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QIAseq[®] UPXome RNA Library Kit Handbook

Low-input RNA-seq library preparation for
complete transcriptome or 3' RNA-seq

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Kit Contents

The QIAseq UPXome RNA Library Kits ship in multiple boxes. It is very important to review the storage temperature of each box and promptly place refrigerated and frozen items into the appropriate storage areas.

Kit	Kit size	Catalog number	Kit contents			
			Component	Component size	Catalog number	Quantity
QIAseq UPXome RNA Lib Kit HMR	(24)	334702	QIAseq RNA Lib	(24)	334782	1
	(96)	334705	Enzymes & Buffers	(96)	334785	
			QIAseq Beads	(10 mL)	333923	1 or 2*
			QIAseq FastSelect -rRNA HMR Kit	(24) (96)	334386 334387	1
			QIAseq Advanced Analysis	(24) (96)	333782 333785	1
QIAseq UPXome Dual RNA-seq Lib Kit	(24)	334712	QIAseq RNA Lib	(24)	334782	1
	(96)	334715	Enzymes & Buffers	(96)	334785	
			QIAseq Beads	(10 mL)	333923	1 or 2*
			QIAseq FastSelect -rRNA HMR Kit	(24) (96)	334386 334387	1
			QIAseq FastSelect -5S/16S/23S Kit	(24) (96)	335925 335927	1
			QIAseq Advanced Analysis	(24) (96)	333782 333785	1

* 1 bottle is provided in the 24 sample kit; 2 bottles in the 96 sample kit.

Kit	Kit size	Catalog number	Kit contents			
			Component	Component size	Catalog number	Quantity
QIAseq UPXome RNA Lib Kit Bacteria	(24)	334762	QIAseq RNA Lib	(24)	334782	1
	(96)	334765	Enzymes & Buffers	(96)	334785	
			QIAseq Beads	(10 mL)	333923	1 or 2*
			QIAseq FastSelect – 5S/16S/23S Kit	(24) (96)	335925 335927	1
QIAseq UPXome RNA Lib Kit Plant	(24)	334722	QIAseq RNA Lib	(24)	334782	1
	(96)	334725	Enzymes & Buffers	(96)	334785	
			QIAseq Beads	(10 mL)	333923	1 or 2*
			QIAseq FastSelect –rRNA Plant	(24) (96)	334315 334317	1
			QIAseq Advanced Analysis	(24) (96)	333782 333785	1
QIAseq UPXome RNA Lib Kit Fish	(24)	334732	QIAseq RNA Lib	(24)	334782	1
	(96)	334735	Enzymes & Buffers	(96)	334785	
			QIAseq Beads	(10 mL)	333923	1 or 2*
			QIAseq FastSelect –rRNA Fish Kit	(24) (96)	333252 333255	1
			QIAseq Advanced Analysis	(24) (96)	333782 333785	1
QIAseq UPXome RNA Lib Kit Worm	(24)	334752	QIAseq RNA Lib	(24)	334782	1
	(96)	334755	Enzymes & Buffers	(96)	334785	
			QIAseq Beads	(10 mL)	333923	1 or 2*
		QIAseq FastSelect –rRNA Worm Kit	(24) (96)	333242 333245	1	

* 1 bottle is provided in the 24 sample kit; 2 bottles in the 96 sample kit.

Kit	Kit size	Catalog number	Kit contents			
			Component	Component size	Catalog number	Quantity
			QIAseq Advanced Analysis	(24) (96)	333782 333785	1
QIAseq UPXome RNA Lib Kit Yeast	(24)	334742	QIAseq RNA Lib	(24)	334782	1
	(96)	334745	Enzymes & Buffers	(96)	334785	
			QIAseq Beads	(10 ml)	333923	1 or 2*
			QIAseq FastSelect -rRNA Yeast	(24) (96)	334215 334217	1
			QIAseq Advanced Analysis	(24) (96)	333782 333785	1
QIAseq UPXome RNA Lib Kit Fly	(24)	334772	QIAseq RNA Lib	(24)	334782	1
	(96)	334775	Enzymes & Buffers	(96)	334785	
			QIAseq Beads	(10 ml)	333923	1 or 2*
			QIAseq FastSelect -rRNA Fly Kit	(24) (96)	333262 333265	1
			QIAseq Advanced Analysis	(24) (96)	333782 333785	1
QIAseq UPXome RNA Lib Kit HMR	(384)	334707	QIAseq RNA Lib	(96)	334785	4
			Enzymes & Buffers			
			QIAseq Beads	(55 ml)	333903	1
			QIAseq FastSelect -rRNA HMR Kit (96)	(96)	334387	4
			QIAseq Advanced Analysis	(96)	333785	4

* 1 bottle is provided in the 24 sample kit; 2 bottles in the 96 sample kit.

Kit	Kit size	Catalog number	Kit contents			
			Component	Component size	Catalog number	Quantity
QIAseq UPXome RNA Lib Kit HMR	(768)	334708	QIAseq RNA Lib Enzymes & Buffers	(96)	334785	8
			QIAseq Beads	(55 ml)	333903	2
			QIAseq FastSelect –rRNA HMR Kit	(384)	334388	2
			QIAseq Advanced Analysis	(96)	333785	8
QIAseq UPXome RNA Lib Kit	(24)	334692	QIAseq RNA Lib Enzymes & Buffers	(24)	334782	1
	(96)	334695		(96)	334785	
			QIAseq Beads	(10 ml)	333923	1 o r 2

QIAseq RNA Lib Enzymes & Buffers
Catalog no.
Number of reactions

(24)
334782
24

(96)
334785
96

US RT Buffer, 5x	150 µL	580 µL
DTT (100 mM)	20 µL	80 µL
dNTP Mix (10 mM)	55 µL	235 µL
N6-T RT Primer	30 µL	120 µL
ODT-T RT Primer	30 µL	120 µL
RNase Inhibitor	20 µL	96 µL
EZ Reverse Transcriptase	2 tubes x 36 µL	2 tubes x 150 µL
Nuclease-free Water	1 tube	2 tubes
QIAseq 2X HiFi MM	100 µL	400 µL
Optical Thin-wall 8-cap Strips (12/bag)	1 bag	2 bags
SIDT-24A	1 plate	–
SIDT-96A	–	1 plate

Kit QIAseq Beads			
Catalog no.	333923		333903
Volume of reagents	(10 mL)		(55 mL)
QIAseq Beads	1 bottle		1 bottle
Kit QIAseq Advanced Analysis			
Catalog no.	333782		333785
Number of analysis credits	(24)		(96)
Analysis credits for GeneGlobe RNA-seq Analysis Portal	24		96
QIAseq Advanced Analysis cards	1 card		1 card
QIAseq FastSelect –rRNA HMR Kit	(24)	(96)	(384)
Catalog no.	334386	334387	334388
Number of reactions	24	96	384
QIAseq FastSelect –rRNA HMR	3 x 12 µL	120 µL	4 x 120 µL
QIAseq FastSelect –5S/16S/23S Kit	(24)		(96)
Catalog no.	335925		335927
Number of reactions	24		96
FastSelect 5S/16S/23S	3 X 8 µL		96 µL
FastSelect FH Buffer	3 x 12 µL		144 µL
Nuclease-free water	1 tube		1 tube
QIAseq Beads	10 mL		10 mL
QIAseq Bead Binding Buffer	10.2 mL		10.2 mL
QIAseq FastSelect –rRNA Plant	(24)		(96)
Catalog no.	334315		334317
Number of reactions	24		96
QIAseq FastSelect –rRNA Plant	3 x 12 µL		120 µL
QIAseq FastSelect –rRNA Fish Kit	(24)		(96)
Catalog no.	333252		333255
Number of reactions	24		96
QIAseq FastSelect –rRNA Fish	3 x 12 µL		120 µL

QIAseq FastSelect –rRNA Worm Kit	(24)	(96)
Catalog no.	333242	333245
Number of reactions	24	96
QIAseq FastSelect –rRNA Worm	3 x 12 µL	120 µL
QIAseq FastSelect –rRNA Yeast	(24)	(96)
Catalog no.	334215	334217
Number of reactions	24	96
QIAseq FastSelect –rRNA Yeast	3 x 12 µL	120 µL
QIAseq FastSelect –rRNA Fly Kit	(24)	(96)
Catalog no.	333262	333265
Number of reactions	24	96
QIAseq FastSelect –rRNA Fly	3 x 12 µL	120 µL

QIAseq UX Index Kits IL UDI (sold separately)

Following cDNA pooling, QIAseq UX Index IL UDI Kits (see table below) are required for library amplification/indexing of RNA-seq libraries made with QIAseq UPXome RNA library Kits. Following the standard protocol, at least 8 cDNA samples and up to 24 cDNA samples may be pooled into a single library. For certain applications, more than 24 cDNA samples can be pooled into a single library.

The number of sequencing indexes required depends on the cDNA pooling strategy. For example, if 96 samples are used for an experiment with 8 samples per pool, then 12 indexes are necessary. If pooling 24 cDNA samples, only four sequencing indexes would be necessary for these 96 samples (24 cDNAs per sequencing index to make four libraries).

With the QIAseq UX 12 Index IL UDI Kit, a maximum of 288 samples can be sequenced together (corresponding to ultraplex pools of 24 cDNAs x 12 sequencing indexes). Each of the QIAseq UX 96 Index Kits facilitates sequencing of 2304 samples (corresponding to ultraplex pools of 24 cDNAs x 96 sequencing indexes).

QIAseq UX 12 Index Kit IL UDI	(12)
Catalog no.	331801
Number of Indexes	12

Index plate* name RUDI-12A

* Twelve wells of each hard plastic 96-well plate contains 9 μ L pre-mixed indexes. The plate has a pierceable foil seal and is sealed in a silver-foil bag. Each index is intended for single use.

QIAseq UX 96 Index Kit IL UDI	-A (96)	-B (96)	-C (96)	-D (96)	-E (96)	-F (96)	-G (96)	-H (96)
Catalog no.	331815	331825	331835	331845	331855	331865	331875	331885
Number of Indexes	96	96	96	96	96	96	96	96

Index plate name	RUDI-96AA	RUDI-96BA	RUDI-96CA	RUDI-96DA	RUDI-96EA	RUDI-96FA	RUDI-96GA	RUDI-96HA
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* Each hard plastic 96-well plate contains 9 μ L pre-mixed indexes. The plate has a pierceable foil seal and is sealed in a silver-foil bag. Each index is intended for single use.

QIAseq UX 96 Index Kit IL UDI	A-D (384)	E-H (384)	A-H (768)
Catalog no.	331817	331857	331818
Number of Indexes	384	384	768

Index plate* names	RUDI-96AA	RUDI-96EA	RUDI-96AA
	RUDI-96BA	RUDI-96FA	RUDI-96BA
	RUDI-96CA	RUDI-96GA	RUDI-96CA
	RUDI-96DA	RUDI-96HA	RUDI-96DA
			RUDI-96EA
			RUDI-96FA
			RUDI-96GA
			RUDI-96HA

* Each hard plastic 96-well plate contains 9 μ L pre-mixed indexes. The plate has a pierceable foil seal and is sealed in a silver-foil bag. Each index is intended for single use.

Shipping and Storage

As described in “Kit Contents” (starting on page 4), QIAseq UPXome RNA Library Kits are shipped in several boxes and may arrive on separate days or in different shipments. Even though two kits have the same number of components, they might be shipped in different number of boxes.

- QIAseq RNA Lib Enzymes & Buffers is shipped on dry ice. Upon receipt, all components should be stored immediately at -30°C to -15°C in a constant-temperature freezer.
- QIAseq FastSelect $-r\text{RNA}$ HMR, QIAseq FastSelect $-r\text{RNA}$ Plant, QIAseq FastSelect $-r\text{RNA}$ Fish, QIAseq FastSelect $-r\text{RNA}$ Worm, QIAseq FastSelect $-r\text{RNA}$ Yeast, and QIAseq FastSelect $-r\text{RNA}$ Fly Kits are shipped on dry ice and should be stored at -30°C to -15°C in a constant-temperature freezer.
- QIAseq FastSelect $-5\text{S}/16\text{S}/23\text{S}$ is shipped on blue ice. Upon receipt, the FastSelect $5\text{S}/16\text{S}/23\text{S}$ tube should be immediately stored at -30°C to -15°C in a constant-temperature freezer. All remaining components should immediately be stored in a refrigerator at $2-8^{\circ}\text{C}$.
- QIAseq Beads are shipped at 4°C and upon receipt should be stored at $2-8^{\circ}\text{C}$ in a refrigerator.

Warning: QIAseq Beads are damaged by freezing.

- QIAseq Advanced Analysis is a paper card that contains a unique code for data analysis using the GeneGlobe RNA-seq Analysis Portal. The card ships at any temperature and should be stored in a safe location at room temperature ($15-25^{\circ}\text{C}$).
- QIAseq UX index kits (sold separately) are shipped on dry ice and should be stored at -30°C to -15°C upon receipt.

When stored correctly, the QIAseq UPXome RNA Library Kit and QIAseq UX index kits can be used until the expiration date printed on the kit box lid. Under these conditions, the components are stable, without showing any reduction in performance and quality, until the date indicated on the label.

Intended Use

All QIAseq UPXome and UX products are intended for molecular biology applications. These products are not intended for the diagnosis, prevention, or treatment of a disease.

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 UPXome RNA Library Kits and QIAseq UX index kits are tested against predetermined specifications to ensure consistent product quality.

Introduction

The QIAseq UPXome RNA Library Kit enables one-day, high-throughput next-generation sequencing (NGS) using Illumina® NGS instruments. This RNA-library kit features ultraplex (UPX) pooling of cDNA and several different workflows, which enables either complete transcription or 3' RNA-seq, simply by varying the reverse transcription reaction and/or the RNA sample. In addition, the kits include QIAseq Beads for fast and efficient reaction cleanup between protocol steps. By using the QIAseq UX Index IL UDI Index Kits, up to 18,432 samples can be sequenced on a single flow cell.

The QIAseq UPXome RNA Library Kits and corresponding data analysis are intended for library construction and gene expression analysis of purified total RNA (500 pg – 100 ng) or enriched mRNA. The kit presents two innovative advantages compared to other protocols.

First, the inclusion of QIAseq FastSelect in the workflow enables rapid and efficient removal of ribosomal RNA during the preparation of the NGS RNA library. In one step, QIAseq FastSelect removes up to 99% of all unwanted rRNA – even when starting with difficult samples or instances where the RNA is already degraded, such as when using formalin-fixed paraffin embedded (FFPE) samples. This significantly increases the number of usable reads during sequencing.

Second, during reverse transcription, a unique sample ID is incorporated into each transcript. Following reverse transcription, cDNAs can be pooled, enabling all subsequent library construction steps to be performed in a reduced number of tubes, potentially even a single tube. This dramatically simplifies RNA-seq library preparation and allows much higher throughput than traditional NGS library preparation methods.

During sample indexing and final library amplification, up to 768 different unique dual indexes (UDIs) can be used, which allows the number of samples to be scaled up significantly for one experiment. When using the standard protocol, up to 24 samples can be pooled for library

amplification and indexing. When combined with the 768 UDIs, up to 18,432 samples can be sequenced together (24 ultraplexed samples x 768 unique dual sequence indexes).

The QIAseq UPXome RNA Library Kits are supported with online, cloud-based pipelines through QIAGEN's GeneGlobe RNA-seq Analysis Portal as well as with on-site software through QIAGEN CLC Genomics Workbench. QIAseq UPXome data analysis includes sample de-multiplexing, primary mapping, differential expression, and sample sequencing quality control.

The Sample to Insight® workflow of the QIAseq UPXome RNA Library Kit defines a new generation of high-throughput NGS technologies for gene expression analysis from eukaryotic cells and isolated RNA samples (Figure 1). The versatile RNA-library kit allows multiple types of RNA-seq libraries to be constructed when starting from total RNA or poly-A enriched RNA.

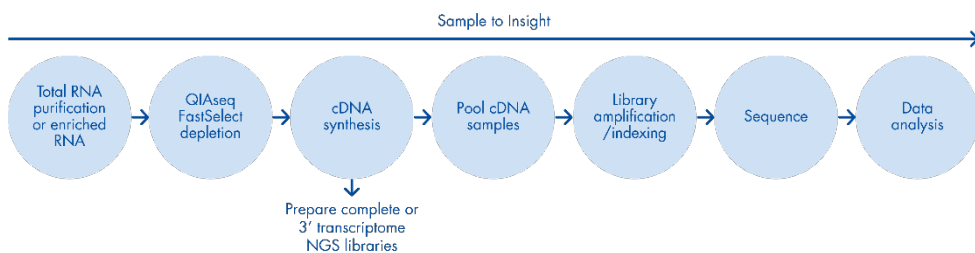


Figure 1. QIAGEN's Sample to Insight QIAseq UPXome RNA Library workflow.

Principle and procedure

The QIAseq UPXome RNA Library Kit workflow is described below (Figure 2 and Figure 3). There are two magnetic bead-based cleanup steps using QIAseq beads (included with the kit).

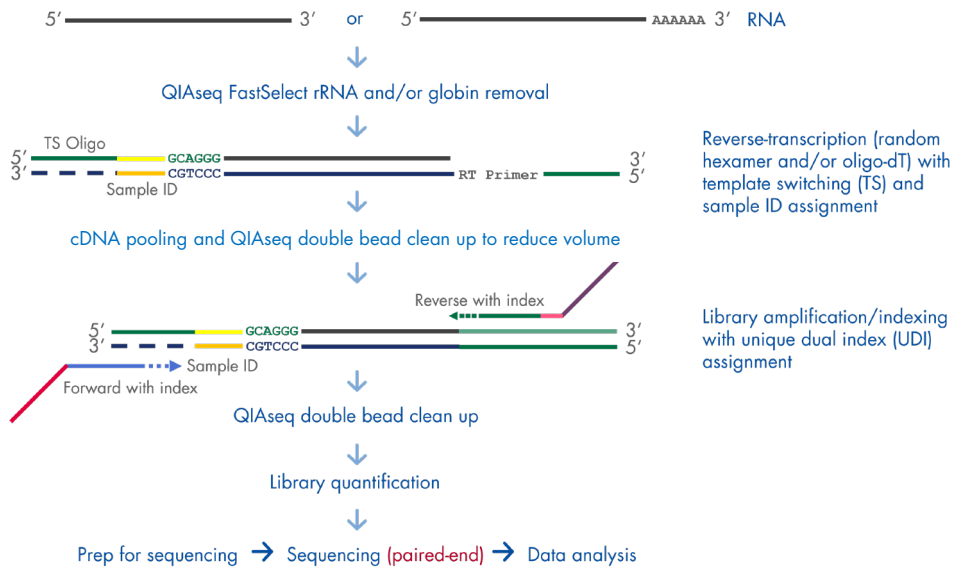


Figure 2. QIAseq UPXome RNA Library Kit workflow.

- Start with purified total RNA, or enriched mRNA: 500 pg – 100 ng of purified total RNA can be used for each sample; 10 ng of total RNA (or greater) is recommended.

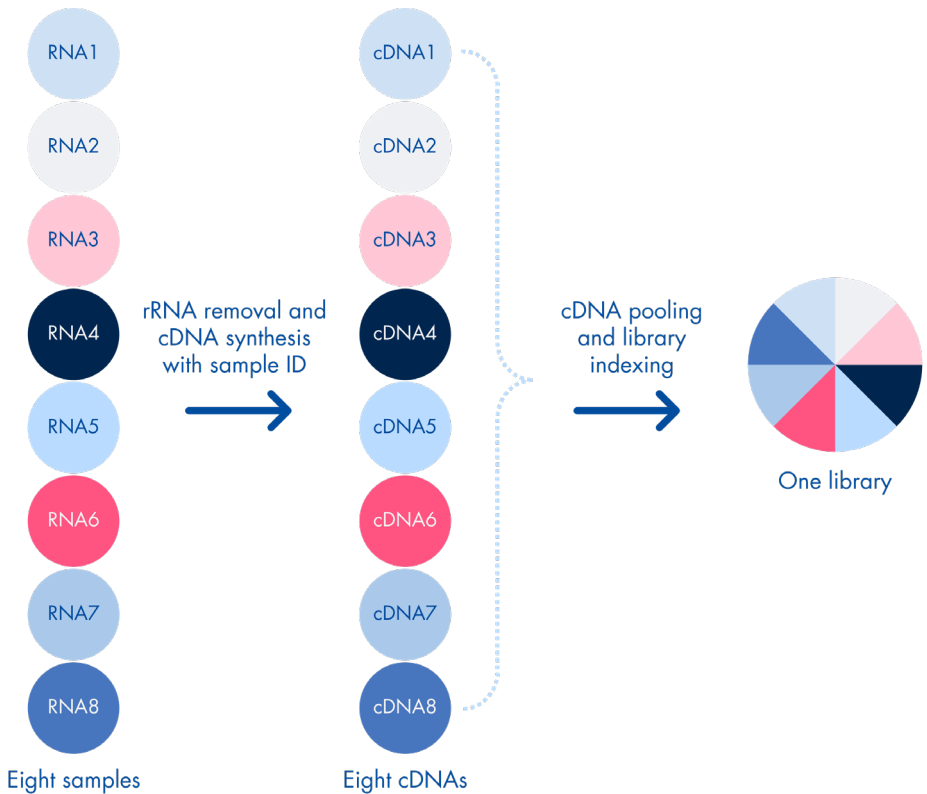


Figure 3. QIAseq UPXome RNA Library Kit workflow. Individual reverse transcription reactions are performed for each sample in the presence of a UPX barcoding oligo, which barcodes each cDNA. During a typical library preparation, up to 24 cDNAs are pooled together into a single library tube. The remaining reactions and library preparation steps associated with those 24 samples are performed in a single tube.

- **FastSelect rRNA depletion:** The FastSelect rRNA reagent is directly combined with the RNA and the US RT Buffer, 5x, enabling a rapid rRNA removal reaction. FastSelect prevents cDNA synthesis of rRNA. Specific heat fragmentation of the RNA is not necessary, as the reverse transcription chemistry is tuned to synthesize a specific range of cDNA sizes, regardless of starting sample quality.
- **cDNA synthesis:** cDNA synthesis can be performed using either the N6-T RT Primer (random hexamer) in combination with the ODT-T RT Primer (oligo-dT primer) for complete

transcriptome analysis or the ODT-T RT Primer exclusively for 3' transcriptome analysis.

The SID-TS-24S RT Plate (Table 1) or SID-TS-96S RT Plate (Table 2) contain 24 or 96 lyophilized template switching oligos, respectively, that facilitate 10 bp sample barcoding during cDNA synthesis; if not all wells are used in an experiment, unused wells can be covered using provided strip caps. At least 8 samples and no more than 24 samples must be pooled together.

Table 1. QIAseq sample Index (SID-TS-24s) RT Plate. Layout of SID-TS-24S plate for 24 samples.

	1	2	3	4	5	6	7	8	9	10	11	12
A	A01	A02	A03	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
B	B01	B02	B03	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
C	C01	C02	C03	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
D	D01	D02	D03	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
E	E01	E02	E03	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
F	F01	F02	F03	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
G	G01	G02	G03	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
H	H01	H02	H03	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty

Table 2. QIAseq Sample Index (SID-TS-96S) RT Plate. Layout of SID-TS-96S Plate for 96 samples.

	1	2	3	4	5	6	7	8	9	10	11	12
A	A01	A02	A03	A04	A05	A06	A07	A08	A09	A10	A11	A12
B	B01	B02	B03	B04	B05	B06	B07	B08	B09	B10	B11	B12
C	C01	C02	C03	C04	C05	C06	C07	C08	C09	C10	C11	C12
D	D01	D02	D03	D04	D05	D06	D07	D08	D09	D10	D11	D12
E	E01	E02	E03	E04	E05	E06	E07	E08	E09	E10	E11	E12
F	F01	F02	F03	F04	F05	F06	F07	F08	F09	F10	F11	F12
G	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
H	H01	H02	H03	H04	H05	H06	H07	H08	H09	H10	H11	H12

- **Library amplification/indexing:** Library amplification using QIAseq UX Index Kits introduces 10-base, UDIs into the library. Up to 768 UDIs are available.
- **NGS:** The QIAseq UPXome RNA Library Kit is compatible with Illumina NGS systems (MiniSeq®, MiSeq®, NextSeq® 500/550, NextSeq 2000, HiSeq® 1000, HiSeq 1500, HiSeq 2000, HiSeq 2500, HiSeq 3000/4000, NovaSeq® 6000, and other Illumina-based sequencing instruments that support paired-end sequencing. For standard expression analysis, 74 bp paired-end sequencing with dual 10 bp indexes should be used. For fusion analysis along with standard expression analysis, 149 bp paired-end sequencing with dual 10 bp indexes should be used. Recommendations for read allocation are found in Table 3; this should be used as a starting point, as read allocation is ultimately dependent on both the application and sample type.

Table 3. Read allocation recommendations per sample

Total RNA input per sample	Number of reads per sample	
	Complete Transcriptome	3' RNA-seq
500 pg RNA	1,000,000	250,000
1 ng RNA	5,000,000	2,000,000
10 ng RNA	20,000,000	5,000,000
100 ng RNA	50,000,000	10,000,000

- **Data analysis:** The QIAseq UPXome RNA Library Kit is supported through two analysis pipelines. The RNA-seq Analysis & Biomarker Discovery Pipeline, is a cloud-based RNA-seq pipeline available in the QIAGEN GeneGlobe Analysis 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.

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 SDSs available from the product supplier.

- Nuclease-free pipette tips and tubes
- Microcentrifuge tubes (2 mL)
- PCR tubes (0.2 mL individual tubes or tubes strips) (VWR cat. no. 20170-012 or 93001-118)
- Ice
- Microcentrifuge
- Thermal cycler
- Magnet for QIAseq Bead Cleanups – MagneSphere® Technology Magnetic Separation Stand (Promega cat. no. Z5342)
- 100% ethanol, ACS-grade

Library QC methods

- QIAxcel® Connect (QIAGEN cat. no. 9003110)
- 2100 Bioanalyzer® (Agilent cat. no. varies)
- Agilent® High Sensitivity DNA Kit (Agilent cat. no. 5067-4626)
- Agilent High Sensitivity D5000 ScreenTape (Agilent cat. no. 5067-5592)
- Library concentration readings:
 - Qubit® Fluorometer (Thermo Fisher Scientific cat. no. varies)
 - Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific cat. no. Q32854)
 - Qubit Assay Tubes (Thermo Fisher Scientific cat. no. Q32856)

Optional RNA spike-in

- ERCC ExFold RNA Spike-In Mixes (Thermo Fisher Scientific cat. no. 4456739)

Optional control total RNA samples for process optimization

- Human XpressRef Universal Total RNA (cat. no. 338112)
- Mouse XpressRef Universal Total RNA (cat. no. 338114)
- Rat XpressRef Universal Total RNA (cat. no. 338116)

Important Notes

- DNase treatment (on-column and in-solution) of total RNA samples is highly recommended.
- When starting with isolated RNA, 500 pg – 100 ng of purified total RNA can be used, with 10 ng total RNA (or greater) being the preferred starting amount. QIAGEN provides a range of solutions for purification of total RNA from different amounts of sample (Table 4).

Table 4. 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 and 74106	Animal and human tissues and cells
RNeasy 96 Kit	74181 and 74182	Animal and 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 RNA samples are of high quality and free of inhibitors that would compromise a reverse transcription or PCR. For more information about recommended laboratory procedures, please consult the handbook with your QIAGEN isolation kit.
- **RNA quantification:** Determine the concentration and purity of total RNA isolated from cells and fresh or frozen tissues by measuring the absorbance in a spectrophotometer. As 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. It is not useful to assess the concentration and purity of total RNA derived from fluids and/or exosomes.
- **RNA integrity:** The integrity and size distribution of total RNA purified from cells and fresh/frozen tissue can be confirmed using an automated analysis system (such as the

QIAxcel® Connect System or the Agilent 2100 Bioanalyzer) that assesses RNA integrity using an RNA integrity score (RIS) or RNA integrity number (RIN). Although the RIN should ideally be ≥ 8 , successful NGS library construction is still possible with samples whose RIN values are ≤ 8 .

- Ensure reactions are thoroughly mixed and that they are prepared and incubated at the recommended temperatures.
- If the workflow is not expected to be completed in one day, convenient stopping points are indicated at the end of the relevant sections.

Protocol: QIAseq UPXome Library Construction

FastSelect rRNA depletion procedure

Important points before starting

- This protocol can be used with low amounts (500 pg – 100 ng) of purified RNA. The recommended starting amount is 10 ng (or greater).
- After cDNA synthesis, at least 8 samples and no more than 24 samples must be pooled together.
- DNase treatment (on-column or in-solution) of total RNA samples is highly recommended.
- 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, the total reaction volume should remain 11 μ L.
- Gently yet thoroughly vortex reactions or reagents unless instructed otherwise. Ensure all reactions are mixed thoroughly and handled at the temperatures recommended in the protocol.
- Use a thermal cycler with a heated lid.

Procedure

1. Thaw template RNA on ice. Gently yet thoroughly mix, then briefly centrifuge to collect residual liquid from the sides of the tubes and return to ice.
2. Prepare the reagents required for FastSelect rRNA depletion.
 - 2a. Thaw the appropriate tube(s) of QIAseq FastSelect, US RT Buffer, 5x, and Nuclease-free Water at room temperature. Briefly centrifuge, gently yet thoroughly vortex to mix, and centrifuge briefly again.
 - 2b. Dilute an aliquot for each FastSelect tube to 0.1x (as an example: using 2 μ L FastSelect tube + 18 μ L Nuclease-free Water). Mix by vortexing and then briefly centrifuge to collect residual liquid from the sides of the tubes.

Note: For 500 pg samples, potential experimental optimization is to dilute FastSelect to 0.005x.
3. Prepare the FastSelect rRNA depletion reaction on as described in Table 5. Briefly centrifuge, gently yet thoroughly vortex to mix, 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. FastSelect rRNA depletion reaction

Component	Volume/reaction
RNA (500 pg – 100 ng)	Variable
QIAseq FastSelect (0.1x)*	1 μ L
ERCC Control†	Optional
US RT Buffer, 5x	4 μ L
Nuclease-free Water	Bring total reaction volume to 11 μ L
Total volume	11 μL

* Any QIAseq FastSelect RNA Removal reagent or combination can be supplemented or substituted here. If additional QIAseq FastSelect RNA Removal reagents are used, the total reaction volume should remain 11 μ L. If using QIAseq FastSelect –5S/16S/23S, which is used to remove bacterial rRNA, refer to Appendix C (page 51). If using QIAseq FastSelect Custom refer to the *QIAseq FastSelect Custom Handbook*.

† 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, the total reaction volume should remain 11 μ L.

4. Incubate as described in Table 6 using a thermal cycler with a heated lid.
5. Proceed to “cDNA synthesis procedure”.

Table 6. FastSelect rRNA depletion incubation

Step	Time
1	2 min at 75°C
2	2 min at 70°C
3	2 min at 65°C
4	2 min at 60°C
5	2 min at 55°C
6	2 min at 37°C
7	2 min at 25°C
8	2 min at 4°C
9	Hold at 4°C

cDNA synthesis procedure

Important points before starting

- Set up the reactions on ice.
- Use a thermal cycler with a heated lid.
- The SID-TS-96S or SID-TS-24S is a 96-well single-use sample ID RT plate. Each well of the SID-TS-96S and only 24 wells of the SID-TS-24S Plate contain pre-dispensed sample ID template switching oligos required for the cDNA synthesis reaction.

Important: If, during the setup of the cDNA synthesis reactions, only some of the wells in the 96-well plate will be used, add 2.5 μ L Nuclease-free Water to each well intended to be used, vortex the plate, centrifuge briefly, and incubate for 10 min at room temperature to fully dissolve the primer. Then transfer the dissolved content of the wells that are going to be used to a new plate. Unused wells should be sealed with provided strip caps.
- Equilibrate the QIAseq Beads to room temperature for 20–30 min before use.

- Vortex the QIAseq Beads thoroughly to ensure they are well resuspended; the solution should be a homogenous brown color. If a delay in the protocol occurs, simply vortex the beads.
- **Important:** Prepare fresh 80% ethanol before performing the procedure.

Procedure

1. Prepare the reagents required for cDNA synthesis.
 - 1a. Thaw DTT (100 mM), dNTP (10 mM), Nuclease-free Water, N6-T RT Primer and/or ODT-T RT Primer at room temperature. Briefly centrifuge, gently yet thoroughly vortex to mix, and briefly centrifuge again.
 - 1b. Keep RNase Inhibitor and EZ Reverse Transcriptase on ice. Mix by flicking the tubes, briefly centrifuge, and return to ice. After use, immediately return the enzymes to the freezer.
 - 1c. Process the SID-TS-96S or SID-TS-24S plate as described in “Important points before starting” if not using all the wells in the plate at one time.
2. Prepare the cDNA synthesis reaction on ice according to Table 7. Briefly centrifuge, gently yet thoroughly vortex to mix, 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. cDNA synthesis reaction

Component	Volume per well
rRNA-depleted sample from previous step	11 μ L
DTT (100 mM)	0.5 μ L
dNTP (10 mM)	2 μ L
Nuclease-free Water*	2.5 μ L
SID-TS-96S or SID-TS-24S RT Plate Dried*	–
N6-T RT Primer†	1 μ L
ODT-T RT Primer†	1 μ L
EZ Reverse Transcriptase	1.5 μ L
RNase Inhibitor	0.5 μ L
Total volume	20 μL

* If not using all the wells in the SID-TS-96S Plate, reduce the 2.5 μ L volume of Nuclease-free Water to 0 μ L in the cDNA synthesis mix, as 2.5 μ L of Nuclease-free water will be used to resuspend the sample ID template switching oligos that will be used.

† For 3' transcriptome analysis, only use the ODT-T RT primer, and replace the N6-T RT primer with an additional 1 μ L of Nuclease-free Water.

3. Add the cDNA synthesis mix to the corresponding wells in the SID-TS-96S plate. Briefly centrifuge, gently yet thoroughly vortex to mix, and briefly centrifuge again.

Important: A unique sample ID must be used for each sample.

4. Incubate as described in Table 8.

Table 8. cDNA synthesis incubation

Step	Time	Temperature
1	1 min	4°C
2	5 min	25°C
3	90 min	42°C
4	10 min	70°C
5	1 min	4°C
6	∞	4°C

5. Upon completion of the cDNA synthesis reactions, pool at least eight samples and no more than 24 samples together into one 1.7 mL (or 2.0 mL) microcentrifuge tube.

Note: The cDNA generated from each well of a SID-TS-96S RT Plate contains a barcoded, specific sample ID.

6. Add the appropriate volume (1.1x the reaction volume) of QIAseq Beads to the combined cDNA synthesis reactions from Step 5 according to Table 9. Addition of QIAseqBeads for cDNA cleanup. Briefly centrifuge, gently yet thoroughly vortex to mix, and centrifuge briefly again.

Note: Vortex the QIAseq Beads thoroughly to ensure they are well resuspended; the solution should be a homogenous brown color.

Table 9. Addition of QIAseqBeads for cDNA cleanup

Number of wells combined	Sample volume	QIAseq Bead volume
8	160 μ L	176 μ L
24	480 μ L	528 μ L

7. Incubate for 5 min at room temperature.
8. Centrifuge the tube until the beads are pelleted (2 min), and then place the plate onto a magnetic rack. After the solution has cleared (2 min or longer), carefully remove and discard the supernatant.

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

Tip: It may be valuable to discard the supernatant twice. The contents settle after the first discard.

9. With the tube still on the magnetic stand, add 200–300 μL freshly prepared 80% ethanol. Wait 2 min and carefully remove and discard the wash. Repeat the wash, for a total of 2 ethanol washes. Remove as much excess ethanol as possible.

Note: When decided between 200 μL or 300 μL , simply ensure that enough is added to submerge the beads.

Important: It is vital to completely remove all traces of ethanol after the second wash. Remove the ethanol with a 200 μL pipette first, briefly centrifuge the tubes immediately, and return the tubes to the magnetic stand. Then use a 10 μL pipette to remove any residual ethanol. This step should be performed quickly.

10. With the tube (cap open) still on the magnetic stand, air-dry at room temperature for 5–10 min (until the beads start to crack and pellet loses its shine).

Note: Visually inspect that the pellet is completely dry.

11. Remove the beads from the magnetic stand and elute the DNA from the beads by adding 22 μL Nuclease-free Water. Gently yet thoroughly vortex (triturate if necessary) to mix, briefly centrifuge, and incubate for 2 min.

12. Centrifuge the tube until the beads are pelleted (2 min), and then return the tube to the magnetic rack until the solution has cleared.

13. Transfer 20 μL of the supernatant to a clean tube.

14. Add 22 μL of QIAseq Beads (1.1x the reaction volume) to the supernatant. Briefly centrifuge, gently yet thoroughly vortex to mix, and briefly centrifuge again.

Note: Vortex the QIAseq Beads thoroughly to ensure they are well resuspended; the solution should be a homogenous brown color.

15. Incubate for 5 min at room temperature.

16. Centrifuge the tube until the beads are pelleted (2 min), and then place the plate onto a magnetic rack. After the solution has cleared (2 min or longer), carefully remove and discard the supernatant.

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

Tip: It may be useful to discard the supernatant twice. The contents settle after the first discard.

17. With the tube still on the magnetic stand, add 200 μL freshly prepared 80% ethanol. Wait 2 min and carefully remove and discard the wash. Repeat the wash, for a total of two ethanol washes. Remove excess ethanol as much as possible.

Important: It is vital to completely remove all traces of the ethanol after the second wash. Remove the ethanol with a 200 μL pipette first, briefly centrifuge the tubes immediately, and return the tubes to the magnetic stand. Then use a 10 μL pipette to remove any residual ethanol. This step should be performed quickly.

18. With the tube (cap open) still on the magnetic stand, air-dry at room temperature for 5–10 min (until the beads start to crack and pellet loses its shine).

Note: Visually inspect that the pellet is completely dry.

19. Remove the beads from the magnetic stand and elute the DNA from the beads by adding 25 μL Nuclease-free Water. Gently yet thoroughly vortex (triturate if necessary) to mix, briefly centrifuge, and incubate for 2 min.

20. Centrifuge the tube until the beads are pelleted (2 min), and then return the tube to the magnetic rack until the solution has cleared.

21. Transfer 23 μL of the supernatant to a clean tube.

Note: From this point forward in the protocol, the procedures are written with the assumption that all cDNA wells (either 8 or 24) have been combined into a single tube. In your experiment, you may be using more tubes.

22. Proceed with “Library amplification/indexing procedure” (page 31). Alternatively, the samples can be stored at -30°C to -15°C .

Library amplification/indexing procedure

Important points before starting

- The QIAseq UX index plates have pierceable foil seals, and the indexes must be pipetted from the plate into separate reaction plates. To prevent cross-contamination, each well is single use.
- A precipitate might be present in the QIAseq 2X HiFi MM. If so, bring the reagent to room temperature for 5 min, and dissolve the precipitate by mixing with pipettor and/or by gentle vortexing.
- Set up the reactions on ice.
- Use a thermal cycler with a heated lid.
- Equilibrate the QIAseq Beads to room temperature for 20–30 min before use.
- Vortex the QIAseq Beads thoroughly to ensure they are well resuspended; the solution should be a homogenous brown color. If a delay in the protocol occurs, simply vortex the beads.
- **Important:** Prepare fresh 80% ethanol before performing this procedure.

Procedure

1. Prepare reagents required for library amplification or indexing.

- 1a. Thaw the QIAseq UX index plates at room temperature. Gently yet thoroughly vortex to mix, and then centrifuge briefly.

Note: The layout and use of QIAseq UX index plates is described in Appendix A: QIAseq UX Index Plates (page 44). During the reaction setup in step 2, indexes are removed to a new plate.

- 1b. Thaw the QIAseq 2X HiFi MM on ice. Mix by gently but thoroughly vortexing the tube, and then centrifuge briefly.

- On ice, prepare the library amplification/indexing reaction according to Table 10. Briefly centrifuge, gently yet thoroughly vortex to mix, and briefly centrifuge again.

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

Table 10. Library amplification/indexing reaction

Component	Volume/reaction
cDNA synthesis bead cleanup supernatant	23 μ L
QIAseq 2X HiFi MM	25 μ L
Index from QIAseq UX index plate	2 μ L
Total volume	50 μL

- In a thermal cycler, perform the cycling program described in Table 11 with the number of cycles determined from Table 12.

Table 11. Library amplification or indexing cycling program

Step	Time	Temperature	Number of cycles
Initial denaturation	30 s	98°C	1
3-step cycling			
Denaturation	5 s	98°C	See Table 12
Annealing	10 s	55°C	
Extension	20 s	72°C	
Final extension	2 min	72°C	1
Hold	1 min	4°C	1
	∞	4°C	Hold

Table 12. PCR cycle number recommendation, based on total RNA input and number of samples pooled

Total RNA input	Number of amplification cycles	
	cDNA pool of 8 samples	cDNA pool of 24 samples
500 pg	24	23
1 ng	20	19
10 ng	18	17

Total RNA input	Number of amplification cycles	
	cDNA pool of 8 samples	cDNA pool of 24 samples
500 pg	24	23
100 ng	15	14
Enriched poly A ⁺	20	19

- Upon completion of library-amplification or indexing reactions, add 40 μL QIAseq Beads (0.8x the reaction volume). Briefly centrifuge, gently yet thoroughly vortex to mix, and briefly centrifuge again.

Note: Vortex the QIAseq Beads thoroughly to ensure they are well resuspended; the solution should be a homogenous brown color.

- Incubate for 5 min at room temperature.
- Centrifuge the tube or plate until the beads are pelleted (2 min), and then place the tubes or plate onto a magnetic rack. After the solution has cleared (2 min or longer), carefully remove and discard the supernatant.

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

Tip: It may be useful to discard the supernatant twice. The contents settle after the first discard.

- With the tube still on the magnetic stand, add 200 μL freshly prepared 80% ethanol. Wait 2 min and carefully remove and discard the wash. Repeat the wash, for a total of two ethanol washes. Remove as much excess ethanol as possible.

Important: It is vital to completely remove all traces of the ethanol after the second wash. Remove the ethanol with a 200 μL pipette first, briefly centrifuge the tubes immediately and return the tubes to the magnetic stand. Then use a 10 μL pipette to remove any residual ethanol. This step should be performed quickly.

- With the tube (cap open) still on the magnetic stand, air-dry at room temperature for 5–10 min (until the beads start to crack and pellet loses its shine).

Note: Visually inspect that the pellet is completely dry.

9. Remove the beads from the magnetic stand and elute the DNA from the beads by adding 22 μL Nuclease-free Water. Gently yet thoroughly vortex to mix, briefly centrifuge, and incubate for 2 min.
10. Centrifuge the tube until the beads are pelleted (2 min), and then return the tube to the magnetic rack until the solution has cleared.
11. Transfer 20 μL of the supernatant to a clean tube.
12. Add 16 μL (0.8x the reaction volume) of QIAseq Beads to the supernatant. Briefly centrifuge, gently yet thoroughly vortex to mix, and centrifuge briefly again.
Note: Vortex the QIAseq Beads thoroughly to ensure they are well resuspended; the solution should be a homogenous brown color.
13. Incubate for 5 min at room temperature.
14. Centrifuge the tube until the beads are pelleted (2 min), and then place the plate onto a magnetic rack. After the solution has cleared (2 min or longer), carefully remove and discard the supernatant.
Important: Do not discard the beads, as they contain the DNA of interest.
Tip: It may be valuable to discard the supernatant twice. The contents settle after the first discard.
15. With the tube still on the magnetic stand, add 200 μL freshly prepared 80% ethanol. Wait 1 min and carefully remove and discard the wash. Repeat the wash, for a total of two ethanol washes. Remove as much excess ethanol as possible.
16. **Important:** It is vital to completely remove all traces of the ethanol after the second wash. Remove the ethanol with a 200 μL pipette first, briefly centrifuge the tubes immediately and return the tubes to the magnetic stand. Then use a 10 μL pipette to remove any residual ethanol. This step should be performed quickly.
17. With the tube (cap open) still on the magnetic stand, air-dry at room temperature for 5–10 min (until the beads start to crack and pellet loses its shine).
Note: Visually inspect that the pellet is completely dry.

18. Remove the beads from the magnetic stand and elute the DNA from the beads by adding 24 μL Nuclease-free Water. Gently yet thoroughly vortex (triturate if necessary) to mix, briefly centrifuge, and incubate for 2 min.
19. Centrifuge the tube until the beads are pelleted (2 min), and then return the tube to the magnetic rack until the solution has cleared.
20. Transfer 22 μL of the supernatant to a clean tube. If not proceeding immediately, the sample can be stored at -30°C to -15°C .
21. Assess the quality of the library using a QIAxcel Connect, Bioanalyzer, TapeStation[®], or Fragment Analyzer. Check for the expected size distribution of library fragments. An example library is shown in Figure 4.

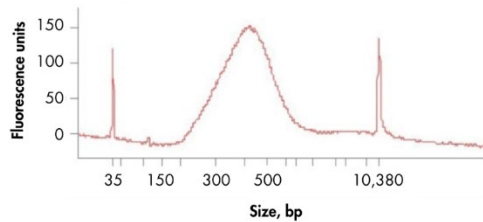


Figure 4. TapeStation trace of library prepared with the QIAseq UPXome RNA Library Kit.

22. Quantify and normalize the individual libraries.

Option 1: Quantification of the libraries using Qubit.

Option 2: The library yield measurements of Qubit or Nanodrop or the Bioanalyzer and TapeStation systems use fluorescence dyes, which intercalate into DNA or RNA and cannot discriminate between cDNA that have and do not have adapter sequences. Real-time PCR-based methods provide an accurate quantification of complete RNA-seq libraries with full adapter sequences. As a result, QIAGEN's QIAseq Library Quant Array Kit (cat. no. 333304) or Assay Kit (cat. no. 333314), which contains laboratory-verified forward and reverse primers together with a DNA standard, is highly recommended for accurate quantification of the prepared library. Please consult the handbook for QIAseq library quant array or assay kits for directions.

23. Proceed to "Protocol: Sequencing Setup", page 37. Alternatively, the purified UPXome RNA library can be safely stored at -30°C to -15°C until ready to use for sequencing.

Protocol: Sequencing Setup

UPXome libraries are compatible with Illumina NGS platforms, including iSeq100, MiniSeq, MiSeq, NextSeq 500/550, NextSeq 1000/2000, HiSeq 2500, HiSeq 3000/4000, and NovaSeq 6000.

Important points before starting

- **Important:** To make sequencing preparation convenient, download Illumina-compatible sample sheets for your sequencing instruments on www.qiagen.com, and refer to Appendix A, page 44.
- **Important:** For standard expression analysis, 74 bp paired-end sequencing with dual 10 bp indexes should be used.
- **Important:** For fusion analysis along with standard expression analysis, 149 bp paired-end sequencing with dual 10 bp indexes should be used.
- **Important:** Ensure that PhiX is included in the sequencing run. Refer to the table below for recommended PhiX amounts. If the system is not listed, refer to the system-specific Illumina documents for recommended PhiX amounts.
- For complete instructions on how to denature sequencing libraries and set up a sequencing run, please refer to the system-specific Illumina documents.
- Sample dilution, pooling, and sequencing:
 - Dilute the individual libraries to a concentration of 4 nM, and then combine libraries with different sample indexes in equimolar amounts. The recommended starting final loading concentration of the pooled UPXome RNA libraries to load onto a MiSeq is 10 pM, or 1.6 pM on a MiniSeq, or 1.6 pM on a NextSeq instrument.
 - Dilute the individual UPXome RNA libraries to a concentration of 10 nM, then combine libraries with different sample indexes in equimolar amounts. The recommended final loading concentration of the pooled UPXome RNA libraries to load onto a NovaSeq instrument is between 175 pM and 265 pM.

Generation of sample sheets for Illumina instruments

Sequences for QIAseq UX UDI indexes are available at www.qiagen.com for download. To make sequencing preparation more convenient, ready-to-use templates that include sample sheets containing all QIAseq UX UDI index sequences are available at www.qiagen.com for different sequencing instruments.

These can be imported and edited using the Illumina Local Run Manager or any text editor. Make sure to download the appropriate sample sheet for the Illumina systems depending on whether you are using Local Run Manager or manually configuring the sequencing run.

All Illumina instruments

1. Go to qiagen.com/us/products/discovery-and-translational-research/next-generation-sequencing/rna-sequencing/ultraplex/qiaseq-upxome-rna-library-kits/ and select **Product Resources** then **Instrument Technical Documents** to find and download the appropriate QIAseq UPXome RNA Library Kit template. Different templates might be available, depending on the instrument.
2. The sample sheet already contains all relevant information to use with the instrument.
3. Open the CSV file, delete any UDI indexes that will not be used in the experiment, and save the file with a new name.
4. Copy the file into the **Sample Sheet** folder on the MiSeq or NextSeq instrument or upload the **Sample Sheet** into Local Run Manager for MiSeq, MiniSeq and NextSeq Illumina instruments.
5. When ready to perform the run, select the file.

6. **Sample dilution and pooling:** Dilute individual libraries to 4 nM unless using for the NovaSeq, in which case, dilute the individual libraries to 10 nM. Then, combine libraries with different sample indexes in equimolar amounts if a similar sequencing depth is needed for each library.

Note: For the NovaSeq, the recommended final pooled library concentration is 1.0–1.5 nM, which will yield a final loading concentration of 200–300 pM on the NovaSeq.

7. Library preparation and loading: Prepare and load the pooled library on an Illumina instrument according to the specific Illumina instrument guide. Dilute the denatured library pool a second time as described in Table 13, to obtain the final library concentration.

Table 13. Recommended final library loading concentrations for Illumina instruments

Illumina sequencing instrument	Illumina-specific documentation	Final library concentration (pM)
iSeq	<i>iSeq 100 System Guide</i>	75
MiSeq	<i>MiSeq System Guide</i>	10
MiniSeq	<i>MiniSeq System Guide</i>	1.2
NextSeq	<i>NextSeq 500 System Guide or NextSeq 550 System Guide</i>	1.2
NovaSeq 6000	<i>NovaSeq 6000 Sequencing System Guide</i>	200–300

8. PhiX Control v3 Spike-in (Illumina, cat. no. FC-110-3001) recommendations are indicated in Table 14, page 40. Follow Illumina guidelines on how to denature, dilute, and combine a PhiX control library with your own pool of libraries. Make sure to use a fresh, reliable stock of PhiX control library. Spike-in 10% PhiX or more of the total library pool.

9. Sequencing run setup: Select **FASTQ Only**.

10. The recommended protocol is 74 bp paired end read with 10 bp dual indexing.

11. Upon completion of the sequencing run, proceed with “Protocol: Data Analysis”, page 41.

Table 14. Summary of Illumina sequencing recommendations for each NGS instrument tested with QIAseq UPXome Kit

Illumina instrument	Flow cell	Recommended sequencing setup	Recommended read 1*	Recommended read 2*	Recommended dual index read	PhiX (percent)
MiSeq	V3 150 cycle	Paired end	74	74	10	10%
NextSeq 500	Mid/high-output 150 cycle	Paired end	74	74	10	10%
NovaSeq 6000	200 cycle SP	Paired end	74	74	10	10%

* The read-length is a recommendation. The read-length can be adjusted, based on the number of cycles of a given flow cell. Additionally, for fusion analysis along with standard expression analysis, 149 bp paired-end sequencing should be used.

Protocol: Data Analysis

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 Biomedical Workbench 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 [GeneGlobe.QIAGEN.com](https://Geneglobe.QIAGEN.com).
2. Click **Analyze Data**.

3. Under **Start Analyzing Your Data**:

- Select analysis type: **Next-Generation Sequencing**
- Select your analyte: **mRNA/lncRNA**
- Select your kit: **QIAseq UPXome**
- Select analysis pipeline: **RNA-seq Analysis Portal**

4. Click **Start Your Analysis**.

Data Analysis using QIAGEN CLC Genomics Workbench

QIAGEN CLC Genomics Workbench (cat. no. 832021) is available for installation on local desktop computers or servers on a subscription basis. QIAGEN CLC Genomics Workbench is a powerful solution that works for everyone, no matter the workflow. Cutting-edge technology, unique features and algorithms widely used by scientific leaders in industry and academia make it easy to overcome challenges associated with data analysis.

QIAseq UPXome RNA analysis is supported by downloading the Biomedical Genomics Analysis plug-in, which provides tools and workflows for NGS panel data analysis, WES, WGS, and RNA-seq.

Troubleshooting Guide

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

Comments and suggestions

Low yield

- | | |
|--|---|
| a) Not enough samples multiplexed per sample index | At least 8 cells must be multiplexed per sample index. |
| b) Using the multi-use Sample ID RT Plate, primers were not properly reconstituted | Prior to use, add 2.5 μ L Nuclease-free Water into each well, vortex the plate, centrifuge briefly, and incubate for 10 min at room temperature to fully dissolve the primer. |
| c) Improper reaction setup | Briefly centrifuge, gently yet thoroughly vortex to mix, and centrifuge briefly again. |
| d) Excess ethanol not removed during bead cleanup steps | After each second ethanol wash, ensure that excess ethanol is removed. Briefly centrifuge and return the tubes or plate to the magnetic stand. Remove the ethanol first with a 200 μ L pipette, and then a 10 μ L pipette to remove any residual ethanol. In addition, allow beads to dry for the appropriate amount of time. |

Sequencing issues

- | | |
|--|---|
| a) Cluster density that is too low or too high | Accurate library quantification is the key for optimal cluster density on any sequencing instrument. A PCR-based quantification method is recommended. Other methods may lead to incorrect quantification of the library, especially when there is overamplification. |
| b) Very low clusters passing filter | Make sure the library is accurately quantified and that the correct amount is loaded onto the sequencing instrument. |

Appendix A: QIAseq UX Index Plates

QIAseq UX index plate layouts

The layouts of the single-use QIAseq UX index plates are shown in Table 15 to Table 23. To make sequencing preparation more convenient, you can download Illumina-compatible sample sheets for different sequencing instruments on www.qiagen.com.

Table 15. QIAseq UX 96 Index Kit IL UDI-A (96) (cat. no. 331815) layout: RUDI-96AA

	1	2	3	4	5	6	7	8	9	10	11	12
A	iRUDI 001	iRUDI 009	iRUDI 017	iRUDI 025	iRUDI 033	iRUDI 041	iRUDI 049	iRUDI 057	iRUDI 065	iRUDI 073	iRUDI 081	iRUDI 089
B	iRUDI 002	iRUDI 010	iRUDI 018	iRUDI 026	iRUDI 034	iRUDI 042	iRUDI 050	iRUDI 058	iRUDI 066	iRUDI 074	iRUDI 082	iRUDI 090
C	iRUDI 003	iRUDI 011	iRUDI 019	iRUDI 027	iRUDI 035	iRUDI 043	iRUDI 051	iRUDI 059	iRUDI 067	iRUDI 075	iRUDI 083	iRUDI 091
D	iRUDI 004	iRUDI 012	iRUDI 020	iRUDI 028	iRUDI 036	iRUDI 044	iRUDI 052	iRUDI 060	iRUDI 068	iRUDI 076	iRUDI 084	iRUDI 092
E	iRUDI 005	iRUDI 013	iRUDI 021	iRUDI 029	iRUDI 037	iRUDI 045	iRUDI 053	iRUDI 061	iRUDI 069	iRUDI 077	iRUDI 085	iRUDI 093
F	iRUDI 006	iRUDI 014	iRUDI 022	iRUDI 030	iRUDI 038	iRUDI 046	iRUDI 054	iRUDI 062	iRUDI 070	iRUDI 078	iRUDI 086	iRUDI 094
G	iRUDI 007	iRUDI 015	iRUDI 023	iRUDI 031	iRUDI 039	iRUDI 047	iRUDI 055	iRUDI 063	iRUDI 071	iRUDI 079	iRUDI 087	iRUDI 095
H	iRUDI 008	iRUDI 016	iRUDI 024	iRUDI 032	iRUDI 040	iRUDI 048	iRUDI 056	iRUDI 064	iRUDI 072	iRUDI 080	iRUDI 088	iRUDI 096

Unique i5 and i7 Index pairs are wet single-use plates. During reaction setup, components are aliquoted into another plate.

Table 16. QIAseq UX 96 Index Kit IL UDI-B (96) (cat. no. 331825) layout – RUDI-96BA

	1	2	3	4	5	6	7	8	9	10	11	12
A	iRUDI 097	iRUDI 105	iRUDI 113	iRUDI 121	iRUDI 129	iRUDI 137	iRUDI 145	iRUDI 153	iRUDI 161	iRUDI 169	iRUDI 177	iRUDI 185
B	iRUDI 098	iRUDI 106	iRUDI 114	iRUDI 122	iRUDI 130	iRUDI 138	iRUDI 146	iRUDI 154	iRUDI 162	iRUDI 170	iRUDI 178	iRUDI 186
C	iRUDI 099	iRUDI 107	iRUDI 115	iRUDI 123	iRUDI 131	iRUDI 139	iRUDI 147	iRUDI 155	iRUDI 163	iRUDI 171	iRUDI 179	iRUDI 187
D	iRUDI 100	iRUDI 108	iRUDI 116	iRUDI 124	iRUDI 132	iRUDI 140	iRUDI 148	iRUDI 156	iRUDI 164	iRUDI 172	iRUDI 180	iRUDI 188
E	iRUDI 101	iRUDI 109	iRUDI 117	iRUDI 125	iRUDI 133	iRUDI 141	iRUDI 149	iRUDI 157	iRUDI 165	iRUDI 173	iRUDI 181	iRUDI 189
F	iRUDI 102	iRUDI 110	iRUDI 118	iRUDI 126	iRUDI 134	iRUDI 142	iRUDI 150	iRUDI 158	iRUDI 166	iRUDI 174	iRUDI 182	iRUDI 190
G	iRUDI 103	iRUDI 111	iRUDI 119	iRUDI 127	iRUDI 135	iRUDI 143	iRUDI 151	iRUDI 159	iRUDI 167	iRUDI 175	iRUDI 183	iRUDI 191
H	iRUDI 104	iRUDI 112	iRUDI 120	iRUDI 128	iRUDI 136	iRUDI 144	iRUDI 152	iRUDI 160	iRUDI 168	iRUDI 176	iRUDI 184	iRUDI 192

Unique i5 and i7 Index pairs are wet single-use plates. During reaction setup, components are aliquoted into another plate.

Table 17. QIAseq UX 96 Index Kit IL UDI-C (96) (cat. no. 331835) layout— RUDI-96CA

	1	2	3	4	5	6	7	8	9	10	11	12
A	iRUDI 193	iRUDI 201	iRUDI 209	iRUDI 217	iRUDI 225	iRUDI 233	iRUDI 241	iRUDI 249	iRUDI 257	iRUDI 265	iRUDI 273	iRUDI 281
B	iRUDI 194	iRUDI 202	iRUDI 210	iRUDI 218	iRUDI 226	iRUDI 234	iRUDI 242	iRUDI 250	iRUDI 258	iRUDI 266	iRUDI 274	iRUDI 282
C	iRUDI 195	iRUDI 203	iRUDI 211	iRUDI 219	iRUDI 227	iRUDI 235	iRUDI 243	iRUDI 251	iRUDI 259	iRUDI 267	iRUDI 275	iRUDI 283
D	iRUDI 196	iRUDI 204	iRUDI 212	iRUDI 220	iRUDI 228	iRUDI 236	iRUDI 244	iRUDI 252	iRUDI 260	iRUDI 268	iRUDI 276	iRUDI 284
E	iRUDI 197	iRUDI 205	iRUDI 213	iRUDI 221	iRUDI 229	iRUDI 237	iRUDI 245	iRUDI 253	iRUDI 261	iRUDI 269	iRUDI 277	iRUDI 285
F	iRUDI 198	iRUDI 206	iRUDI 214	iRUDI 222	iRUDI 230	iRUDI 238	iRUDI 246	iRUDI 254	iRUDI 262	iRUDI 270	iRUDI 278	iRUDI 286
G	iRUDI 199	iRUDI 207	iRUDI 215	iRUDI 223	iRUDI 231	iRUDI 239	iRUDI 247	iRUDI 255	iRUDI 263	iRUDI 271	iRUDI 279	iRUDI 287
H	iRUDI 200	iRUDI 208	iRUDI 216	iRUDI 224	iRUDI 232	iRUDI 240	iRUDI 248	iRUDI 256	iRUDI 264	iRUDI 272	iRUDI 280	iRUDI 288

Unique i5 and i7 Index pairs are wet single-use plates. During reaction setup, components are aliquoted into another plate.

Table 18. QIAseq UX 96 Index Kit IL UDI-D (96) (cat.no. 331845) layout – RUDI-96DA

	1	2	3	4	5	6	7	8	9	10	11	12
A	iRUDI 289	iRUDI 297	iRUDI 305	iRUDI 313	iRUDI 321	iRUDI 329	iRUDI 337	iRUDI 345	iRUDI 353	iRUDI 361	iRUDI 369	iRUDI 377
B	iRUDI 290	iRUDI 298	iRUDI 306	iRUDI 314	iRUDI 322	iRUDI 330	iRUDI 338	iRUDI 346	iRUDI 354	iRUDI 362	iRUDI 370	iRUDI 378
C	iRUD2 91	iRUDI 299	iRUDI 307	iRUDI 315	iRUDI 323	iRUDI 331	iRUDI 339	iRUDI 347	iRUDI 355	iRUDI 363	iRUDI 371	iRUDI 379
D	iRUDI 292	iRUDI 300	iRUDI 308	iRUDI 316	iRUDI 324	iRUDI 332	iRUDI 340	iRUDI 348	iRUDI 356	iRUDI 364	iRUDI 372	iRUDI 380
E	iRUDI 293	iRUDI 301	iRUDI 309	iRUDI 317	iRUDI 325	iRUDI 333	iRUDI 341	iRUDI 349	iRUDI 357	iRUDI 365	iRUDI 373	iRUDI 381
F	iRUDI 294	iRUDI 302	iRUDI 310	iRUDI 318	iRUDI 326	iRUDI 334	iRUDI 342	iRUDI 350	iRUDI 358	iRUDI 366	iRUDI 374	iRUDI 382
G	iRUDI 295	iRUDI 303	iRUDI 311	iRUDI 319	iRUDI 327	iRUDI 335	iRUDI 343	iRUDI 351	iRUDI 359	iRUDI 367	iRUDI 375	iRUDI 383
H	iRUDI 296	iRUDI 304	iRUDI 312	iRUDI 320	iRUDI 328	iRUDI 336	iRUDI 344	iRUDI 352	iRUDI 360	iRUDI 368	iRUDI 376	iRUDI 384

Unique i5 and i7 Index pairs are wet single-use plates. During reaction setup, components are aliquoted into another plate.

Table 19. QIAseq UX 96 Index Kit IL UDI-E (96) (cat. no. 331855) layout – RUDI-96EA

	1	2	3	4	5	6	7	8	9	10	11	12
A	iRUDI 385	iRUDI 393	iRUDI 401	iRUDI 409	iRUDI 417	iRUDI 425	iRUDI 433	iRUDI 441	iRUDI 449	iRUDI 457	iRUDI 465	iRUDI 473
B	iRUDI 386	iRUDI 394	iRUDI 402	iRUDI 410	iRUDI 418	iRUDI 426	iRUDI 434	iRUDI 442	iRUDI 450	iRUDI 458	iRUDI 466	iRUDI 474
C	iRUDI 387	iRUDI 395	iRUDI 403	iRUDI 411	iRUDI 419	iRUDI 427	iRUDI 435	iRUDI 443	iRUDI 451	iRUDI 459	iRUDI 467	iRUDI 475
D	iRUDI 388	iRUDI 396	iRUDI 404	iRUDI 412	iRUDI 420	iRUDI 428	iRUDI 436	iRUDI 444	iRUDI 452	iRUDI 460	iRUDI 468	iRUDI 476
E	iRUDI 389	iRUDI 397	iRUDI 405	iRUDI 413	iRUDI 421	iRUDI 429	iRUDI 437	iRUDI 445	iRUDI 453	iRUDI 461	iRUDI 469	iRUDI 477
F	iRUDI 390	iRUDI 398	iRUDI 406	iRUDI 414	iRUDI 422	iRUDI 430	iRUDI 438	iRUDI 446	iRUDI 454	iRUDI 462	iRUDI 470	iRUDI 478
G	iRUDI 391	iRUDI 399	iRUDI 407	iRUDI 415	iRUDI 423	iRUDI 431	iRUDI 439	iRUDI 447	iRUDI 455	iRUDI 463	iRUDI 471	iRUDI 479
H	iRUDI 392	iRUDI 400	iRUDI 408	iRUDI 416	iRUDI 424	iRUDI 432	iRUDI 440	iRUDI 448	iRUDI 456	iRUDI 464	iRUDI 472	iRUDI 480

Unique i5 and i7 Index pairs are wet single-use plates. During reaction setup, components are aliquoted into another plate.

Table 20. QIAseq UX 96 Index Kit UDI-F (96) (cat. no. 331865) layout – RUDI-96FA

	1	2	3	4	5	6	7	8	9	10	11	12
A	iRUDI 481	iRUDI 489	iRUDI 497	iRUDI 505	iRUDI 513	iRUDI 521	iRUDI 529	iRUDI 537	iRUDI 545	iRUDI 553	iRUDI 561	iRUDI 569
B	iRUDI 482	iRUDI 490	iRUDI 498	iRUDI 506	iRUDI 514	iRUDI 522	iRUDI 530	iRUDI 538	iRUDI 546	iRUDI 554	iRUDI 562	iRUDI 570
C	iRUDI 483	iRUDI 491	iRUDI 499	iRUDI 507	iRUDI 515	iRUDI 523	iRUDI 531	iRUDI 539	iRUDI 547	iRUDI 555	iRUDI 563	iRUDI 571
D	iRUDI 484	iRUDI 492	iRUDI 500	iRUDI 508	iRUDI 516	iRUDI 524	iRUDI 532	iRUDI 540	iRUDI 548	iRUDI 556	iRUDI 564	iRUDI 572
E	iRUDI 485	iRUDI 493	iRUDI 501	iRUDI 509	iRUDI 517	iRUDI 525	iRUDI 533	iRUDI 541	iRUDI 549	iRUDI 557	iRUDI 565	iRUDI 573
F	iRUDI 486	iRUDI 494	iRUDI 502	iRUDI 510	iRUDI 518	iRUDI 526	iRUDI 534	iRUDI 542	iRUDI 550	iRUDI 558	iRUDI 566	iRUDI 574
G	iRUDI 487	iRUDI 495	iRUDI 503	iRUDI 511	iRUDI 519	iRUDI 527	iRUDI 535	iRUDI 543	iRUDI 551	iRUDI 559	iRUDI 567	iRUDI 575
H	iRUDI 488	iRUDI 496	iRUDI 504	iRUDI 512	iRUDI 520	iRUDI 528	iRUDI 536	iRUDI 544	iRUDI 552	iRUDI 560	iRUDI 568	iRUDI 576

Unique i5 and i7 Index pairs are wet single-use plates. During reaction setup, components are aliquoted into another plate.

Table 21. QIAseq UX 96 Index Kit IL UDI-G (96) (cat. no. 331875) layout – RUDI-96GA

	1	2	3	4	5	6	7	8	9	10	11	12
A	iRUDI 577	iRUDI 585	iRUDI 593	iRUDI 601	iRUDI 609	iRUDI 617	iRUDI 625	iRUDI 633	iRUDI 641	iRUDI 649	iRUDI 657	iRUDI 665
B	iRUDI 578	iRUDI 586	iRUDI 594	iRUDI 602	iRUDI 610	iRUDI 618	iRUDI 626	iRUDI 634	iRUDI 642	iRUDI 650	iRUDI 658	iRUDI 666
C	iRUDI 579	iRUDI 587	iRUDI 595	iRUDI 603	iRUDI 611	iRUDI 619	iRUDI 627	iRUDI 635	iRUDI 643	iRUDI 651	iRUDI 659	iRUDI 667
D	iRUDI 580	iRUDI 588	iRUDI 596	iRUDI 604	iRUDI 612	iRUDI 620	iRUDI 628	iRUDI 636	iRUDI 644	iRUDI 652	iRUDI 660	iRUDI 668
E	iRUDI 581	iRUDI 589	iRUDI 597	iRUDI 605	iRUDI 613	iRUDI 621	iRUDI 629	iRUDI 637	iRUDI 645	iRUDI 653	iRUDI 661	iRUDI 669
F	iRUDI 582	iRUDI 590	iRUDI 598	iRUDI 606	iRUDI 614	iRUDI 622	iRUDI 630	iRUDI 638	iRUDI 646	iRUDI 654	iRUDI 662	iRUDI 670
G	iRUDI 583	iRUDI 591	iRUDI 599	iRUDI 607	iRUDI 615	iRUDI 623	iRUDI 631	iRUDI 639	iRUDI 647	iRUDI 655	iRUDI 663	iRUDI 671
H	iRUDI 584	iRUDI 592	iRUDI 600	iRUDI 608	iRUDI 616	iRUDI 624	iRUDI 632	iRUDI 640	iRUDI 648	iRUDI 656	iRUDI 664	iRUDI 672

Unique i5 and i7 Index pairs are wet single-use plates. During reaction setup, components are aliquoted into another plate.

Table 22. QIAseq UX 96 Index Kit IL UDI-H (96) (cat. no. 331885) layout — RUDI-96HA

	1	2	3	4	5	6	7	8	9	10	11	12
A	iRUDI 673	iRUDI 681	iRUDI 689	iRUDI 697	iRUDI 705	iRUDI 713	iRUDI 721	iRUDI 729	iRUDI 737	iRUDI 745	iRUDI 753	iRUDI 761
B	iRUDI 674	iRUDI 682	iRUDI 690	iRUDI 698	iRUDI 706	iRUDI 714	iRUDI 722	iRUDI 730	iRUDI 738	iRUDI 746	iRUDI 754	iRUDI 762
C	iRUDI 675	iRUDI 683	iRUDI 691	iRUDI 699	iRUDI 707	iRUDI 715	iRUDI 723	iRUDI 731	iRUDI 739	iRUDI 747	iRUDI 755	iRUDI 763
D	iRUDI 676	iRUDI 684	iRUDI 692	iRUDI 700	iRUDI 708	iRUDI 716	iRUDI 724	iRUDI 732	iRUDI 740	iRUDI 748	iRUDI 756	iRUDI 764
E	iRUDI 677	iRUDI 685	iRUDI 693	iRUDI 701	iRUDI 709	iRUDI 717	iRUDI 725	iRUDI 733	iRUDI 741	iRUDI 749	iRUDI 757	iRUDI 765
F	iRUDI 678	iRUDI 686	iRUDI 694	iRUDI 702	iRUDI 710	iRUDI 718	iRUDI 726	iRUDI 734	iRUDI 742	iRUDI 750	iRUDI 758	iRUDI 766
G	iRUDI 679	iRUDI 687	iRUDI 695	iRUDI 703	iRUDI 711	iRUDI 719	iRUDI 727	iRUDI 735	iRUDI 743	iRUDI 751	iRUDI 759	iRUDI 767
H	iRUDI 680	iRUDI 688	iRUDI 696	iRUDI 704	iRUDI 712	iRUDI 720	iRUDI 728	iRUDI 736	iRUDI 744	iRUDI 752	iRUDI 760	iRUDI 768

Unique i5 and i7 Index pairs are wet single-use plates. During reaction setup, components are aliquoted into another plate.

Table 23. QIAseq UX 12 Index Kit IL UDI (12) (cat. no. 331801) layout — RUDI-12A

	1	2	3	4	5	6	7	8	9	10	11	12
A	iRUDI001	iRUDI009	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
B	iRUDI002	iRUDI010	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
C	iRUDI003	iRUDI011	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
D	iRUDI004	iRUDI012	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
E	iRUDI005	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
F	iRUDI006	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
G	iRUDI007	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
H	iRUDI008	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty

Unique i5 and i7 Index pairs are wet single-use plates. During reaction setup, components are aliquoted into another plate.

Appendix B: mRNA Enrichment using RNeasy Pure mRNA Bead Kit

Below is a brief protocol for mRNA enrichment. For further information, visit www.qiagen.com/HB-1783 for the RNeasy Pure mRNA Bead Handbook.

Important points before starting

- This protocol is for mRNA enrichment, and the starting material is 100 ng total RNA.
- Vortex the bottle containing Pure mRNA Beads for 3 min (before first use) or 1 min (before subsequent uses) to ensure that the magnetic particles are fully resuspended.
- Heat a heating block to 70°C, and heat Buffer OEB to 70°C.
- Unless otherwise indicated, all protocol steps – including centrifugation – should be performed at 20–30°C. Steps 5–8 are processed using a magnetic rack.

Procedure

1. Determine the amount of starting RNA. Pipette 100 ng total RNA into an RNase-free 1.5 mL tube and adjust the volume with RNase-free Water (if necessary) to a volume of 250 μ L.
2. Add 1 μ L RNase Inhibitor (4U/ μ L), 250 μ L Buffer mRBB and 25 μ L Pure mRNA Beads, and vortex.
3. Incubate the sample for 3 min at 70°C in a heating block. This step disrupts the secondary structure of RNA.
4. Remove the sample from the heating block and place at room temperature for 10 min. This step allows hybridization between the oligo-dT of the Pure mRNA Beads and the poly-A tail of the mRNA.

5. Briefly pellet the mRNA–bead complex by centrifugation for 2 min at maximum speed, and place the tube on a magnetic rack. Wait for 1 min, making sure the bead separation is complete, and remove the supernatant.

Note: Save the supernatant until certain that satisfactory binding and elution of poly A⁺ mRNA has occurred.

6. Resuspend the mRNA–bead pellet in 400 μ L Buffer OW2 by vortexing and pipette the solution into a 1.5 mL Eppendorf[®] tube. Briefly centrifuge the tube to pellet the mRNA–bead pellet prior to placing on a magnetic rack. Wait 1 min until the bead separation has been completed and remove the supernatant.
7. Apply another 400 μ L Buffer OW2, mix by vortexing and pipette the solution into a 1.5 mL Eppendorf tube. Briefly centrifuge the tube to pellet the mRNA–bead pellet prior to placing on a magnetic rack. Wait 1 min until the bead separation has been completed and remove the supernatant.
8. Pipette 10 μ L hot (70°C) Buffer OEB into the 1.5 mL containing mRNA–bead and pipette up and down 3–4 times to resuspend the beads thoroughly. Briefly centrifuge and place the tube on a magnetic rack and wait 1 min until bead separation has been completed. Remove the clear supernatant and transfer it to a new 1.5 mL Eppendorf tube as this contains the enriched mRNA.
9. With this enriched poly A⁺ mRNA, proceed to “FastSelect rRNA depletion procedure”, page 23.

Appendix C: QIAseq FastSelect 5S/16S/23S with the QIAseq UPXome RNA Library Kit

Important points before starting

- This protocol is for incorporation of QIAseq FastSelect –5S/16S/23S rRNA removal with the QIAseq UPXome Library Kit. The starting material is 10 ng to 100 ng total RNA. Using less than 10 ng input is not recommended when using QIAseq FastSelect 5S/16S/23S.
- After cDNA synthesis, at least 8 samples and no more than 24 samples must be pooled together.
- We highly recommend DNase treatment (on-column and in-solution) of total RNA samples.
- 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, the total reaction volume should remain 11 μ L.
- Bring FastSelect 5S/16S/23S, FastSelect FH Buffer, and Nuclease-free Water to room temperature (15-25°C).

Important: Only for QIAseq FastSelect 5S/16S/23S, incubate the tube at 37°C for 5 min, and then vortex to dissolve the precipitate.

- Dilute an aliquot for each FastSelect tube to 0.1x using 2 μ L FastSelect tube and 18 μ L Nuclease-free Water. Mix by vortexing and then briefly centrifuge to collect residual liquid from the sides of the tubes.

Procedure

1. Thaw template RNA on ice. Gently yet thoroughly mix, then briefly centrifuge to collect residual liquid from the sides of the tubes and return to ice.

2. Prepare the reagents required for FastSelect rRNA depletion.
 - 2a. Thaw the appropriate tube(s) of QIAseq FastSelect, FastSelect FH Buffer, and Nuclease-free Water at room temperature (15–25°C). Briefly centrifuge, gently yet thoroughly vortex to mix, and centrifuge briefly again.

Note: For QIAseq FastSelect 5S/16S/23S, incubate the tube at 37°C for 5 min, and then vortex to dissolve the precipitate.
 - 2b. Prepare a 1:10 dilution of QIAseq FastSelect 5S/16S/23S tube (as an example: using 2 µL FastSelect tube and 18 µL Nuclease-free Water). Mix by vortexing and then briefly centrifuge to collect residual liquid from the sides of the tubes.
3. Prepare the FastSelect rRNA depletion reaction on as described in Table 24. Briefly centrifuge, gently yet thoroughly vortex to mix, 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 24. Preparation of FastSelect 5S/16S/23S depletion reaction

Component	Volume/reaction
RNA (10 ng – 100 ng)	Variable
QIAseq FastSelect 5S/16S/23S (0.1x)*	1 µL
ERCC Control†	Optional
FastSelect FH Buffer	1.5 µL
Nuclease-free Water	Bring total reaction volume to 15 µL
Total volume	15 µL

* Any QIAseq FastSelect RNA Removal reagent or combination can be supplemented or substituted here. If additional QIAseq FastSelect RNA Removal reagents are used, the total reaction volume should remain 11 µL.

† ERCC Control RNA (see “Equipment and Reagents to be Supplied by User”, page 19) can be added according to the concentrations specified by the manufacturer. If added, the total reaction volume should remain 11 µL.

4. Incubate as described in Table 25 using a thermal cycler with a heated lid.

Table 25. FastSelect 5S/16S/23S rRNA depletion incubation

Step	Time
1	2 min at 75°C
2	2 min at 70°C
3	2 min at 65°C
4	2 min at 60°C
5	2 min at 55°C
6	2 min at 37°C
7	2 min at 25°C
8	2 min at 4°C
9	Hold at 4°C

5. Upon completion of the rRNA depletion reaction, add 19.5 μL (1.3x volume) QIAseq Beads to the 15 μL reaction. Briefly centrifuge, gently yet thoroughly vortex to mix, and centrifuge briefly again.

Note: Vortex the QIAseq Beads thoroughly to ensure they are well resuspended; the solution should be a homogenous brown color.

6. Incubate for 5 min at room temperature.
7. Centrifuge the tube or plate until the beads are pelleted (2 min), and then place the plate onto a magnetic rack. After the solution has cleared (2 min or longer), carefully remove and discard the supernatant.

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

Tip: It may be useful to discard the supernatant twice. The contents settle after the first discard.

8. Add 15 μL of Nuclease-free Water and 19.5 μL QIAseq Bead Binding Buffer (prewarmed to room temperature and mixed by vortexing). Briefly centrifuge, gently yet thoroughly vortex to mix, and centrifuge briefly again.
9. Incubate for 5 min at room temperature.

10. Centrifuge the tube or plate until the beads are pelleted (2 min), and then place the tube or plate onto a magnetic rack. After the solution has cleared (2 min or longer), carefully remove and discard the supernatant.
Important: do not discard the beads, as they contain the DNA of interest.
Tip: It may be useful to discard the supernatant twice. The contents settle after the first discard.
11. With the tube still on the magnetic stand, add 200 μL freshly prepared 80% ethanol. Wait 2 min and carefully remove and discard the wash. Repeat the wash, for a total of two ethanol washes. Remove as much excess ethanol as possible.
12. **Important:** It is vital to completely remove all traces of the ethanol after the second wash. Remove the ethanol with a 200 μL pipette first, briefly centrifuge the tubes immediately and return the tubes to the magnetic stand. Then use a 10 μL pipette to remove any residual ethanol. This step should be performed quickly.
13. With the tube (cap open) still on the magnetic stand, air-dry at room temperature for 5–10 min (until the beads start to crack and pellet loses its shine).
Note: visually inspect that the pellet is completely dry.
14. Remove the beads from the magnetic stand and elute the DNA from the beads by adding 9 μL Nuclease-free Water. Gently yet thoroughly vortex (triturate if necessary) to mix, briefly centrifuge, and incubate for 2 min.
15. Centrifuge the tube until the beads are pelleted (2 min), and then return the tube to the magnetic rack until the solution has cleared.
16. Transfer 7 μL of the supernatant to a clean tube.
17. Thaw the US RT Buffer, 5x and add 4 μL to the 7 μL supernatant. Briefly centrifuge, gently yet thoroughly vortex to mix, and centrifuge briefly again.
18. Proceed to “cDNA synthesis procedure”, page 25. Alternatively, the samples can be stored at -30°C to -15°C in a constant-temperature freezer.

Ordering Information

Product	Contents	Cat. no.
QIAseq UPXome RNA Library Kit (24)	For 24 reactions: Contains all buffers and reagents for either whole or 3' transcriptome library prep of 24 ultra-low input RNA samples	Varies
QIAseq UPXome RNA Library Kit (96)	For 96 reactions: Contains all buffers and reagents for either whole or 3' transcriptome library prep of 96 ultra-low input RNA samples	Varies
QIAseq FastSelect Custom RNA Removal Kits	Includes QIAseq FastSelect Custom RNA Removal Kit for 1536 standard samples	Varies
QIAseq FastSelect -rRNA/Globin Kit (96)	Includes one tube of QIAseq FastSelect reagent for rRNA removal and one tube of QIAseq FastSelect reagent for globin mRNA removal; sufficient for 96 reactions from human, mouse, and rat samples	335377
QIAseq UX 12 Index Kit IL UDI (12)	Sample Index Kit for 12 libraries using unique dual indexes on Illumina NGS instruments; indexes are supplied as liquid in a 96-well plate with a pierceable foil seal	331801
QIAseq UX 96 Index Kit IL UDI A-H	Sample Index Kits sold in sets of 96 libraries using unique dual indexes on Illumina NGS instruments; indexes are supplied as liquid in a 96-well plate with a pierceable foil seal. 8 different sets of 96 indexes are available for a total of 768 UDIs.	Varies

Product	Contents	Cat. no.
Related products		
QIAseq Library Quant Array Kit	Reagents for quantification of libraries prepared for Illumina or Ion Torrent® platforms; array format	333304
QIAseq Library Quant Assay Kit	Reagents for quantification of libraries prepared for Illumina or Ion Torrent platforms; assay format	333314
Human XpressRef Universal Total RNA	2 tubes, each with 100 µg of total RNA prepared from 20 different human adult and fetal normal major organs.	338112
Mouse XpressRef Universal Total RNA	2 tubes, each with 100 µg of total RNA prepared from several male and female mice (Balb/c strain), whole bodies without fur	338114
Rat XpressRef Universal Total RNA	2 tubes, each with 100 µg of total RNA prepared from several male and female rats (SD Wistar strain), whole bodies without fur	338116
RNeasy Micro Kit (50)	50 RNeasy MinElute® Spin Columns, collection tubes (1.5 mL and 2 mL), RNase-free DNase I, carrier RNA, RNase-free reagents, and buffers	74004
RNeasy Mini Kit (50)*	50 RNeasy Mini Spin Columns, collection tubes (1.5 mL and 2 mL), RNase-free reagents, and buffers	74104
RNeasy 96 Kit (4)*	For 4 x 96 total and cytoplasmic RNA preps: 4 RNeasy 96 Plates, Elution Microtubes CL, caps, S-Blocks, AirPore tape sheets, RNase-free reagents, and buffers	74181
QIAGEN CLC Genomics Workbench	Comprehensive analysis package for the analysis and visualization of data from all major next-generation sequencing (NGS) platforms. The workbench supports and seamlessly integrates	832021

Product**Contents****Cat. no.**

into a typical NGS workflow. CLC Genomics Workbench is available for Windows, Mac OS X, and Linux platforms

* Larger kit sizes available; visit www.qiagen.com

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Document Revision History

Revision	Description
July 2022	Initial release
October 2022	Changed QIAseq DIRECT Unique UDIs product name to QIAseq UX Index Kits UDI Table layout changes on Table 12 Change to product catalog numbers on Tables 12–20 Updated procedure steps in Appendix C Updated Ordering Information to add QIAseq UX 12 Index Kit IL UDI (12) and QIAseq UX 96 Index Kit IL UDI A–H (96)
February 2023	Corrected Table numbers and cross-referencing

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