA enrichment**
  Oligo-dT probes are covalently attached to the surface of the magnetically charged QIAGEN Pure mRNA Beads. The mRNA binds rapidly and efficiently to the oligo-dT probes in the presence of Buffer mRBB. The bound mRNA is then washed and eluted, providing a highly enriched pool of mRNA.
- **RNA fragmentation/FastSelect RNA removal**
  Prior to RNA heat fragmentation, the FastSelect reagent is directly combined with the enriched mRNA and the library prep-specific buffers. Heat fragmentation (if necessary) is then performed and the reaction temperature is gradually cooled to room temperature (15–25°C).
- **First-strand synthesis**
  First-strand synthesis is performed using an RNase H- Reverse Transcriptase (RT) in combination with random primers.
- **Second-strand synthesis, end-repair, and A-addition**
  Second-strand synthesis is performed using 5′ phosphorylated random primers. This enables subsequent strand-specific ligation, as only one strand of the library is 5′ phosphorylated.

- **Strand-specific ligation**
  QIAseq adapters are efficiently asymmetrically ligated to the inserts due to the 5′ phosphate that results from 5′ phosphorylated second-strand synthesis reaction.
- **Library amplification**
  QIAseq HiFi PCR reagents ensure high fidelity library amplification regardless of the GC content.

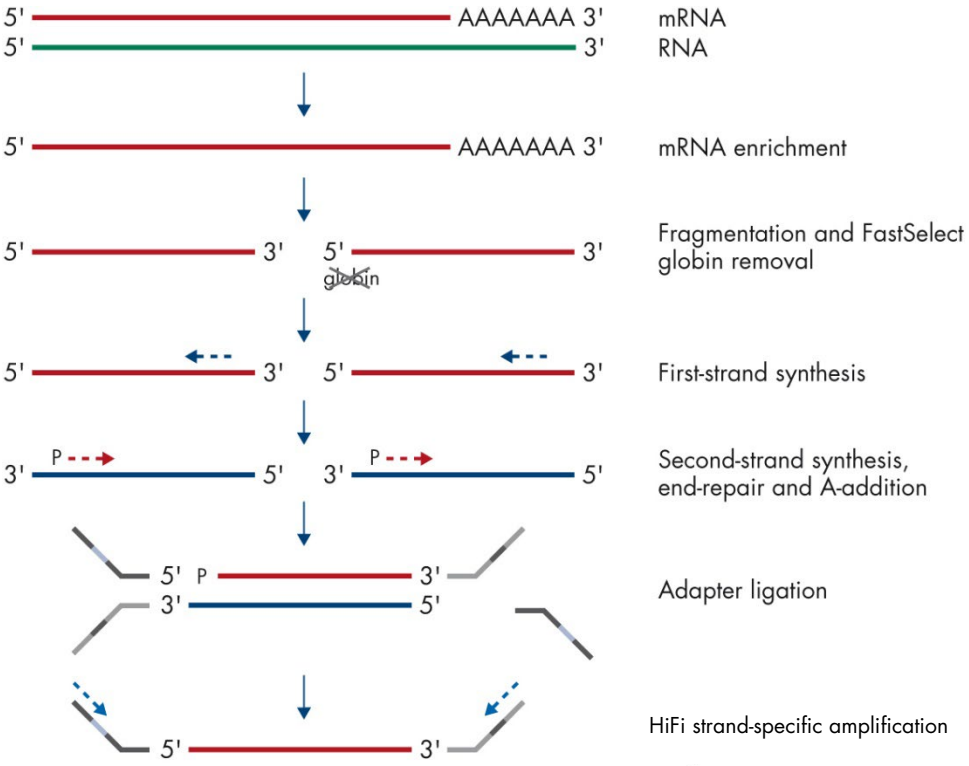

Figure 2. QIAseq Stranded mRNA Library Kit with integrated QIAseq FastSelect globin mRNA removal.

Library Amplification

Important: When preparing libraries for use on illumina NGS instruments, care must be taken to identify the type of flow cells which will be utilized. For sequencing using newer illumina instruments that utilize patterned flow cells, we recommend that the final library should be amplified using HiFi PCR Mastermix and following the Hi-Fi PCR Library amplification protocol.

The CleanStart Library amplification ensures that amplified library is not able to contaminate subsequent rounds of library preparation. We recommend using this protocol if labs are worried about PCR contamination in the results. Researchers should test if the CleanStart library amplification method is compatible with their sequencing instrument and flow cell configurations.

Next-generation sequencing

Libraries prepared with the QIAseq Stranded mRNA Library Kits can be sequenced with Illumina NGS systems (NextSeq® 500/550, HiSeq® 1000, HiSeq 1500, HiSeq 2000, HiSeq 2500, HiSeq 3000/4000, and NovaSeq™ 6000). When using unique dual indexes (cat. nos. 180440, 180441, 180442, 180443, 180445), 74 bp paired-end reads and dual 10 bp index reads are required. When using combinatorial indexes (cat. no. 180773 or 180775), 76 bp paired-end reads and dual 8 bp index reads are required.

Data analysis

The QIAseq Stranded RNA Library Kit is supported through two analysis pipelines: The RNA-seq Analysis & Biomarker Discovery Pipeline, a cloud-based RNAseq pipeline available in the QIAGEN GeneGlobe Analysis Portal: **https://geneglobe.qiagen.com/us/analyze/rnaseq-analysis-and-biomarker-discovery-portal**

Additionally, the QIAGEN CLC Genomics Workbench can be used. The pipelines automatically perform all steps necessary for primary mapping, sample cluster analysis, and differential expression for bulk sequencing applications.

When performing read alignment, the QIAseq Stranded Libraries represent the sense strand (or positive DNA strand) of the RNA sequence due to the strand-specific ligation during the second strand cDNA synthesis.

Equipment and Reagents to be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

- 100% ethanol (ACS grade)
- Nuclease-free pipette tips and tubes
- PCR tubes (0.2 mL individual tubes or tube strips) (VWR cat. no. 20170–012 or 93001–118) or plates
- 1.5 mL LoBind® tubes (Eppendorf cat. no. 022431021)
- Ice
- Microcentrifuge
- Thermal cycler
- Vortexer
- Magnet for bead cleanups:
  - Tubes: MagneSphere® Technology Magnetic Separation Stand (Promega cat. no. Z5342)
  - Plates: DynaMag™-96 Side Magnet (Thermo Fisher Scientific cat. no. 12331D)
- Optional spike-in: ERCC RNA Spike-In Mix (Thermo Fisher Scientific cat. no. 4456740)
- Library QC: 2100 Bioanalyzer® (Agilent cat. no. G2939BA), Agilent High Sensitivity DNA Kit (Agilent cat. no. 5067-4626)
- Preferred qPCR library quantification method: QIAseq Library Quant Array (cat. no. 333304) or QIAseq Library Quant Assay Kit (cat. no. 333314)

Important Notes

High-quality RNA is essential for robust library preparation and sequencing. QIAGEN provides a range of solutions for purification of total RNA (Table 1).

Table 1. Recommended kits for purification of total RNA

Kit	Cat. no.	Starting material
RNeasy® Micro Kit	74004	Small amounts of cells and tissue
RNeasy Mini Kit	74104, 74106	Animal/human tissues and cells
RNeasy 96 Kit	74181, 74182	Animal/human tissues and cells
RNeasy FFPE Kit	73504	FFPE tissue samples
QIAamp® ccfDNA/RNA Kit	55184	Animal and human plasma and serum
exoRNeasy Midi Kit	77144	Animal and human plasma and serum
exoRNeasy Maxi Kit	77164	Animal and human plasma and serum

- Ensure that total RNA samples are of high quality relative to their sample type. For additional information, see "Appendix D: General Remarks on Handling RNA".
  - **RNA quantification**: The concentration and purity of total RNA isolated from cells and fresh/frozen tissues should be determined by measuring the absorbance in a spectrophotometer, such as the QIAxpert®. Since the spectral properties of nucleic acids are highly dependent on pH, we recommend preparing dilutions and measuring absorbance in 10 mM Tris·Cl, pH 7.5, instead of RNase-Free Water. Pure RNA has an A_{260}:A_{280} ratio of 1.9–2.1 in 10 mM Tris·Cl, pH 7.5.
  - **RNA integrity**: The integrity and size distribution of total RNA from cells and fresh/frozen tissue can be confirmed using an automated analysis system (such as the QIAGEN QIAxcel or Agilent 2100 Bioanalyzer) that assesses RNA integrity by monitoring the ribosomal RNA bands. Although the RNA integrity number (RIN) should ideally be ≥8, successful library prep is still possible with samples with RIN values <8. In situations where the RNA is highly degraded, we recommend to consider using QIAseq FastSelect in combination with the QIAseq Stranded RNA Library Kit UDI instead of using an mRNA enrichment strategy, if possible.

- **Indexing recommendations**:

The QIAseq Stranded mRNA Library Kits include a QIAseq Y-Adapter plate with either CDI adapters or UDI adapters. We recommend using the QIAseq Y-Adapter plates delivered with the kit. Each QIAseq Stranded mRNA Library includes one of the following:

  - QIAseq Unique Dual-Index (UDI) Y-Adapter Plate (24)
  - QIAseq Unique Dual-Index (UDI) Y-Adapter Plate A, B, C, or D (96)
  - QIAseq Combinatorial Dual-Index (CDI) Y-Adapter Plate (24)
  - QIAseq Combinatorial Dual-Index (CDI) Y-Adapter Plate (96)

- Sample multiplexing is one of the most important NGS tools for increasing throughput and reducing costs. It works by combining multiple samples to be processed together in a single sequencing run; as a consequence, sequencing reads need to be demultiplexed by reassigning each single read to its original source library. This is facilitated by the integration of index sequences into the individual adapter molecules.
- CDI adapters use twelve i7 and eight i5 barcode motifs that can be combined to form up to 96 combinatory dual indices. In contrast, QIAseq UDI Adapters use a fixed combination of 2 unique barcode motifs per adapter molecule. Therefore, each single-index motif is only used once on any UDI adapter plate. To multiplex more than 96 libraries in a single sequencing run, combine kits with different UDI Y-adapter plates. Importantly, usage of UDI adapters effectively mitigates the risk of read misassignment due to index hopping. This is enabled by filtering misassigned reads during the demultiplexing of individual samples, thus generating highly accurate output data. For this reason, usage of UDI adapters is highly recommended. For more information on QIAseq Y-Adapter plates, please refer to “Appendix A: QIAseq Dual-Index Y-Adapters”, page 41.
- The protocol can be stopped at several steps and picked up on the following day. The stopping points are as follows:
  - End of “Protocol: mRNA Enrichment“
  - End of “Protocol: First-strand Synthesis”
  - End of “Protocol: Second-strand Synthesis, End-repair, and A-addition”
  - End of “Protocol: Strand-specific Ligation”

Protocol: mRNA Enrichment

Important points before starting

- This protocol is optimized for enriching RNA originating from all eukaryotic species with a poly-A tail.
- The recommended total RNA input is 100 ng – 1 µg.
- See "Appendix C: mRNA Enrichment in 200 µL Plates" for enrichment of mRNA using 200 µL strip tubes or 96-well plates.

Things to do before starting

- All buffers and reagents should be vortexed before use to ensure thorough mixing.
- Vortex the Pure mRNA Beads for 3 min before the first use or for 1 min before subsequent uses.
- Heat a water bath or heating block to 70°C, and heat Buffer OEB to 70°C.
- Unless otherwise indicated, all protocol steps, including centrifugation, should be performed at room temperature.

Procedure: mRNA enrichment in 1.5 mL tubes

1. Vortex Pure mRNA Beads for 1 min to thoroughly resuspend.
2. Prepare the enrichment reaction according to Table 2. Briefly centrifuge, vortex, and centrifuge briefly again.

Table 2. Setup of enrichment reaction

Component	Volume/reaction
Total RNA (100 ng – 1 µg)	Variable
RNase Inhibitor	1 µL
Buffer mRBB	250 µL
Thoroughly resuspended Pure mRNA Beads	25 µL
Nuclease-Free Water	Bring total reaction volume to 526 µL
Total volume	**526 µL**

3. Incubate for 3 min at 70°C, followed by 10 min at room temperature.
4. Briefly centrifuge, and then place the tubes onto a magnetic rack. After the solution has cleared (~2 min), discard the supernatant.
5. Add 400 µL of Buffer OW2. Vortex, centrifuge briefly, and place the tubes onto a magnetic rack. After the solution has cleared, discard the supernatant.
6. Repeat step 5.
7. Add 50 µL Buffer OEB. Vortex, centrifuge briefly, and incubate at 70°C for 3 min.
8. Remove the sample from 70°C and place at room temperature for 5 min.
9. Add 50 µL of Buffer mRBB and vortex. Briefly centrifuge, and incubate at room temperature for 10 min.
10. Briefly centrifuge, and then place the tubes onto a magnetic rack. After the solution has cleared, carefully discard the supernatant. Leave any residual liquid in the tube to minimize bead loss.
11. Add 400 µL of Buffer OW2. Vortex, centrifuge briefly, and place tubes onto a magnetic rack. After the solution has cleared, discard the supernatant.

12. Add 29 µL of Buffer OEB heated to 70°C to the bead pellet, and vortex.
13. Briefly centrifuge, and place the tubes onto a magnetic rack. After the solution has cleared, transfer 27 µL of the supernatant to a clean tube. The supernatant contains enriched poly(A)+ RNA.
14. Proceed to "Protocol: Fragmentation/FastSelect RNA Removal". Alternatively, the samples can be stored at –90 to –65°C.

Protocol: Fragmentation/FastSelect RNA Removal

Important points before starting

- The entire 27 µL product from "Protocol: mRNA Enrichment" is the starting material for fragmentation/FastSelect RNA removal.
- FastSelect removal of globin mRNA (sold separately, see "Ordering Information") is recommended when mRNA has been enriched from whole blood samples.
- To generate optimal insert sizes, fragmentation time needs to be determined for each experiment, depending on the quality and origin of the RNA. Follow the recommendations in Table 4.

Procedure

1. Thaw previously enriched mRNA on ice. Gently mix, then briefly centrifuge to collect residual liquid from the sides of the tubes, and return to ice.
2. Prepare the reagents required for the RNA fragmentation and QIAseq FastSelect globin mRNA removal.
   - 2a. Thaw RT Buffer, 5x, Nuclease-Free Water, and the tube(s) from the appropriate QIAseq FastSelect RNA Removal Kits at room temperature.
   - 2b. Mix by vortexing and then briefly centrifuge to collect residual liquid from the sides of the tubes.
3. On ice, prepare the fragmentation/RNA removal reaction according to Table 3. Briefly centrifuge, mix by pipetting up and down 10 times, and centrifuge briefly 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. Setup of fragmentation/RNA removal reactions

Component	Volume/reaction
mRNA enrichment reaction (already in tube)	27 µL
RT Buffer, 5x	8 µL
QIAseq FastSelect –Globin*	1 µL
ERCC Control†	Optional
Nuclease-Free Water	1 µL
Total volume	**37 µL**

* From QIAseq FastSelect RNA Removal Kit. If not performing, add 1 µL of Nuclease-Free Water instead.
† ERCC Control RNA (see "Equipment and Reagents to be Supplied by User") can be added according to the concentrations specified by the manufacturer. If added, replace the Nuclease-Free Water (1 µL) with ERCC.

4. Incubate as described in Table 4, according to your input RNA quality and approximate insert size.

Table 4. Fragmentation/RNA removal protocol

Input RNA quality	Step	Insert size ~ 150–250 bp	Insert size 251- 350 bp
High quality (RIN >9)	1*	15 min at 95°C	3 min at 95°C
Moderate quality (RIN 5–6)	1*	10 min at 95°C	3 min at 95°C
FFPE or degraded sample (RIN <3)	1*	No fragmentation†	No fragmentation†
Important: Only include steps 2–9 if performing FastSelect Globin mRNA Removal	2	2 min at 75°C	2 min at 75°C
	3	2 min at 70°C	2 min at 70°C
Steps 2–9 are performed when using FastSelect –Globin, regardless of Input RNA quality. They need to be performed whether the RNA is high quality, moderate quality, FFPE, or degraded.	4	2 min at 65°C	2 min at 65°C
	5	2 min at 60°C	2 min at 60°C
	6	2 min at 55°C	2 min at 55°C
	7	2 min at 37°C	2 min at 37°C
	8	2 min at 25°C	2 min at 25°C
	9	Hold at 4°C	Hold at 4°C

* Choose one option for the Step 1 time, according to the input RNA quality and desired insert size.
† Also suitable for exosomal RNA or RNA of other origin with a size between 80–500 bp.

5. Proceed immediately to "Protocol: First-strand Synthesis".

Protocol: First-strand Synthesis

Important points before starting

- The entire 37 µL product from "Protocol: Fragmentation/FastSelect RNA Removal" is the starting material for first-strand synthesis.
- Set up first-strand synthesis on ice.
- Do not vortex any first-strand synthesis reagents or reactions.
- Use a thermal cycler with a heated lid.
- Ensure that QIAseq Beads are brought to room temperature before using.
- 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 first-strand synthesis.

   1a. Thaw 1 M DTT at room temperature.

   1b. Mix by flicking the tube.

   1c. Centrifuge to collect residual liquid from the sides of the tube.

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

2. Dilute 1 M DTT to 0.4 M for use in Table 5.

3. On ice, prepare the first-strand reaction according to Table 5. Briefly centrifuge, mix by pipetting up and down 10 times, and centrifuge briefly 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. Setup of first-strand reaction

Component	Volume/reaction (µL)
Fragmentation/RNA removal reaction (already in tube)	37
Diluted DTT (0.4 M)	1
RT Enzyme	1
RNase Inhibitor	1
Total volume	**40**

4. Incubate as describe in Table 6.

Table 6. First-strand protocol

Step	Temperature (°C)	Incubation time
1	25	10 min
2	42	15 min
3	70	15 min
4	4	Hold

5. Add 56 µL of resuspended QIAseq Beads. Vortex for 3 seconds and briefly centrifuge.
6. Incubate for 5 min at room temperature.
7. Place the tubes/plate onto a magnetic rack. After the solution has cleared (~10 min or longer), carefully remove and discard the supernatant.

   Important: Do not discard the beads, because they contain the DNA of interest.

8. With the tubes still on the magnetic stand, add 200 µL of 80% ethanol. Rotate the tubes/plate 3 times to wash the beads. Carefully remove and discard the wash.
9. Repeat the ethanol wash.

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

10. With the tubes/plate (caps opened) still on the magnetic stand, air-dry at room temperature for 5–10 min.

    Note: Visually inspect that the pellet is completely dry and that all residual ethanol has evaporated.

11. Remove the tubes/plate from the magnetic stand. Eluate the DNA from the beads by adding 40 µL Nuclease-Free Water. Mix well by pipetting.
12. Return the tubes/plate to the magnetic rack until the solution has cleared.
13. Transfer 38.5 µL supernatant to clean tubes/plate.
14. Proceed to "Protocol: Second-strand Synthesis, End-repair, and A-addition". Alternatively, the samples can be stored at –30 to –15°C in a constant-temperature freezer.

Protocol: Second-strand Synthesis, End-repair, and A-addition

Important points before starting

- The entire 38.5 µL product from "Protocol: First-strand Synthesis" is the starting material for the second-strand synthesis, end-repair, and A-addition procedure.
- Set up reaction on ice.
- Do not vortex any second-strand synthesis, end-repair, and A-addition reagents or reactions.
- Use a thermal cycler with a heated lid.
- Ensure the QIAseq Beads are brought to room temperature before using.
- Ensure 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 required reagents.

   1a. Thaw Second Strand Buffer, 10x, at room temperature.

   1b. Mix by vortexing.

   1c. Centrifuge to collect residual liquid from the sides of the tubes.

   Note: Second Strand Enzyme Mix should be removed from the freezer just before use and placed on ice. After use, immediately return the enzyme to the freezer.

2. On ice, prepare the first-strand reaction according to Table 7. Briefly centrifuge, mix by pipetting up and down 10 times, and centrifuge briefly 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. Setup of second-strand synthesis, end repair, and A-addition reaction

Component	Volume/reaction (µL)
Product from "Protocol: First-strand Synthesis"	38.5
Second Strand Buffer, 10x	5
Second Strand Enzyme Mix	6.5
Total volume	**50**

3. Incubate as described in Table 8.

Table 8. Second-strand synthesis, end-repair, and A-addition protocol

Step	Temperature (°C)	Incubation time
1	25	30 min
2	65	15 min
3	4	Hold

4. Add 70 µL of resuspended QIAseq Beads. Vortex for 3 s and briefly centrifuge.
5. Incubate for 5 min at room temperature.
6. Place the tubes/plate onto a magnetic rack. After the solution has cleared (~10 min or longer), carefully remove and discard the supernatant.

   Important: Do not discard the beads, because they contain the DNA of interest.
7. With the tubes still on the magnetic stand, add 200 µL of 80% ethanol. Rotate the tubes/plate 3 times to wash the beads. Carefully remove and discard the wash.
8. Repeat the ethanol wash.

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

9. With the tubes/plate (caps opened) still on the magnetic stand, air-dry at room temperature for 5–10 min.

   Note: Visually inspect that the pellet is completely dry and that all ethanol has evaporated.

10. Remove the tubes/plate from the magnetic stand and elute the DNA from the beads by adding 52 µL Nuclease-Free Water. Mix well by pipetting.
11. Return the tubes/plate to the magnetic rack until the solution has cleared.
12. Transfer 50 µL supernatant to clean tubes/plate.
13. Proceed to "Protocol: Strand-specific Ligation". Alternatively, the samples can be stored at –30 to –15°C in a constant-temperature freezer.

Protocol: Strand-specific Ligation

Important points before starting

- The entire 50 µL product from "Protocol: Second-strand Synthesis, End-repair, and A-addition" is the starting material for the strand-specific ligation.
- Set up reaction on ice.
- Do not vortex any strand-specific ligation enzymes or reactions.
- Use a thermal cycler without a heated lid.
- For UDI adapter plates, the layout of the 24-plex and 96-plex plates is described in "Appendix A: QIAseq Dual-Index Y-Adapters". The index motifs used in the QIAseq Unique Dual-Index Kits are listed at **www.qiagen.com**, QIAseq UDI Y-Adapter Sequences.
- For CDI adapter plates, the layout and barcode sequences are described in Appendix A: QIAseq Dual-Index Y-Adapters.
- Unused, undiluted adapters can be stored at –30 to –15°C. If desired, residual diluted adapter can be removed and discarded before plate storage.
- Do not reuse diluted adapter due to the risk of barcode cross-contamination and lower-than-expected adapter concentration after storage of diluted material.
- Ensure the QIAseq Beads are brought to room temperature before using.
- Ensure 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 required reagents.
   1a. Thaw Ultralow Input Ligation Buffer, 4x, and Ligation Initiator at room temperature.
   1b. Mix by vortexing.
   1c. Centrifuge to collect residual liquid from the sides of the tubes.

Note: Ultralow Input Ligase should be removed from the freezer just before use and placed on ice. After use, immediately return the enzyme to the freezer.

2. Prepare the adapter plate as follows. The layouts of the 24-plex and 96-plex single-use adapter plates are displayed in Appendix A: QIAseq Dual-Index Y-Adapters.

   Note: If multiplexing 1–6 samples, consult the *Low-Plex Pooling Guidelines for Enrichment Protocols* document from Illumina to select the ideal adapter combinations.

   2a. Thaw the adapter plate at room temperature. Vortex and centrifuge briefly before use.

   2b. Remove the clear protective adapter plate lid and carefully pierce only the foil seal for each adapter well to be used, using a fresh tip to pierce each well.

   2c. Remove an aliquot and dilute the adapters as suggested in Table 9.

Table 9. Dilution of QIAseq adapters

Total RNA starting amount	Adapter dilution
100 ng	1:100
500 ng	1:25
1 µg	1:12.5
5 µg	1:5

   2d. Replace the plate lid and freeze unused, undiluted adapter at –30 to –15°C. Remove and discard residual diluted adapter before plate storage.

3. On ice, prepare the strand-specific ligation reaction according to Table 10. Briefly centrifuge. Mix by pipetting up and down 15–20 times and centrifuge briefly again.

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

   Important: Pipet slowly to mix. The Ligation Initiator and the reaction mix are very viscous.

Table 10. Setup of Strand-specific ligation reaction

Component	Volume/reaction (µL)
Product from "Protocol: Second-strand Synthesis, End-repair, and A-addition"	50
Diluted adapter*	2
Ultralow Input Ligation Buffer, 4x	25
Ultralow Input Ligase	5
Ligation Initiator	6.5
Nuclease-Free Water	11.5
Total volume	**100**

*Choose a unique adapter for each sample.

4. Incubate at 25°C for 10 min.
   Important: Do not use a heated lid.
5. Add 80 µL of resuspended QIAseq Beads. Vortex for 3 s and briefly centrifuge.
6. Incubate for 5 min at room temperature.
7. Place the tubes/plate onto a magnetic rack. After the solution has cleared (~10 min or longer), carefully remove and discard the supernatant.
   Important: Do not discard the beads, because they contain the DNA of interest.
8. With the tubes still on the magnetic stand, add 200 µL of 80% ethanol. Rotate the tubes/plate 3 times to wash the beads. Carefully remove and discard the wash.
9. Repeat the ethanol wash.
   Important: Completely remove all traces of the ethanol after this second wash. Briefly centrifuge and return the tubes to the magnetic stand. Remove the ethanol with a 200 µL pipette first, and then use a 10 µL pipette to remove any residual ethanol.
10. With the tubes/plate (caps opened) still on the magnetic stand, air-dry at room temperature for 5–10 min.
    Note: Visually inspect that the pellet is completely dry and that all ethanol has evaporated.

11. Remove the tubes from the magnetic stand, and elute the DNA from the beads by adding 92 µL Nuclease-Free Water. Mix well by pipetting.
12. Return the tubes/plate to the magnetic rack until the solution has cleared.
13. Transfer 90 µL supernatant to clean tubes/plate.
14. Add 108 µL of resuspended QIAseq Beads. Vortex for 3 s and briefly centrifuge.
15. Incubate for 5 min at room temperature.
16. Place the tubes/plate onto a magnetic rack. After the solution has cleared (~10 min or longer), carefully remove and discard the supernatant.

    Important: Do not discard the beads, because they contain the DNA of interest.
17. With the tubes still on the magnetic stand, add 200 µL of 80% ethanol. Rotate the tubes/plate 3 times to wash the beads. Carefully remove and discard the wash.
18. Repeat the ethanol wash.

    Important: Completely remove all traces of the ethanol after this second wash. Briefly centrifuge and return the tubes to the magnetic stand. Remove the ethanol with a 200 µL pipette first, and then use a 10 µL pipette to remove any residual ethanol.
19. With the tubes/plate (caps opened) still on the magnetic stand, air-dry at room temperature for 5–10 min.

    Note: Visually inspect that the pellet is completely dry and that all ethanol has evaporated.
20. Remove the tubes/plate from the magnetic stand, and elute the DNA from the beads by adding 25 µL Nuclease-Free Water. Mix well by pipetting.
21. Return the tubes/plate to the magnetic rack until the solution has cleared.
22. Transfer 23.5 µL of supernatant to clean tubes/plate.
23. Proceed to "Protocol: HiFi PCR Library Amplfication". Alternatively, the samples can be stored at –30 to –15°C in a constant-temperature freezer.

Protocol: HiFi PCR Library Amplfication

Important points before starting

- This protocol is compatible with all Illumina NGS instruments that use patterned and non-patterned flow cells.
- The entire 23.5 µL product from "Protocol: Strand-specific Ligation" is the starting material for the HiFi PCR Library Amplification.
- Set up the reaction on ice.
- Use a thermal cycler with a heated lid.
- Ensure the QIAseq Beads are brought to room temperature before using.
- Ensure 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 HiFi PCR library amplification.
   1a. Thaw Primer Mix, 10uM at room temperature and thaw HiFi PCR Master Mix, 2x, on ice.
   1b. Mix by flicking the tube.
   1c. Centrifuge to collect residual liquid from the sides of the tubes.

Table 11. Setup of library amplification

Component	Volume/reaction (µL)
Product from "Protocol: Strand-specific Ligation"	23.5
HiFi PCR MasterMix, 2x	25
Primer Mix 10uM	1.5
Total volume	**50**

2. On ice, prepare the library amplification reaction according to Table 11. Briefly centrifuge, mix by pipetting up and down 10 times, and centrifuge briefly again.

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

3. Select the number of PCR cycles, based on total RNA input, according to Table 12.

   Important: When removing globin mRNA with QIAseq FastSelect –Globin Kit, 2 additional cycles of library amplification need to be performed.

Table 12. Recommended number of PCR cycles, based on total RNA input

Total RNA input	Number of amplification cycles*
100 ng	14–16†
500 ng	11–13†
1 µg	9–11†
5 µg	7–9†

* Use selected number of cycles for amplification in Table 13.
† **Important**: When removing globin mRNA with QIAseq FastSelect -Globin RNA Removal, 2 additional cycles of library amplification need to be performed.

4. Incubate as described in Table 13.

Table 13. HiFi PCR library amplification cycling conditions

Step	Time	Temperature (°C)	Number of cycles
Initial denaturation	2 min	98	1
PCR	20 s 30 s 30 s	98 60 72	See Table 12
Final extension	1 min	72	1
Hold	∞	4	Hold

5. After amplification, add 60 µL of QIAseq Beads. Vortex for 3 s and briefly centrifuge.
6. Incubate for 5 min at room temperature.

7. Place the tubes/plate onto a magnetic rack. After the solution has cleared (~10 min or longer), carefully remove and discard the supernatant.

   Important: Do not discard the beads, because they contain the DNA of interest.

8. With the tubes/plate still on the magnetic stand, add 200 µL of 80% ethanol. Rotate the tube 3 times to wash the beads. Carefully remove and discard the wash.
9. Repeat the ethanol wash.

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

10. With the tubes/plate (caps opened) still on the magnetic stand, air-dry at room temperature for 5–10 min.

    Note: Visually inspect that the pellet is completely dry and that all ethanol has evaporated.

11. Remove the tubes/plate from the magnetic stand, and elute the DNA from the beads by adding 22 µL Nuclease-Free Water. Mix well by pipetting.
12. Return the tubes/plate to the magnetic rack until the solution has cleared.
13. Transfer 20 µL to clean tubes/plate. This is the QIAseq Stranded Sequencing Library.
14. Proceed to "Recommendations: Library QC, Quantification, and Sequencing". Alternatively, the samples can be stored at –30 to –15°C in a constant-temperature freezer.

Recommendations: Library QC, Quantification, and Sequencing

NGS library QC

QC can be performed with the Agilent Bioanalyzer or TapeStation. Check for the correct size distribution of library fragments (~300–500 bp median size) and for the absence of adapters or adapter-dimers (~130 bp). Figure 3 shows the library size distributions for 1 ng and 50 ng libraries prepared from UHRR input material. Both results show no traces of adapter-dimers. The 1 ng input RNAseq library shows optimal size distribution, while the 50 ng input RNAseq library shows broader library size distribution (but does not affect the NGS sequencing quality).

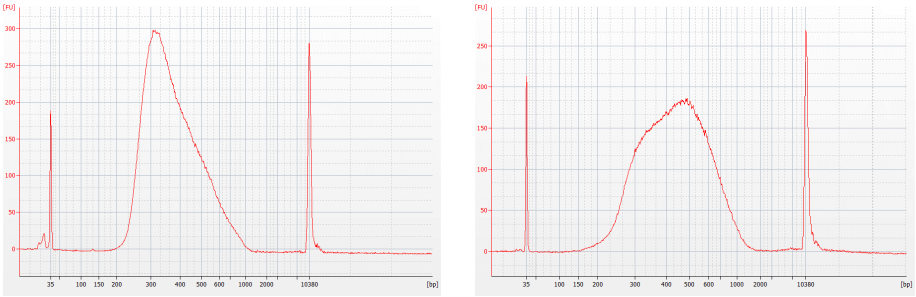

Figure 3. QIAseq Stranded RNAseq library size distributions measured with the Agilent Bioanalyzer High Sensitivity DNA Chip using standard protocol conditions with different input amounts. Left: 1 ng, right: 50 ng UHRR input material.

Important: If excessive adapter-dimers (~130 bp) are prominent after library QC (greater than 1–2% of total library yields), perform a second purification with QIAseq Beads. This can be accomplished by bringing the sample to a final volume of 55 µL and repeating steps 5–13 of the HIFi PCR library amplification.

Preferred library quantification method

The library yield measurements from the Bioanalyzer or TapeStation rely on fluorescence dyes that intercalate into DNA or RNA. These dyes cannot discriminate between cDNA with or without adapter sequences, so only complete QIAseq Stranded mRNA Select libraries with full adapter sequences will be sequenced. As a result, QIAGEN's QIAseq Library Quant Array Kit or Assay Kit, which contains laboratory-verified forward and reverse primers, together with a DNA standard, is highly recommended for accurate quantification of the prepared QIAseq Stranded mRNA Select library.

Sample dilution, pooling, sequencing, and data analysis

When the QIAseq Stranded libraries have been quantified with the QIAseq Library Quant Array or Assay Kit, typical QIAseq Stranded library yields are approximately 8–10 nM in 20 µL volume, depending on the quality of the input starting RNA used. This yield is sufficient for an NGS sequencing run. For sequencers other than the MiSeq and our NextSeq, please refer to Illumina specific documents. Dilute the individual QIAseq Stranded libraries to a concentration of 4 nM. Then, combine libraries with different sample indexes in equimolar amounts. The recommended starting concentration of the pooled QIAseq Stranded libraries to load onto a MiSeq® is 9 pM, while it is 1.6 pM on a NextSeq.

The recommended starting point for mRNA-enriched samples is 25 M reads/sample. When using UDIs, 74 bp paired-end reads and dual 10 bp index reads are required. When using CDIs, 76 bp paired-end reads and dual 8 bp index reads are required. Longer paired-end reads (UDI libraries: 149 bp paired-end reads and dual 10 bp index reads, and CDI libraries: 151 bp paired-end reads and dual 8 bp index reads) are recommended for rare/novel transcripts, splice site isoforms, and fusion gene detection.

Data analysis recommendations are outlined in "Appendix B: Data Analysis Recommendations".

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page in our Technical Support Center: **www.qiagen.com/FAQ/FAQList.aspx** The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information or protocols in this handbook (for contact information, visit **support.qiagen.com**).

	Comments and suggestions
Low library yields	
a) Suboptimal PCR cycle number	An increased number of PCR cycles in the HiFi or CleanStart PCR library amplification step can increase library yields.
b) Insufficient RNA input amount	Higher RNA input amounts can lead to higher library yields; however, more RNA input could lead to a higher adapter dilution (see "Appendix C: mRNA Enrichment in 200 µL Plates").
c) Not enough adapter molecules in ligation (only if no adapter-dimers are visible)	Decrease adapter dilution. An increased number of adapter molecules during ligation can increase ligation efficiency and library yields but can also increase adapter-dimer formation.
d) Low mRNA enrichment performance	Use higher-quality RNA or increase the amount of total RNA input for the mRNA enrichment procedure, to increase resulting mRNA material for the RNAseq library preparation.
High Bioanalyzer peak at 120–140 bp (adapter-dimers)	
a) Increase adapter dilution during ligation	Higher adapter dilutions decrease the adapter-dimer formation during the ligation step.
b) Decrease QIAseq Beads volume in cleanup after ligation	Lower QIAseq Bead volumes in cleanup step after ligation (e.g., 0.6x/1.1x instead of the standard volumes with 0.8x/1.2x) can increase adapter-dimer depletion but can lead to lower library yields.
c) Increase RNA input amount	Higher RNA input amounts can lead to higher library yields.
Bioanalyzer peaks at higher molecular weight (>1000 bp; PCR overamplification)	
Reduce PCR cycle number in the HIFi PCR or CleanStart PCR Library amplification step.	Single-stranded library products can self-anneal after too many PCR cycles when free PCR primers are no longer available. Reduced PCR cycle numbers are only necessary when the molarity of the high molecular peak is significantly elevated (>50% compared to library yields <700 bp).

Comments and suggestions	
Strong bias in transcript coverage plots after NGS data analysis	
a) Keep input RNA on ice as much as possible	Degraded RNA can lead to stronger transcript coverage bias after NGS data analysis. Degradation can be caused by RNase contamination or prolonged storage of RNA at elevated temperatures (>4°C).
b) Add additional RNase Inhibitor into reactions	In cases of strong RNAseq contaminants, add additional RNase Inhibitor into the reactions to inhibit enzymatic activity of QIAseq Stranded RNA library enzymes.
Very broad library size distributions	
Increase fragmentation time	If the library size distribution is very broad with library sizes >700 bp, increase RNA fragmentation time to 20 min, depending on the used RNA input material.

Appendix A: QIAseq Dual-Index Y-Adapters

Generation of sample sheets for Illumina instruments

Index sequences for QIAseq UDI and CDI Y-Adapters are available for download at **www.qiagen.com**

Sequencing on the NextSeq, HiSeq X™, or HiSeq 3000/4000 system follows a different dual-indexing workflow than other Illumina systems. If you are manually creating sample sheets for these instruments, enter the reverse complement of the i5 index adapter sequence. If you are using Illumina Experiment Manager, BaseSpace, or Local Run Manager for run planning, the software will automatically reverse complement index sequences when necessary.

Ready-to-use sample sheets containing all QIAseq CDI and UDI Y-Adapter barcode sequences are available for MiSeq, NextSeq, MiniSeq®, and HiSeq instruments. These can be conveniently downloaded from the product pages on **www.qiagen.com**

These can be imported and edited using the Illumina Experiment Manager Software, Illumina Local Run Manager, or any text editor. Make sure to download the appropriate sample sheet for NextSeq, HiSeq X, or HiSeq 3000/4000 systems depending on whether you are using Local Run Manager or manually configuring the sequencing run.

Unique Dual-Index Y-Adapters

The layout of the 24-plex and 96-plex (A/B/C/D) single-use UDI adapter plate is shown in Figure 4 and Figure 5. The index motifs used in the QIAseq Unique Dual-Index Kits are listed at **www.qiagen.com**

To make sequencing preparation more convenient, you can download Illumina-compatible sample sheets for different sequencing instruments at **www.qiagen.com**

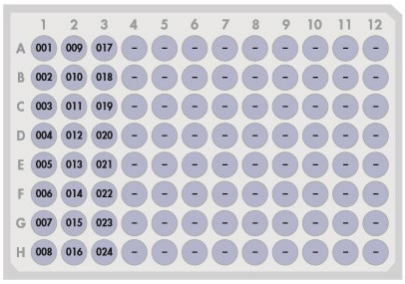

Figure 4. QIAseq UDI Y-Adapter Plate (24) layout (UDI 1–24).

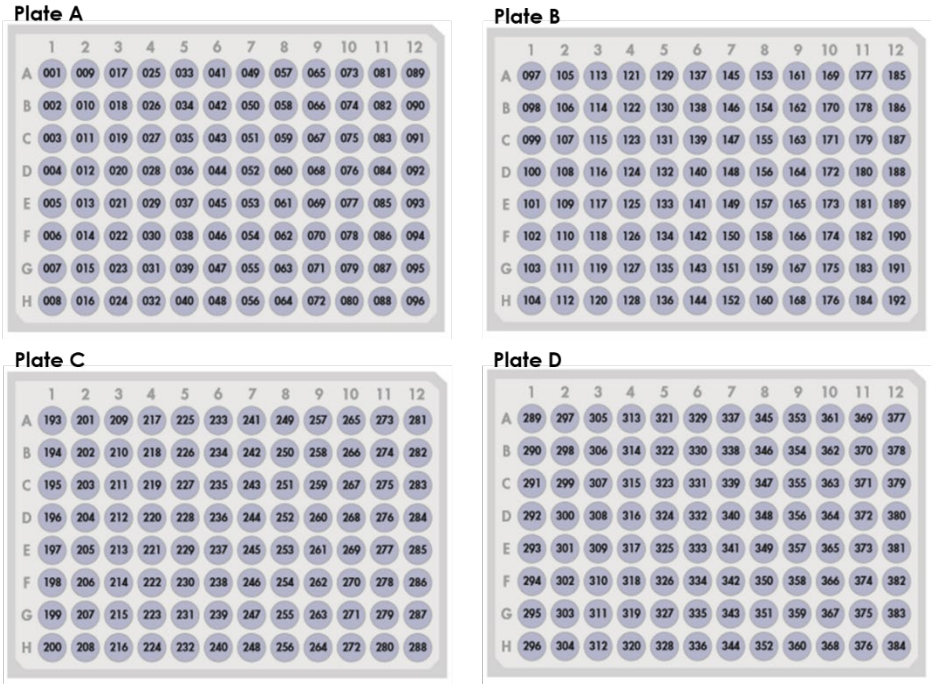

Figure 5. QIAseq UDI Y-Adapter Plates: Plate A (96) layout (UDI 1–96), Plate B (96) layout (UDI 97–192), Plate C (96) layout (UDI 193–288), and Plate D (96) layout (UDI 289–384).

Combinatorial Dual-Index Y-Adapters

The layout for the 96-plex and 24-plex single-use CDI adapter plates is shown in Figure 6 and Figure 7. The barcode sequences used in the QIAseq Combinatorial Dual-Index Kits are listed in Table 14. Indices 501-508 and 701-712 correspond to the respective Illumina adapter barcodes, Illumina TruSeq® CD indexes (formerly TruSeq HT). To make sequencing preparation more convenient, you can download Illumina-compatible sample sheets for different sequencing instruments from the product pages on **www.qiagen.com**

Follow *Low-Plex Pooling Guidelines for Enrichment Protocols* from Illumina to choose the correct combinations of D50x/D70x adapters for the corresponding instrument if loading between 1–6 samples onto one flow-cell lane.

Table 14. CDI adapter barcodes sequences used in the QIAseq CDI Y-Adapter Kits (24- and 96-plex Adapter Plates)

D50X barcode name	i5 barcode sequence*	D70X barcode name	i7 barcode sequence
D501	TATAGCCT	D701	ATTACTCG
D502	ATAGAGGC	D702	TCCGGAGA
D503	CCTATCCT	D703	CGCTCATT
D504	GGCTCTGA	D704	GAGATTCC
D505	AGGCGAAG	D705	ATTCAGAA
D506	TAATCTTA	D706	GAATTCGT
D507	CAGGACGT	D707	CTGAAGCT
D508	GTACTGAC	D708	TAATGCGC
		D709	CGGCTATG
		D710	TCCGCGAA
		D711	TCTCGCGC
		D712	AGCGATAG

* Sequencing on the MiniSeq, NextSeq, and HiSeq 3000/4000 systems follow a different dual-indexing workflow than other Illumina systems, which requires the reverse complement of the i5 index adapter sequence.

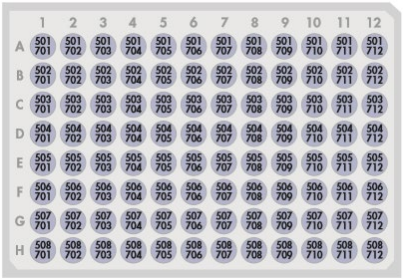

Figure 6. QIAseq CDI Y-Adapter Plate (96) layout (CDI 1–96).

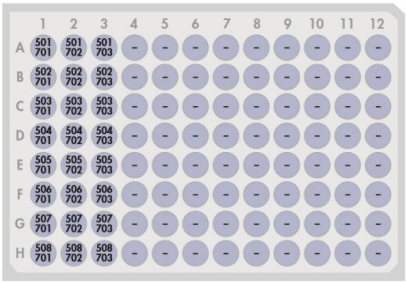

Figure 7. QIAseq CDI Y-Adapter Plate (24) layout (CDI 1–24).

Appendix B: Data Analysis Recommendations

RNAseq Analysis & Biomarker Discovery pipeline

RNA-seq Analysis & Biomarker Discovery pipeline Primary and secondary analysis tools are available at **geneglobe.qiagen.com**

The RNA-seq Analysis & Biomarker Discovery Pipeline uses QIAGEN CLC BiomedicalWorkbench for read alignment, and differential expression. QIAGEN Ingenuity® Pathway Analysis (IPA®) returns the top hits from the QIAGEN Knowledge Base for canonical pathways, upstream regulators, and diseases.

Using the RNA-seq Analysis & Biomarker Discovery Pipeline, FASTQ files can be uploaded, and RNA sequences aligned. Differential RNA expression will be calculated and visualized using interactive volcano plots. Differentially expressed RNAs will be queried against the QIAGEN Knowledge Base for canonical pathways, upstream regulators, and diseases and biological functions. Important RNAs can then be identified, and digital PCR and qPCR assays easily found for biological verification.

For each alignment, a credit is deducted from your account. Credits for using the RNA-seq Analysis & Biomarker Discovery Pipeline are included with QIAseq library kits. Credits can also be purchased for using the RNA-seq Analysis Portal with non-QIAGEN kits at **www.qiagen.com**

Procedure

1. Go to **https://geneglobe.qiagen.com/**
2. Click **Analyze Data.**
3. Under Start Analyzing Your Data:

3a. Select analysis type: Next-Generation Sequencing
3b. Select your analyte: mRNA/lncRNA
3c. Select your kit: QIAseq Stranded RNA Kit

4. Click **Start Your Analysis**

RNAseq alignment using CLC Genomics Workbench

Downstream NGS data can be analyzed with CLC Genomics Workbench (see **digitalinsights.qiagen.com**). When doing alignment, the QIAseq Stranded libraries represent the sense strand of the RNA sequence.

CLC Genomics Workbench is a comprehensive analysis package for the analysis and visualization of data from all major NGS platforms. The workbench supports and seamlessly integrates into a typical NGS workflow. CLC Genomics Workbench is available for Windows®, Mac OS® X, and Linux® platforms. Incorporating cutting-edge technology and algorithms, CLC Genomics Workbench supports key NGS features within genomics, transcriptomics, and epigenomics research fields. Additionally, it includes all the classical analysis tools of CLC Main Workbench.

Gene expression interpretation

Ingenuity® Pathway Analysis (IPA®) is an all-in-one, web-based software application that enables analysis, integration, and understanding of data from gene expression, miRNA, and SNP microarrays, as well as metabolomics, proteomics, and RNAseq experiments. This application is a great tool for interpreting the data you generate from the new QIAseq Stranded RNAseq kit. IPA is the market leader in gene expression analysis, having been cited in over 18,000 scientific publications to date.

You will find that IPA data analysis and search capabilities help you understand the significance of data, specific targets, or candidate biomarkers in the context of larger biological or chemical systems. The software is backed by the Ingenuity Knowledge Base of

highly structured, detail-rich biological and chemical findings. For information on IPA, visit **digitalinsights.qiagen.com**

Appendix C: mRNA Enrichment in 200 µL Plates

This protocol is used to enrich poly(A)+ RNA from total RNA using 200 µL strip tubes or 96-well plates.

Important points before starting

- The recommended total RNA input is 100 ng to 1 µg.

Things to do before starting

- All buffers and reagents should be vortexed before use to ensure thorough mixing.
- Vortex the Pure mRNA Beads for 3 min before the first use or for 1 min before subsequent uses.
- Heat a water bath or heating block to 70°C, and heat Buffer OEB to 7°C.
- Unless otherwise indicated, all protocol steps – including centrifugation – should be performed at room temperature.

Procedure

1. Vortex Pure mRNA Beads for 1 min to thoroughly resuspend.
2. Prepare the enrichment reaction according to Table 15. Briefly centrifuge, vortex, and centrifuge briefly again.

Table 15. Setup of enrichment reaction for 200 µL plates

Component	Volume/reaction
Total RNA (100 ng – 1 µg)	Variable
RNase Inhibitor	1 µL
Buffer mRBB	71 µL
Thoroughly resuspend Pure mRNA Beads	25 µL
Nuclease-Free Water	Bring total reaction volume to 150 µL
Total volume	**150 µL**

3. Incubate for 3 min at 70°C, followed by 10 min at room temperature.
4. Briefly centrifuge, and then place tubes onto a magnetic rack. After the solution has cleared (~2 min), discard the supernatant.
5. Add 150 µL Buffer OW2. Vortex, centrifuge briefly, and place the tubes onto a magnetic rack. After the solution has cleared, discard the supernatant.
6. Repeat step 5.
7. Add 50 µL Buffer OEB. Vortex, centrifuge briefly, and incubate at 70°C for 3 min.
8. Remove the sample from 70°C and place at room temperature for 5 min.
9. Add 50 µL of Buffer mRBB and vortex. Briefly centrifuge, and incubate at room temperature for 10 min.
10. Briefly centrifuge, and then place the tubes/plates onto a magnetic rack. After the solution has cleared, carefully discard the supernatant.
11. Add 150 µL of Buffer OW2. Vortex, centrifuge briefly, and place the tubes/plates onto a magnetic rack. After the solution has cleared, discard the supernatant.
12. Add 29 µL of Buffer OEB heated to 70°C to the bead pellet and vortex.
13. Briefly centrifuge, and place the tubes/plates onto a magnetic rack. After the solution has cleared, transfer 27 µL of the supernatant to a clean tube. The supernatant contains enriched, poly(A)+ RNA.
14. Proceed to "Protocol: Fragmentation/FastSelect RNA Removal" or store the samples at –90 to –65°C.

Appendix D: General Remarks on Handling RNA

Handling RNA

Ribonucleases (RNases) are very stable and active enzymes that generally do not require cofactors to function. Since RNases are difficult to inactivate and even minute amounts are sufficient to degrade RNA, do not use any plasticware or glassware without first eliminating possible RNase contamination. Care should be taken to avoid inadvertently introducing RNases into the RNA sample during or after the purification procedure. To create and maintain an RNase-free environment, the following precautions must be taken during the pretreatment and use of disposable and nondisposable vessels and solutions while working with RNA.

General handling

Proper microbiological, aseptic technique should always be used when working with RNA. Hands and dust particles may carry bacteria and molds and are the most common sources of RNase contamination. Always wear latex or vinyl gloves while handling reagents and RNA samples, to prevent RNase contamination from the surface of the skin or from dusty laboratory equipment. Change gloves frequently, and keep tubes closed whenever possible. Keep purified RNA on ice when aliquots are pipetted for downstream applications.

For removal of RNase contamination from bench surfaces, nondisposable plasticware, and laboratory equipment (e.g., pipettes and electrophoresis tanks), general laboratory reagents can be used. To decontaminate plasticware, rinse with 0.1 M NaOH, 1 mM EDTA* followed by RNase-Free Water, or rinse with chloroform* if the plasticware is chloroform-resistant. To decontaminate electrophoresis tanks, clean with detergent (e.g., 0.5% SDS),* rinse with RNase-Free Water, rinse with ethanol (if the tanks are ethanol-resistant), and allow to dry.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material data sheets (SDSs), available from the product supplier.

Disposable plasticware

The use of sterile, disposable polypropylene tubes is recommended throughout the procedure. These tubes are generally RNase-free and do not require pretreatment to inactivate RNases.

Glassware

Glassware should be treated before use to ensure that it is RNase-free. Glassware used for RNA work should be cleaned with a detergent*, thoroughly rinsed, and oven baked at 240°C for 4 hours or more (overnight, if more convenient) before use. Autoclaving alone will not fully inactivate many RNases. Alternatively, glassware can be treated with DEPC* (diethyl pyrocarbonate), as described in "Solutions" below.

Solutions

Solutions (water and other solutions)* should be treated with 0.1% DEPC. DEPC is a strong, but not absolute, inhibitor of RNases. It is commonly used at a concentration of 0.1% to inactivate RNases on glass or plasticware or to create RNase-free solutions and water. DEPC inactivates RNases by covalent modification. Add 0.1 mL DEPC to 100 mL of the solution to be treated and shake vigorously to bring the DEPC into solution. Let the solution incubate for 12 hours at 37°C. Autoclave for 15 minutes to remove any trace of DEPC. DEPC will react with primary amines and cannot be used directly to treat Tris* buffers. DEPC is highly unstable in the presence of Tris buffers and decomposes rapidly into ethanol and CO_2. When preparing Tris buffers, treat water with DEPC first, and then dissolve Tris to make the appropriate buffer. Trace amounts of DEPC will modify purine residues in RNA by carbethoxylation. Carbethoxylated RNA is translated with very low efficiency in cell-free systems. However, its ability to form DNA:RNA or RNA:RNA hybrids is not seriously affected unless a large fraction of the purine residues have been modified. Residual DEPC must always be eliminated from solutions or vessels by autoclaving or heating to 100°C for 15 minutes.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material data sheets (SDSs), available from the product supplier.

Appendix E: Protocol: CleanStart Library Amplification

Important points before starting

- The CleanStart library protocol should be used when amplified library contamination may be an issue.
- Amplified libraries using the CleanStart protocol may exhibit abnormal clustering on illumina patterned flowcells and should be tested for compatibility with the specific instrument, flowcell and data analysis software.
- The entire 23.5 µL product from "Protocol: Strand-specific Ligation" is the starting material for the CleanStart Library Amplification.
- QIAseq CleanStart PCR reagents use a proprietary PCR reaction, in conjunction with modification enzymes, to ensure that previously constructed NGS libraries are removed.

  Important: If a previously amplified CleanStart Library needs to be re-amplified - for instance, when an additional library is needed to replace a failed NGS run- omit the decontamination step of the PCR protocol (incubation for 15 min at 37°C) to disable selective degradation.
- Set up the reaction on ice.
- Do not vortex any CleanStart library amplification reagents or reactions.
- Use a thermal cycler with a heated lid.
- Ensure the QIAseq Beads are brought to room temperature before using.
- Ensure 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 CleanStart library amplification.
   1a. Thaw CleanStart PCR Primer Mix at room temperature and thaw CleanStart PCR Mix, 2x, on ice.
   1b. Mix by flicking the tube.
   1c. Centrifuge to collect residual liquid from the sides of the tubes.

2. On ice, prepare the library amplification reaction according to Table 16Table 13. Briefly centrifuge, mix by pipetting up and down 10 times, and centrifuge briefly again.

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

Table 16. Setup of library amplification

Component	Volume/reaction (µL)
Product from "Protocol: Strand-specific Ligation"	23.5
CleanStart PCR Mix, 2x	25
CleanStart PCR Primer Mix	1.5
Total volume	**50**

3. Select the number of PCR cycles, based on total RNA input, according to Table 17.

   Important: When removing globin with QIAseq FastSelect Globin RNA Removal Kit, 2 additional cycles of library amplification need to be performed.

Table 17. Recommended number of PCR cycles, based on total RNA input

Total RNA Input	Number of amplification cycles*
100 ng	14–16†
500 ng	11–13†
1 µg	9–11†
5 µg	7–9†

* Use selected number of cycles for amplification in Table 13.

† **Important**: When removing globin with QIAseq FastSelect Globin RNA Removal Kit, add 2 additional cycles of amplification.

4. Incubate as described in Table 18.

Table 18. CleanStart library amplification cycling conditions

Step	Time	Temperature (°C)	Number of cycles
CleanStart decontamination*	15 min	37	1
Initial denaturation	2 min	98	1
PCR	20 s 30 s 30 s	98 60 72	See Table 17
Final extension	1 min	72	1
Hold	∞	4	Hold

* For the reamplification of libraries, omit the CleanStart decontamination step. Start with incubation at step 2: 98°C for 2 min.

5. After amplification, add 60 µL QIAseq Beads. Vortex for 3 s and briefly centrifuge.
6. Incubate for 5 min at room temperature.
7. Place the tubes/plate onto a magnetic rack. After the solution has cleared (~10 min or longer), carefully remove and discard the supernatant.

   Important: Do not discard the beads, because they contain the DNA of interest.

8. With the tubes still on the magnetic stand, add 200 µL of 80% ethanol. Rotate the tubes/plate 3 times to wash the beads. Carefully remove and discard the wash.
9. Repeat the ethanol wash.

   Important: Completely remove all traces of the ethanol after this second wash. Briefly centrifuge and return the tubes to the magnetic stand. Remove the ethanol with a 200 µL pipette first, and then use a 10 µL pipette to remove any residual ethanol.
10. With the tubes/plate (caps opened) still on the magnetic stand, air-dry at room temperature for 5–10 min.

    Note: Visually inspect that the pellet is completely dry, and that all ethanol is evaporated.
11. Remove the tubes/plate from the magnetic stand and elute the DNA from the beads by adding 22 µL Nuclease-Free Water. Mix well by pipetting.
12. Return the tubes/plate to the magnetic rack until the solution has cleared.
13. Transfer 20 µL to clean tubes/plate. This is the QIAseq Stranded Sequencing Library.
14. Proceed to "Recommendations: Library QC, Quantification, and Sequencing". Alternatively, the samples can be stored at –30 to –15°C in a constant-temperature freezer.

Notes

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Ordering Information

Product	Contents	Cat. no.
QIAseq Stranded mRNA Library UDI Kits		
QIAseq Stranded mRNA Library Kit UDI-A (96)	For 96 stranded RNAseq sequencing library prep reactions: mRNA enrichment of total RNA, fragmentation, reverse transcription, second-strand synthesis + end-repair + A-addition, adapter ligation, CleanStart PCR enrichment and QIAseq Beads for library cleanups; for library cleanups for use with Illumina instruments; includes a plate containing 96 adapters with different barcodes (pierceable foil seal allowing usage of defined parts of the plate)	180441
QIAseq Stranded mRNA Library Kit UDI-B (96)	For 96 stranded RNAseq sequencing library prep reactions: mRNA enrichment of total RNA, fragmentation, reverse transcription, second-strand synthesis + end-repair + A-addition, adapter ligation, CleanStart PCR enrichment and QIAseq Beads for library cleanups; for library cleanups for use with Illumina instruments; includes a plate containing 96 adapters with different barcodes (pierceable foil seal allowing usage of defined parts of the plate)	180442
QIAseq Stranded mRNA Library Kit UDI-C (96)	For 96 stranded RNAseq sequencing library prep reactions: mRNA enrichment of total RNA, fragmentation, reverse transcription, second-strand synthesis + end-repair + A-addition, adapter ligation, CleanStart PCR enrichment and QIAseq Beads for library cleanups; for library cleanups for use with Illumina instruments; includes a plate containing 96 adapters with different barcodes (pierceable foil seal allowing usage of defined parts of the plate)	180443

Product	Contents	Cat. no.
QIAseq Stranded mRNA LibraryKit UDI-D (96)	For 96 stranded RNAseq sequencing library prep reactions: mRNA enrichment of total RNA, fragmentation, reverse transcription, second-strand synthesis + end-repair + A-addition, adapter ligation, CleanStart PCR enrichment and QIAseq Beads for library cleanups; for library cleanups for use with Illumina instruments; includes a plate containing 96 adapters with different barcodes (pierceable foil seal allowing usage of defined parts of the plate)	180445
QIAseq Stranded mRNA Library Kit UDI (24)	For 24 stranded RNAseq sequencing library prep reactions: mRNA enrichment of total RNA, fragmentation, reverse transcription, second-strand synthesis + end-repair + A-addition, adapter ligation, CleanStart PCR enrichment and QIAseq Beads for library cleanups; for library cleanups for use with Illumina instruments; includes a plate containing 96 adapters with different barcodes (pierceable foil seal allowing usage of defined parts of the plate)	180440
QIAseq FastSelect Kits		
QIAseq FastSelect – Globin Kit	Globin mRNA removal reagent: supports human, mouse, and rat; available in 24, 96, or 384 reactions	334376 334377 334378
QIAseq FastSelect – rRNA/Globin Kit	Cytoplasmic and mitochondrial rRNA removal reagent and globin mRNA removal reagent: supports human, mouse, and rat; available in 24, 96, or 384 reactions	335376 335377 335378
QIAseq Y-Adapter Kits for Illumina		
QIAseq UDI Y-Adapter Kit A (96)	Unique Dual-Index Adapters for Illumina (1–96)	180312
QIAseq UDI Y-Adapter Kit B (96)	Unique Dual-Index Adapters for Illumina (97–192)	180314

QIAseq UDI Y-Adapter Kit C (96)	Unique Dual-Index Adapters for Illumina (193–288)	180316
QIAseq UDI Y-Adapter Kit D (96)	Unique Dual-Index Adapters for Illumina (289–384)	180318
QIAseq UDI Y-Adapter Kit (24)	Unique Dual-Index Adapters for Illumina (1–24)	180310
QIAseq Library Quantification Kits for use with Illumina instruments		
QIAseq Library Quant Array Kit	Plate containing dried assay reagents for quantification of libraries prepared for Illumina; SYBR® Green Master Mix (1.35 mL x 2)	333304
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); SYBR Green Master Mix (1.35 mL x 5)	333314

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Document Revision History

Date	Changes
05/2020	Corrected the volume of QIAseq Beads for step 5 in “Protocol: CleanStart Library Amplification”.
02/2021	Changed the main title to “QIAseq Stranded mRNA Library Kit Handbook”. Incorporated the UDIs in this handbook. Added QIAseq Stranded mRNA Library Kit in the “Kit Contents” section. Revised the “Shipping and Storage”, “Introduction”, “Important Notes” sections. Added 100% ethanol (ACS grade) in the “Equipment and Reagents to Be Supplied by User” section. Updated tables and added new tables. Deleted Figure 3 and added new figures. Updated “Procedure” in “Protocol: First-strand Synthesis” and “Protocol: Second-strand Synthesis, End-repair, and A-addition”. Revised “Important points before starting” in “Protocol: Strand specific Ligation” and “Protocol: CleanStart Library Amplification”. Updated Appendix A and changed the title to “QIAseq Dual-Index Y Adapters”. Updated the “Ordering Information” section.
05/2023	Modified HB into new template and style guide. Updated preferred method of library amplification for Illumina patterned flowcells, warning on cleanstart for patterned flowcells; Change CleanStart Library Amplification and incorporated new. Moved Protocol: CleanStart Library Amplification to Appendix E.

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