January 2021

QIAseq® UPX 3' Transcriptome Handbook

For high-throughput 3' transcriptome analysis from up to 10 ng of purified RNA, cell lysates, and single cells, using next-generation sequencing

Contents

Kit Contents

QIAseq UPX 3' Transcriptome Kit	(96)	(96-M)	(384)
Catalog no.	333088	333089	333090
Number of reactions	96	96 x 4	384
Box 1 of 2			
Cell Lysis Buffer	1 ml	1 ml	1 ml
RNase Inhibitor	96 µl	150 µl	150 µl
3' Trans RT Buffer	144 µl	576 µl	576 µl
EZ Reverse Transcriptase	36 µl	144 µl	144 µl
Nuclease-free Water	1 tube	1 tube	1 tube
UPX AMP Primer	100 µl	323 µl	100 µl
2X Quant AMP MM	144 µl	576 µl	144 µl
2X QIAGEN® HiFi PCR MM	360 µl	1440 µl	360 µl
UL Adapter	25 µl	85 µl	25 µl
Fragmentation Buffer, 10x	40 µl	192 µl	40 µl
Fragmentation Enzyme Mix	90 µl	384 µl	90 µl
FERA Solution	15 µl	60 µl	15 µl
Ligation Buffer, 5x	160 µl	600 µl	160 µl
DNA Ligase	120 µl	360 µl	120 µl
uQuant Buffer, 5x	75 µl	300 µl	75 µl
UPCR Buffer, 5x	60 µl	300 µl	60 µl
HotStarTaq® DNA Polymerase	60 µl	240 µl	60 µl
Optical adhesive film	–	–	1
384-EasyLoad Covers	–	–	1
Box 2 of 2			
Indented flat 12-cap strips	1 bag	1 bag	–
Cell Index (CID) RT Plate	CID-96S Plate 96-well single-use plate	CID-96M Plate 96-well multi-use plate	CID-384 Plate 384-well single-use plate
Ligation Solution	970 µl	970 µl	970 µl
QIAseq Beads	10 ml	26 ml	10 ml
QIAseq NGS Bead Binding Buffer	10.2 ml	34 ml	10.2 ml

QIAseq UPX 3' Trans. 12-index (48) **Catalog no.** **Number of reactions**	**(12 sample index for 48 samples on Illumina platform)** **333074** **48**
3' Trans P1	25 µl
3' Trans P2	25 µl
3' Trans P3	25 µl
3' Trans P4	25 µl
3' Trans P5	25 µl
3' Trans P6	25 µl
3' Trans P7	25 µl
3' Trans P8	25 µl
3' Trans P9	25 µl
3' Trans P10	25 µl
3' Trans P11	25 µl
3' Trans P12	25 µl
QIAseq D Read 2 Primer I	24 µl

QIAseq UPX 3' Trans. 48-Index (192) **Catalog no.** **Number of reactions**	**(48 sample index for 192 samples on Illumina® platform)** **333075** **192**
Box containing 4 tubes of QIAseq D Read 2 Primer I (24 µl), indented flat 12-cap strips (48) and arrays. Each array well contains one PCR primer pair for amplification and sample indexing – enough for a total of 192 samples (for indexing up to 48 samples per run) for 3' transcriptome sequencing on Illumina platforms.	4

Shipping and Storage

The QIAseq UPX 3' Transcriptome Kit is shipped in 2 boxes:

- Box 1 is shipped on dry ice. Upon receipt, all components in Box 1 should be stored immediately at –30 to –15°C in a constant-temperature freezer.
- Box 2 is shipped on blue ice. Upon receipt, all components in Box 2 should be stored immediately at 2–8°C in a refrigerator.
  - QIAseq Beads should never be frozen.
- QIAseq Index Kits (sold separately) are shipped on dry ice and should be stored at –30 to –15°C upon receipt.

When stored correctly, QIAseq UPX 3' Transcriptome Kit is good 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 UPX products are intended for molecular biology applications. These products are not intended for the diagnosis, prevention, or treatment of a disease.

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

Safety Information

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

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of QIAseq UPX 3' Transcriptome Kit is tested against predetermined specifications to ensure consistent product quality.

Introduction

Tissues are heterogeneous mixtures of different cell populations, with each cell contributing a unique proteome and transcriptome. Normal and disease-related biology are both inherently heterogeneous, and cells can respond individually and in concert to internal and external stimuli. In addition, individual cells can differ due to epigenetics, circadian clock, cell cycle, microenvironment, cell-to-cell contacts, and intrinsic transcriptional noise. While bulk transcriptomic analysis of mRNA, lncRNA, and miRNA expression is critical for understanding biological systems, the consequential cellular averages mask intrinsic transcriptional variability across individual cell subpopulations. In fact, the contribution of rare cell subtypes may be completely obscured when cells are assessed in bulk. Single-cell expression analysis brings into focus the individual contribution of every cell, providing a complete, granular understanding of a specific biological response.

The QIAseq UPX 3' Transcriptome Kit enables high-throughput next-generation sequencing (NGS) of polyadenylated RNAs using Illumina NGS instruments. The kit and data analysis are intended for library construction and gene expression analysis of purified RNA (10 pg – 10 ng), cell lysates (up to 1000 cells), and single cells. The QIAseq UPX 3' Transcriptome Kit presents an innovative advantage in that during reverse transcription, each cell is tagged with a unique ID (referred to as Cell-ID) and each RNA molecule is tagged with a unique molecular index (UMI). Since each sample is indexed during the reverse transcription reaction, the cDNAs from each sample can be combined and all subsequent library construction steps are performed in a single tube. This simplifies RNA-seq library preparation resulting in a much higher throughput than traditional NGS library preparation methods. This increased productivity can be achieved by simply performing manual library preparations or further magnified through automation of the protocols.

During final library amplification, up to 48 different sample IDs can be utilized, which allows further scalability. Together, the combination of cell IDs and sample IDs enables up to 4608

samples to be sequenced together when starting with a 96-well plate, or 18,432 libraries when starting with a 384-well plate.

The QIAseq UPX 3’ Transcriptome Kits are supported with both online, cloud-based pipelines through QIAGEN GeneGlobe and with on-site software through QIAGEN CLC Genomics Workbench. QIAseq UPX data analysis enables primary mapping, single-cell clustering analysis, and differential expression analysis for both single-cell and bulk sequencing applications.

For applications involving blood, QIAseq FastSelect –rRNA Globin can be used to remove the globin mRNA from the RNA-seq library. The globin genes may represent up to 90% of the mRNA component, and removal of this ubiquitous mRNA during cDNA synthesis can result in RNA-seq libraries with more sensitivity.

Collectively, the Sample to Insight workflow of the QIAseq UPX 3' Transcriptome Kit defines a new generation of high-throughput NGS technologies for gene expression analysis from eukaryotic cells and RNA samples (Figure 1).

Figure 1. QIAGEN’s Sample to Insight QIAseq UPX 3' Transcriptome workflow.

Principle and procedure

The QIAseq UPX 3' Transcriptome Kit workflow (Figure 2 and Figure 3) is described below. A magnetic bead-based cleanup using QIAseq beads (included with the kit) is performed between each step.

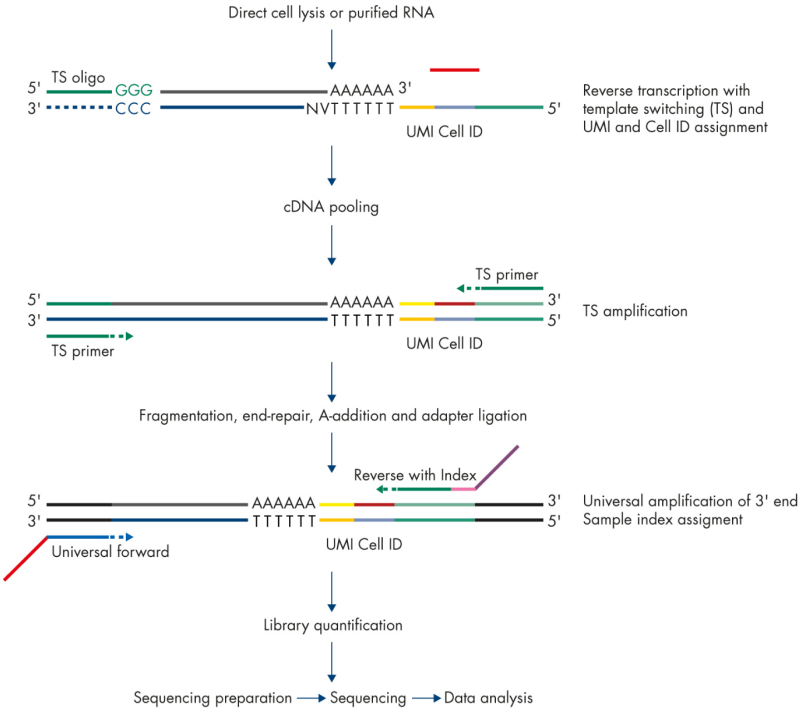

Figure 2. QIAseq UPX 3' Transcriptome Kit workflow.

- **Start with purified total RNA, cell lysates, or single cells**: 10 pg – 10 ng of purified RNA can be used for each sample, or alternatively single cells or cell lysates up to 1000 cells can be lysed using the UPX cell lysis buffer.

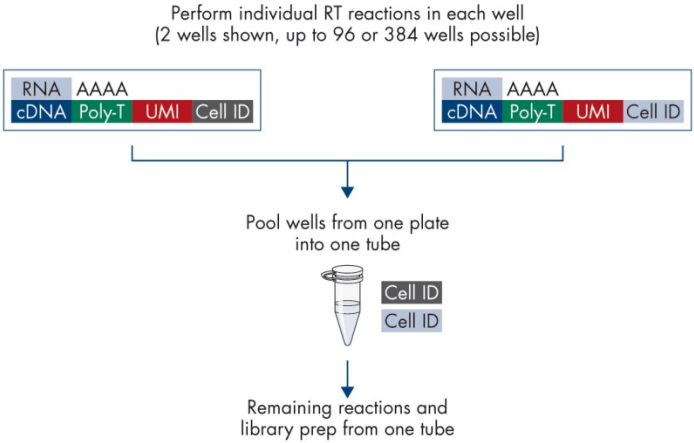

Figure 3. QIAseq UPX 3' Transcriptome Kit handling. Individual reverse transcription reactions are performed for each sample that barcodes the cDNA with a unique ID (Cell ID) and each molecule with a unique molecular index (UMI). Following reverse transcription, all wells from a plate can be pooled into a single tube. The remaining reactions and library prep associated with that plate are performed in a single tube.

- **Rebuffer QIAseq Beads**: QIAseq Beads are re-buffered with NGS Binding Buffer to create RQ Beads. RQ Beads are stable for 1 week when stored at 2–8°C.
- **Reverse transcription**: The LNA-enhanced anchored oligo-dT reverse transcription (RT) primer contains an integrated cell ID and UMI (Figure 4A); the UMI is a 12-base, fully random sequence. Statistically, this provides 4^{12} possible UMIs per RT primer, and each cDNA molecule in the sample receives a unique UMI sequence. Following reverse transcription, all cDNAs from a single plate, whether it is 96-well or 384-well, can be combined in a single tube due to the unique cell IDs. The UMI is used during data analysis to remove PCR duplication. A template-switching oligonucleotide is included in the reaction, which in combination with the RT primer, enables subsequent universal amplification of every cDNA molecule.
- **Reverse Transcription Primer Plate formats and recommended uses**: The RT primers are provided lyophilized in three different plate formats for different uses: Breakable 96-well plates (cat. no. 333088), standard-hard shell 96-well plates (for 384 samples) (cat. no. 333089), or a single-use 384-well plate (cat. no. 333090) (Figure 4B and Figure 4C).

Each well in the breakable 96-well plate (cat. no. 333088) is intended to be directly used for the reverse transcription reaction, one well for each sample. Researchers can cut the plate by row or column to use as little as 8 samples per library and store the remaining lyophilized RT primers until they have additional samples to process.
The standard-hard shell multi-use 96-well plate (cat. no. 333089) contains RT primers, which are resuspended before use. This format is intended for researchers who want to distribute the RT primer into another container for the reverse transcription reaction. Each resuspended RT primer can be distributed up to 4 times for a total of 384 reactions (cat. no. 333088).
The single-use 384-well format (cat. no. 333090) is intended to be directly for the reverse transcription reaction. As a note, the full plate must be used together.
For the 96-well plates, 96 different RT primers are provided, each with a unique cell ID.
For the 384-well plates, 384 different RT primers are provided, each with a unique cell ID.

A

NVTTTTTT(n)–[UMI]–[cell ID]————

B

	1	2	3	4	5	6	7	8	9	10	11	12
A	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
B	C13	C14	C15	C16	C17	C18	C19	C20	C21	C22	C23	C24
C	C25	C26	C27	C28	C29	C30	C31	C32	C33	C34	C35	C36
D	C37	C38	C39	C40	C41	C42	C43	C44	C45	C46	C47	C48
E	C49	C50	C51	C52	C53	C54	C55	C56	C57	C58	C59	C60
F	C61	C62	C63	C64	C65	C66	C67	C68	C69	C70	C71	C72
G	C73	C74	C75	C76	C77	C78	C79	C80	C81	C82	C83	C84
H	C85	C86	C87	C88	C89	C90	C91	C92	C93	C94	C95	C96

C

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17	C18	C19	C20	C21	C22	C23	C24
B	C25	C26	C27	C28	C29	C30	C31	C32	C33	C34	C35	C36	C37	C38	C39	C40	C41	C42	C43	C44	C45	C46	C47	C48
C	C49	C50	C51	C52	C53	C54	C55	C56	C57	C58	C59	C60	C61	C62	C63	C64	C65	C66	C67	C68	C69	C70	C71	C72
D	C73	C74	C75	C76	C77	C78	C79	C80	C81	C82	C83	C84	C85	C86	C87	C88	C89	C90	C91	C92	C93	C94	C95	C96
E	C97	C98	C99	C100	C101	C102	C103	C104	C105	C106	C107	C108	C109	C110	C111	C112	C113	C114	C115	C116	C117	C118	C119	C120
F	C121	C122	C123	C124	C125	C126	C127	C128	C129	C130	C131	C132	C133	C134	C135	C136	C137	C138	C139	C140	C141	C142	C143	C144
G	C145	C146	C147	C148	C149	C150	C151	C152	C153	C154	C155	C156	C157	C158	C159	C160	C161	C162	C163	C164	C165	C166	C167	C168
H	C169	C170	C171	C172	C173	C174	C175	C176	C177	C178	C179	C180	C181	C182	C183	C184	C185	C186	C187	C188	C189	C190	C191	C192
I	C193	C194	C195	C196	C197	C198	C199	C200	C201	C202	C203	C204	C205	C206	C207	C208	C209	C210	C211	C212	C213	C214	C215	C216
J	C217	C218	C219	C220	C221	C222	C223	C224	C225	C226	C227	C228	C229	C230	C231	C232	C233	C234	C235	C236	C237	C238	C239	C240
K	C241	C242	C243	C244	C245	C246	C247	C248	C249	C250	C251	C252	C253	C254	C255	C256	C257	C258	C259	C260	C261	C262	C263	C264
L	C265	C266	C267	C268	C269	C270	C271	C272	C273	C274	C275	C276	C277	C278	C279	C280	C281	C282	C283	C284	C285	C286	C287	C288
M	C289	C290	C291	C292	C293	C294	C295	C296	C297	C298	C299	C300	C301	C302	C303	C304	C305	C306	C307	C308	C309	C310	C311	C312
N	C313	C314	C315	C316	C317	C318	C319	C320	C321	C322	C323	C324	C325	C326	C327	C328	C329	C330	C331	C332	C333	C334	C335	C336
O	C337	C338	C339	C340	C341	C342	C343	C344	C345	C346	C347	C348	C349	C350	C351	C352	C353	C354	C355	C356	C357	C358	C359	C360
P	C361	C362	C363	C364	C365	C366	C367	C368	C369	C370	C371	C372	C373	C374	C375	C376	C377	C378	C379	C380	C381	C382	C383	C384

Figure 4. UPX anchor oligo-dT RT primer and Cell Index (ID) RT Plate. A: The LNA-enhanced anchored oligo-dT reverse transcription (RT) primer contains an integrated cell ID and UMI. **B**: Layout of CID-96S and CID-96M Plates. **C**: Layout of CID-384 Plate.

- **Template amplification**: Following cDNA pooling, amplification of the cDNA is performed to ensure cDNA containing UMIs are sufficiently enriched for subsequent library preparation. In this reaction, QIAGEN HiFi PCR Master Mix is used to ensure efficient, accurate amplification of the cDNA.
- **Fragmentation, end repair, A-addition, and adapter ligation**: Amplified pooled cDNA is first fragmented, end repaired, and A-tailed within a single, controlled multi-enzyme reaction. The prepared cDNA fragments are then ligated at their 5' ends with a sequencing-platform–specific adapter.
- **Universal PCR and Sample ID assignment**: Library amplification introduces up to 48 sample indices using a single indexing approach. This universal amplification approach ensures that the DNA fragments containing the cell ID and UMI are sufficiently amplified for NGS. When using 96-well plates (cat. nos. 333088 or 333089), up to 48 plates can be multiplexed together for a total of 4608 samples per flow cell lane. When starting with the 384-well formats (cat. no. 333090), a total of 18,432 samples can be sequenced together in a single flow cell lane.

- **Library quantification**: QIAGEN's QIAseq Library Quant Array Kit or Assay Kit (cat. no. 333304 or 333314), which contains laboratory-verified forward and reverse primers together with a DNA standard, is highly recommended for accurate quantification of the prepared library. Real-time PCR-based methods provide an accurate quantification of complete RNA-seq libraries with full adapter sequences and improves the ability to generate high-quality data.
  The library yield measurements of Qubit® or Nanodrop® or the Agilent® Bioanalyzer® and TapeStation® systems use fluorescence dyes, which intercalate into DNA or RNA and cannot discriminate between cDNA with or without adapter sequences and can also be inaccurate due to the inability to accurately estimate a library with diverse sizes.
- **NGS**: The QIAseq UPX 3' Transcriptome 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 of at least 150 cycles). Recommendations for read allocation is dependent on RNA or cell input, and gene expression levels.

Table 1. Read allocation recommendations per sample

RNA or cell input per sample	Number of paired-end reads per sample
10 pg RNA	100,000
1 ng RNA	2,500,000
10 ng RNA	5,000,000
Single cell	100,000
100 cells	2,500,000
1000 cells	5,000,000

- **Data analysis**: The QIAseq UPX 3' Transcriptome Kit is supported using an online, cloud-based RNA-seq pipeline in QIAGEN GeneGlobe Analysis and through on-site software provided by QIAGEN CLC Genomics Workbench. The pipelines automatically perform all steps necessary for primary mapping, single-cell cluster analysis, and differential expression for single-cell and 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.

- **Recommended Library Quantification Method**: QIAseq Library Quant System: QIAseq Library Quant Array Kit (QIAGEN cat. no. 333304) or QIAseq Library Quant Assay Kit (QIAGEN cat. no. 333314)
- UPX Lysis Buffer and RNA-seq Inhibitor (optional – 6 ml of Cell Lysis Buffer, RNase Inhibitor, and Nuclease-free Water for applications involving cell lysates or custom applications, QIAGEN cat. no. 333076)
- 100% ethanol, ACS-grade
- QIAGEN QIAseq FastSelect –Globin Kit, QIAseq FastSelect –rRNA Kit, QIAseq FastSelect –rRNA/Globin Kit (optional – useful for removal of globin mRNA or rRNA for RNA-seq applications from blood and other samples)
- 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
- MagneSphere® Technology Magnetic Separation Stand (Promega cat. no. Z5342)
- Optional DNA/RNA Concentration:
  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)

- Library QC methods:
  2100 Bioanalyzer (Agilent cat. no. varies)

  Agilent High Sensitivity DNA Kit (Agilent cat. no. 5067-4626)
- Optional RNA samples for process optimization:
  Human XpressRef Universal Total RNA (QIAGEN cat. no. 338112)

  Mouse XpressRef Universal Total RNA (QIAGEN cat. no. 338114)

  Rat XpressRef Universal Total RNA (QIAGEN cat. no. 338116)
- Optional RNA spike-in:
  ERCC ExFold RNA Spike-In Mixes (Thermo Fisher Scientific cat. no. 4456739)

Important Notes

- When starting with live cells, single cells or pellets, start with Section 1, then follow Section 3, Section 4, and Section 5.
  Note: Up to 1000 cells will be lysed using the provided Cell Lysis Buffer and RNase Inhibitor
- When starting with isolated RNA, start with Section 2, then follow Section 3, Section 4, and Section 5.
  Note: 10 pg – 10 ng of purified total RNA can be used. QIAGEN provides a range of solutions for purification of total RNA from different amounts of sample (Table 2).

Table 2. Recommended kits for purification of total RNA

Kit	Cat. no.	Starting material
QIAGEN RNeasy® Micro Kit	74004	Small amounts of cells and tissue
QIAGEN RNeasy Mini Kit	74104 and 74106	Animal/human tissues and cells
QIAGEN RNeasy 96 Kit	74181 and 74182	Animal/human tissues and cells

- Ensure that RNA samples are of high quality and free of inhibitors that would compromise a reverse transcription or PCR reactions. For more information on 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/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® Advanced 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 as well as 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 indicated sections.

Section 1: QIAseq UPX 3′ Transcriptome Libraries When Starting From Cells

Protocol: Cell Lysis

Important points before starting

- This protocol is for starting with cells as the input material. If starting with purified RNA, please proceed to "Section 2: QIAseq UPX 3′ Transcriptome Libraries When Starting from Purified RNA".
- The recommended starting amount is 1 to 1000 cells per reverse transcription reaction.
- At least eight samples should be used to build a QIAseq UPX 3′ Transcriptome library to ensure enough sequence diversity is available for proper sequencing on Illumina NGS instruments.
- Three options are available for cell-based protocols (described in Figure 4, page 13):
  - CID-96S Plate: 96-well single-use Cell ID RT Plate (cat. no. 333088)
  - CID-384 Plate: 384-well single-use Cell ID RT Plate (cat. no. 333090)
  - Empty 96-well plates
- **Important**: When cells have been collected into empty 96-well plates, RT primers must be dispensed from CID-96M Plate, the multi-use 96-well Cell ID RT Plate (cat. no. 333089, described in Figure 4, page 13), during setup of the reverse transcription reactions.
- Ensure reactions are thoroughly mixed, prepared at recommended temperatures, and incubated at recommended temperatures.
- Do not vortex reactions or reagents unless instructed.

Procedure

1. Thaw Cell Lysis Buffer and Nuclease-free Water at room temperature (15–25°C). Mix by flicking the tube. Centrifuge the tubes briefly to collect residual liquid from the sides of the tubes and keep at room temperature.
   Remove RNase Inhibitor from the –30 to –15°C freezer just before preparation of the Master Mix, and place on ice. Return RNase inhibitor to the freezer immediately after use.
2. Prepare the Cell Lysis Premix on ice as described in Table 3. Briefly centrifuge, mix by pipetting up and down 12 times, and briefly centrifuge.
   Note: The cell lysis buffer contains all components required for cell lysis (25% greater volume than what is required for the total number of reactions).

Table 3. Guide for preparation of cell lysis premix

Component	Volume 8 wells	Volume 24 wells	Volume 96 wells	Volume 384 wells
Cell Lysis Buffer	10 µl	30 µl	120 µl	480 µl
RNase Inhibitor	2.5 µl	7.5 µl	30 µl	120 µl
Nuclease-free Water	17.5 µl	52.5 µl	210 µl	840 µl
Total volume	**30 µl**	**90 µl**	**360 µl**	**1440 µl**

3. Aliquot 3 µl Cell Lysis Premix into the required wells of the Cell ID RT Plate (CID-96S Plate or CID-384 Plate) or empty 96-well plates.

   Note: The CID-96S Plate (cat. no. 333088) is breakable, allowing cells to be processed in batches. The CID-384 (cat. no. 333090) should be completely used at 1 time.
4. Capture cells into the plate containing the Cell Lysis Premix.
5. Cap or seal the plate/selected wells and gently spin down and collect cells in to the lysis buffer at the bottom on the well.
6. Incubate for 15 min on ice to lyse the cells. After 15 min, you may stop the protocol by freezing the cells at –90 to –65°C.
7. Prepare RQ Beads by rebuffering QIAseq Beads according to "Appendix A: Protocol: Rebuffering of QIAseq Beads (RQ Beads)". RQ Beads are stable for 1 week at 2–8°C from the day of preparation.
8. Proceed with "Protocol: Reverse Transcription of Lysed Cells".

Protocol: Reverse Transcription of Lysed Cells

Important points and setup before starting

- **Important**: Prepare RQ Beads prior to starting this protocol. See "Appendix A: Protocol: Rebuffering of QIAseq Beads (RQ Beads)".
- The 3 µl cell lysates from "Protocol: Cell Lysis" are the starting materials for the reverse transcription reactions.
- If using the CID-96M Plate (cat. no. 333089), add 2.5 µl Nuclease-free Water to each well, vortex the plate, centrifuge briefly, and incubate for 10 min at room temperature to fully dissolve the primer.
- **Important**: When using the CID-96M Plate (cat. no. 333089), each cell lysate must use a different reverse transcription primer in order to properly label each sample and for proper sample demultiplexing during data analysis.
- Ensure all reactions are mixed thoroughly and handled at the temperatures recommended in the protocol.
- Do not vortex reactions or reagents unless instructed.

Procedure

1. Prepare reagents required for the reverse transcription reactions. Thaw 3' Trans RT Buffer and Nuclease-free Water at room temperature. Mix by flicking the tube. Centrifuge the tubes briefly to collect residual liquid from the sides of the tubes, and keep at room temperature.

   Remove EZ Reverse Transcriptase from the –30 to –15°C freezer just before preparation of the Master Mix, and place on ice. Return the enzyme to the freezer immediately after use.

2. Prepare the RT Premix on ice as described in Table 4.

   Note: For single-cell analysis, ERCC can be added as a control. Prepare the ERCC by diluting the stock 1.25 x 10^6–fold using 0.1x TE Buffer. If ERCC control is omitted, add an additional 2.5 µl of Nuclease-free Water.

Table 4. Preparation of RT premix

Component	Volume 8 wells	Volume 24 wells	Volume 96 wells	Volume 384 wells
3' Trans RT Buffer	10 µl	30 µl	120 µl	480 µl
EZ Reverse Transcriptase	2.5 µl	7.5 µl	30 µl	120 µl
Nuclease-free Water	5 µl	15 µl	60 µl	240 µl
ERCC diluted 1.25 x 10^6-fold* (optional)	2.5 µl	7.5 µl	30 µl	120 µl
Total volume	**20 µl**	**60 µl**	**240 µl**	**960 µl**

* For single-cell analysis, ERCC can be added as a control. Prepare the ERCC by diluting the stock 1.25 x 10^6-fold using 0.1x TE Buffer. If ERCC will not be used, replace with extra Nuclease-free Water.

3. Aliquot the RT Premix

- **When using CID-96S and CID-384 Plates**: For Cell ID RT Plates (CID-96S Plate or CID-384 Plate), add 2 µl of RT Premix into 3 µl of cell lysate (Table 5a).

Table 5a. Preparation of RT Reaction for the CID-96S Plate or CID-384 Plate

Reagent	Volume per well
RT Premix	2.0 µl
Cell Lysate	3.0 µl
Total volume	**5.0 µl**

- **When using CID-96M Plates**: Aliquot 1.5 µl of RT Premix into the plate wells containing 3 µl of cell lysate. Subsequently aliquot 0.5 µl of each Cell ID RT primer from the CID-96M Plate into the appropriate well (Table 5b).
  Note: A different Cell ID RT Primer must be used for every sample.

Table 5b. Preparation of RT Reaction for CID-96M plates

Reagent	Volume per well
RT Premix	1.5 µl
Cell Lysate	3.0 µl
Cell ID RT Primer (A different Cell ID RT Primer must be used for every sample)	0.5 µl

4. Briefly centrifuge, mix by pipetting up and down, and then centrifuge briefly again.
5. Incubate as described in Table 6.

Table 6. Reverse transcription incubation

Step	Time	Temperature
1	10 min	25°C
2	90 min	42°C
3	15 min	70°C
4	∞	4°C

6. Upon completion of reverse transcription reactions, combine the synthesized cDNA from the different wells into one 2 ml microcentrifuge tube. Up to 96 wells can be combined in one tube as per Table 7.

   Note: The cDNA generated from each well of a Cell ID RT Plate contains a specific cell ID that enables tracking of that particular sample.

   Important: When working with 384 wells, perform the cleanup as 4 sets of 96 wells. The supernatants will be combined after step 14, prior to the second cleanup.

   Note: Minimally, the volume of the combined sample must be 100 µl. If the combined sample is not 100 µl, add Nuclease-free Water to bring the volume to 100 µl (indicated in Table 11 for 8 combined wells).

Table 7. Addition of RQ Beads for cDNA cleanup

Number of wells combined	Nuclease-free Water	RQ Bead volume
8	60 µl	90 µl
24	0 µl	108 µl
96*	0 µl	432 µl

* When working with 384 wells, perform the cleanup as 4 sets of 96 wells. The supernatants will be combined after step 14, prior to the second cleanup.

7. Add 0.9x (volume) of RQ Beads to the combined cDNA synthesis reactions from step 5 (e.g., 90 µl RQ Beads to 100 µl cDNA synthesis reactions). Mix well by pipetting up and down 12 times.

8. Incubate for 10 min at room temperature.

   Place the tubes/plate on a magnetic rack for 5 min. After the solution has cleared, carefully remove and discard the supernatant.

   Note: Keep the tubes with the RQ Beads on the magnetic stand while handling the supernatant and during the washing steps.

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

9. Add 200–300 µl freshly prepared 80% ethanol so as to submerge the beads. As an option, please rotate the tube (3 times) to wash the beads while sitting on the magnetic rack. Carefully remove and discard the wash.
10. Repeat the ethanol wash.

    Important: Completely remove all traces of ethanol after the second wash. To do this, briefly centrifuge and return the tubes or plate to the magnetic stand. Remove the ethanol first with a 200 µl pipette, and then with a 10 µl pipette to remove any residual ethanol.

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

    Note: Visually inspect that the pellet is completely dry.

12. Remove the tube from the magnetic rack and elute the DNA from the beads by adding 25 µl Nuclease-free Water. Mix well by pipetting.
13. Return the tube/plate to the magnetic rack until the solution has cleared.

14. Transfer 23 µl of the supernatant to clean tubes.

    Important: When working with 384 wells (CID-384 plates), combine all 4 eluates to give 92 µl.

15. Adjust the supernatant volume to 100 µl using Nuclease-free Water.
16. Add 90 µl (0.9x volume) of RQ Beads to the 100 µl of supernatant. Mix well by pipetting up and down 12 times.
17. Incubate for 10 min at room temperature.

    Place the tubes/plate on a magnetic rack for 5 min. After the solution has cleared, carefully remove and discard the supernatant.

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

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

18. Add 200 µl of 80% ethanol. Rotate the tube (3 times) to wash the beads. Carefully remove and discard the wash.
19. Repeat the ethanol wash.

    Important: Completely remove all traces of ethanol after this second wash.
    To do this, briefly centrifuge and return the tubes or plate to the magnetic stand.
    Remove the ethanol first with a 200 µl pipette, and then a 10 µl pipette to remove any residual ethanol.

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

    Note: Visually inspect that the pellet is completely dry. Ethanol carryover to the next step will negatively affect reaction efficiency.

21. Remove the beads from the magnetic stand and elute the DNA from the beads by adding 13 µl Nuclease-free Water. Mix well by pipetting.
22. Return the tube/plate to the magnetic rack until the solution has cleared.
23. Transfer 11 µl of the supernatant to clean tubes.
24. From this point forward, the procedures assume that all cDNA wells (either 8, 24, 96, or 384) have been combined into a single tube.

25. Proceed with “Optional Protocol: Quantitative Determination of Template Amplification”, page 32. Alternatively, the samples can be stored at –30 to –15°C in a constant-temperature freezer.

    Note: If the number of cycles required for template amplification has already been determined, proceed with “Protocol: Template Amplification”, page 35.

Section 2: QIAseq UPX 3′ Transcriptome Libraries When Starting from Purified RNA

Protocol: Reverse Transcription of Purified RNA

Important points before starting

- This protocol can be used with low amounts of purified RNA (10 pg – 10 ng).
- When working with low amounts of purified RNA, 3 options are available:
  CID-96S Plate: 96-well single-use Cell ID RT Plate

  CID-384 Plate: 384-well single-use Cell ID RT Plate

  Empty 96-well plates
- **Important**: When using empty 96-well plates, RT primers must be dispensed from the CID-96M Plate (cat. no. 333089) during setup of the reverse transcription reactions. Each CID-96M Plate well contains a pre-dispensed, anchored oligo-dT primer containing a UMI and cell ID required for the reverse transcriptase reaction. Prior to use, add 2.5 µl Nuclease-free Water to each well, vortex the plate, centrifuge briefly, and incubate for 10 min at room temperature to fully dissolve the primer.
- Ensure all reactions are mixed thoroughly and handled at the temperatures recommended in the protocol.
- **Important**: Prepare RQ Beads prior to starting the Protocol: Reverse Transcription of Purified RNA. See "Appendix A: Protocol: Rebuffering of QIAseq Beads (RQ Beads)".
- Do not vortex reactions or reagents unless instructed.

Procedure

1. Prepare reagents required for the reverse transcription reactions. Thaw Cell Lysis Buffer, 3' Trans RT Buffer, and Nuclease-free Water at room temperature. Mix by flicking the tube. Centrifuge the tubes briefly to collect residual liquid from the sides of the tubes and keep at room temperature.

Remove the EZ Reverse Transcriptase and the RNase Inhibitor from the –30 to –15°C freezer just before preparation of the Master Mix, and place on ice. Return both enzymes to the freezer immediately after use.

2. Prepare the RT Premix on ice as described in Table 8.

Table 8. Preparation of RT premix

Component	Volume 8 wells	Volume 24 wells	Volume 96 wells	Volume 384 wells
Cell Lysis Buffer	10 µl	30 µl	120 µl	480 µl
RNase Inhibitor	2.5 µl	7.5 µl	30 µl	120 µl
3' Trans RT Buffer	10 µl	30 µl	120 µl	480 µl
EZ Reverse Transcriptase	2.5 µl	7.5 µl	30 µl	120 µl
Total volume	**25 µl**	**75 µl**	**300 µl**	**1200 µl**

Table 9a: Preparation of RT Reaction for single-use Cell ID RT Plates (CID-96S Plate or CID-384 Plate)

Component	Volume per well
RT Premix	2.5 µl
Purified RNA	2.5 µl

Table 9b: Preparation of RT Reaction for CID-96M plates

Component	Volume per well
RT Premix	2.5 µl
Purified RNA	2.0 µl
Cell ID RT Primer (A different Cell ID RT Primer must be used for every sample)	0.5 µl

3. Aliquot the RT Premix and purified RNA

   3a. For CID-96S and CID-384 plates, follow Table 9a
   Aliquot 2.5 µl of RT Premix into the wells of the plate. Subsequently add 2.5 µl of purified RNA to each well.

3b. For CID-96M Plates, follow Table 9b

Aliquot 2.5 µl of RT Premix into each well of an empty 96- or 384-well plate. Subsequently add 2 µl of purified RNA and 0.5 µl of each Cell ID RT primer from the CID-96M plate into the appropriate well.

Note: A different Cell ID RT Primer must be used for every sample.

4. Briefly centrifuge, mix by pipetting up and down, and then centrifuge briefly again.
5. Incubate as described in Table 10.

Table 10. Reverse transcription incubation

Step	Time	Temperature
1	10 min	25°C
2	90 min	42°C
3	15 min	70°C
4	∞	4°C

6. Upon completion of the reverse transcription reactions, combine the synthesized cDNA from the different wells into one 2 ml microcentrifuge tube. Up to 96 wells can be combined in one tube.

   Note: The cDNA generated from each well of a Cell ID RT Plate contains a specific cell ID that enables tracking of that particular sample.

   Note: Minimally, the volume of the combined sample must be 100 µl. If the combined sample is not 100 µl, add Nuclease-free Water to bring the volume to 100 µl (indicated in Table 11 for 8 combined wells).

Table 11. Addition of RQ Beads for cDNA cleanup

Number of wells combined	Nuclease-free Water	QIAseq Bead volume
8	60 µl	90 µl
24	0 µl	108 µl
96*	0 µl	432 µl

* When working with 384 wells, perform the cleanup as 4 sets of 96 wells. The supernatants will be combined after step 14, prior to the second cleanup.

Important: When working with 384 wells, perform the cleanup as 4 sets of 96 wells. The supernatants will be combined after step 14, prior to the second cleanup.

7. Add 0.9x (volume) of RQ Beads to the combined cDNA synthesis reactions from step 5 (e.g., 90 µl beads to 100 µl synthesis reactions). Mix well by pipetting up and down 12 times.
8. Incubate for 10 min at room temperature.

   Place the tubes/plate on a magnetic rack for 5 min. After the solution has cleared, carefully remove and discard the supernatant.

   Note: Keep the beads on the magnetic stand while handling the supernatant and during the washing steps.

   Important: Do not discard the beads, because they contain the DNA of interest.
9. Add 200–300 µl freshly prepared 80% ethanol (enough to submerge the beads). As an option, please rotate the tube (3 times) to wash the beads while sitting on the magnetic rack. Carefully remove and discard the wash.
10. Repeat the ethanol wash.

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

    Note: Visually inspect that the pellet is completely dry.
12. Remove the beads from the magnetic stand and elute the DNA from the beads by adding 25 µl Nuclease-free Water. Mix well by pipetting.
13. Return the tube/plate to the magnetic rack until the solution has cleared.
14. Transfer 23 µl of the supernatant to clean tubes.

    Important: When working with 384 wells, combine all 4 eluates to give 92 µl.
15. Adjust the supernatant volume to 100 µl using Nuclease-free Water.
16. Add 0.9x (volume) of RQ Beads. Mix well by pipetting up and down 12 times.

17. Incubate for 10 min at room temperature.

    Place the tubes/plate on a magnetic rack for 5 min. After the solution has cleared, carefully remove and discard the supernatant.

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

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

18. Add 200 µl 80% ethanol. Rotate the tube (3 times) to wash the beads. Carefully remove and discard the wash.

19. Repeat the ethanol wash.

    Important: Completely remove all traces of ethanol after this second wash. To do this, briefly centrifuge and return the tubes or plate to the magnetic stand. Remove the ethanol first with a 200 µl pipette, and then a 10 µl pipette to remove any residual ethanol.

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

    Note: Visually inspect that the pellet is completely dry. Ethanol carryover to the next step will affect reaction efficiency.

21. Remove the beads from the magnetic stand and elute the DNA from the beads by adding 13 µl Nuclease-free Water. Mix well by pipetting.

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

23. Transfer 11 µl of the supernatant to clean tubes.

24. From this point forward in the protocol, the procedures assume that all cDNA wells (either 8, 24, 96, or 384) have been combined into a single tube.

25. Proceed with "Optional Protocol: Quantitative Determination of Template Amplification", page 32. Alternatively, the samples can be stored at –30 to –15°C in a constant-temperature freezer.

    Note: If the number of cycles required for template amplification has already been determined, proceed with "Protocol: Template Amplification", page 35. Alternatively, the samples can be stored at –30 to –15°C in a constant-temperature freezer.

Section 3: QIAseq UPX 3′ Transcriptome Library Completion Following Pooling of cDNA

Optional Protocol: Quantitative Determination of Template Amplification

Important points before starting

- The starting material is 1 µl of the product from the cDNA cleanup in "Protocol: Reverse Transcription of Lysed Cells" or "Protocol: Reverse Transcription of Purified RNA".
- This protocol is used to determine the number of cycles required in "Protocol: Template Amplification". If the number of cycles required for template amplification has already been determined, proceed to "Protocol: Template Amplification", page 35.
- 2x Quant AMP MM contains a green fluorescent dye that intercalates into double-stranded DNA molecules. Avoid exposure to light for a prolonged period of time.

Procedure

1. Prepare reagents required for the quantitative amplification reaction. Thaw UPX AMP Primer and 2x Quant AMP MM at room temperature. Mix by flicking the tube. Centrifuge the tubes briefly to collect residual liquid from the sides of the tubes.
2. On ice, prepare the library amplification reaction according to Table 12. Briefly centrifuge, mix by pipetting up and down 12 times, and centrifuge briefly again.

Table 12. Setup of quantitative amplification reactions

Component	Volume/reaction
Product from reverse transcription cleanup	1 µl
2x Quant AMP MM	10 µl
UPX AMP Primer	1.6 µl
Nuclease-free Water	7.4 µl
Total volume	**20 µl**

3. Incubate the reaction in a real-time PCR instrument as described in Table 13.

   Important: Do not collect data during the first 4 cycles.

Table 13. Quantitative amplification protocol

Step	Time	Temperature
Hold	2 min	98°C
3-step cycling		
Denaturation	20 s	98°C
Annealing	45 s	65°C
Extension	3 min 30 s	72°C
Cycle number	**4 cycles**	
3-step cycling		
Denaturation	20 s	98°C
Annealing	20 s	67°C
Extension*	3 min 30 s	72°C
Cycle number	**40 cycles**	
Hold	∞	4°C

* Perform fluorescence data collection using an excitation of 490–500 nm and emission at 515–527 nm.

4. When the run has finished, observe the amplification plot in **Log View** and define the baseline using **auto baseline**. Using the **Log View** of the amplification plot, determine the cycle in which the amplification curve reaches its Plateau Phase, and plan to use 3 cycles fewer for the actual amplification step.

   A typical amplification curve is shown in Figure 5. The arrow indicates the point when the curve reaches the plateau phase. If 100 pg of isolated RNA is used as a template, the curve should reach its plateau around cycle 24, which means ~21 cycles should be used for the amplification. Variations can occur, but the cycle numbers should not deviate greatly from this. If more or less RNA is used, the cycle number should increase or decrease by ~3 cycles per 10-fold change in concentration.

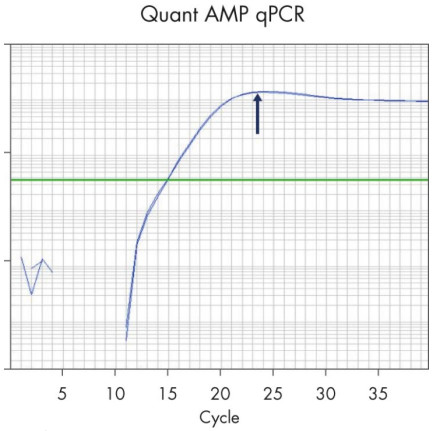

Figure 5. Screenshot of a typical amplification curve. The PCR reaches its peak at 24 cycles (as indicated by the arrow). The correct cycle number for amplification would therefore be 21 cycles (100 pg of RNA were used).

5. Proceed with “Protocol: Template Amplification”, page 35.

Protocol: Template Amplification

Important points before starting

- Use 10 µl of the product from the cDNA cleanup in "Protocol: Reverse Transcription of Lysed Cells" or "Protocol: Reverse Transcription of Purified RNA" as the starting material.
- The number of cycles of template amplification should be determined using "Optional Protocol: Quantitative Determination of Template Amplification".

Procedure

1. Prepare reagents required for the amplification reaction. Thaw UPX AMP Primer and 2x QIAGEN HiFi PCR MM at room temperature. Mix by flicking the tube. Centrifuge the tubes briefly to collect residual liquid from the sides of the tubes.
2. On ice, prepare the library amplification reaction according to Table 14. Briefly centrifuge, mix by pipetting up and down 12 times, and centrifuge briefly again.

Table 14. Setup of template amplification reactions

Component	Volume/reaction
Product from reverse transcription cleanup	10 µl
2x QIAGEN HiFi PCR MM	25 µl
UPX AMP Primer	4 µl
Nuclease-free Water	11 µl
Total volume	**50 µl**

3. Incubate the reaction in a thermal cycler, as described in Table 15.

Table 15. Template amplification protocol

Step	Time	Temperature
Hold	2 min	98°C
3-step cycling		
Denaturation	20 s	98°C
Annealing	45 s	65°C
Extension	3 min 30 s	72°C
Cycle number	**4 cycles**	
3-step cycling		
Denaturation	20 s	98°C
Annealing	20 s	67°C
Extension	3 min 30 s	72°C
Cycle number	**Based on results from "Optional Protocol: Quantitative Determination of Template Amplification"**	
Hold	∞	4°C

4. Once the amplification has finished, add 50 µl Nuclease-free Water to bring each sample to 100 µl.
5. Add 60 µl RQ Beads. Mix well by pipetting up and down 12 times.
6. Incubate for 10 min at room temperature.

   Place the tubes/plate on a magnetic rack for 5 min. After the solution has cleared, carefully remove and discard the supernatant.

   Note: Keep the beads on the magnetic stand while handling the supernatant and during the washing steps.

   Important: Do not discard the beads, because they contain the DNA of interest.
7. Add 200 µl 80% ethanol. Rotate the tube (3 times) to wash the beads. Carefully remove and discard the wash.
8. Repeat the ethanol wash.

Important: Completely remove all traces of ethanol after this second wash. To do this, briefly centrifuge and return the tubes or plate to the magnetic stand. Remove the ethanol first with a 200 µl pipette, and then a 10 µl pipette to remove any residual ethanol.

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

   Note: Visually inspect that the pellet is completely dry. Ethanol carryover will affect reaction efficiency.

10. Remove the beads from the magnetic stand and elute the DNA from the beads by adding 13 µl Nuclease-free Water. Mix well by pipetting.
11. Return the tube/plate to the magnetic rack until the solution has cleared.
12. Transfer 11 µl of the supernatant to clean tubes/plate.
13. Determine the concentration of the sample using a Qubit Fluorometer.
14. Proceed with "Protocol: Fragmentation, End Repair, and A-Addition", page 38. Alternatively, the completed library amplification product can be stored at –30 to –15°C in a constant-temperature freezer.

Protocol: Fragmentation, End Repair, and A-Addition

Important point before starting

- Before setting up the reaction, it is critical to accurately determine the amount of input DNA. 50 ng is recommended. A Qubit fluorometer should be used for quantification of the DNA.

Procedure

1. Prepare reagents required for the amplification reaction. Thaw 10x Fragmentation Buffer and FERA Solution at room temperature. Mix by flicking the tube. Centrifuge the tubes briefly to collect residual liquid from the sides of the tubes.

   Remove the Fragmentation Enzyme Mix from the –30 to –15°C freezer just before preparation of the Master Mix, and place on ice. After use, immediately return the enzyme to the freezer.
2. Prepare the library amplification reaction on ice, according to Table 16. Briefly centrifuge, mix by pipetting up and down 12 times, and centrifuge briefly again.

Table 16. Setup of fragmentation, end repair, and A-addition reactions

Component	Volume/reaction
Product from template amplification cleanup (50 ng)	Variable
Fragmentation Buffer, 10x	2.5 µl
FERA Solution	0.75 µl
Nuclease-free Water	Variable
Fragmentation Enzyme Mix	5 µl
Total volume	**25 µl**

3. Program the thermal cycler according to Table 17.

Table 17. Fragmentation, end repair, and A-addition incubation

Step	Incubation temperature	Incubation time
1	4°C	1 min
2	32°C	5 min
3	65°C	30 min
4	4°C	Hold

4. Prior to adding the tubes/plate to a thermal cycler, start the program. When the thermal cycler reaches 4°C, pause the program.

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

5. Transfer the tubes/plate prepared in step 2 to the prechilled thermal cycler and resume the cycling program.
6. Upon completion, allow the thermal cycler to return to 4°C.
7. Place the samples on ice and immediately proceed with “Protocol: Adapter Ligation”, page 40.

Protocol: Adapter Ligation

Important point before starting

- The entire reaction from the fragmentation, end repair, and A-addition reaction is the template for the adapter ligation reaction.

Procedure

1. Prepare reagents required for the ligation reaction. Thaw 5x Ligation Buffer, UL Trans Adapter, and Ligation Solution at room temperature. Mix by flicking the tube. Centrifuge the tubes briefly to collect residual liquid from the sides of the tubes.
   Remove the DNA Ligase from the –30 to –15°C freezer just before preparation of the Master Mix, and place on ice. After use, immediately return the enzyme to the freezer.
2. On ice, prepare the library amplification reaction according to Table 18. Briefly centrifuge, mix by pipetting up and down 12 times, and centrifuge briefly again.

Table 18. Setup of adapter ligation reactions

Component	Volume/reaction
Fragmentation, end repair, and A-addition reaction (already in tube)	25 µl
Ligation Buffer, 5x	10 µl
DNA Ligase	5 µl
UL Adapter	1.4 µl
Ligation Solution	7.2 µl
Nuclease-free Water	1.4 µl
Total volume	**50 µl**

3. Incubate for 15 min at 20°C.

   Important: Do not use a heated lid during the ligation.
4. Add 50 µl Nuclease-free Water to bring each sample to 100 µl.
5. Add 60 µl RQ Beads. Mix well by pipetting up and down 12 times.
6. Incubate for 10 min at room temperature.

Place the tubes/plate on a magnetic rack for 5 min. After the solution has cleared, carefully remove and discard the supernatant.

Note: Keep the beads on the magnetic stand while handling the supernatant and during the washing steps.

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

7. Add 200 µl 80% ethanol. Rotate the tube (3 times) to wash the beads. Carefully remove and discard the wash.
8. Repeat the ethanol wash.

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

   Note: Visually inspect that the pellet is completely dry. Ethanol carryover will negatively affect reaction efficiency.
10. Remove the beads from the magnetic stand and elute the DNA from the beads by adding 13 µl Nuclease-free Water. Mix well by pipetting.
11. Return the tube/plate to the magnetic rack until the solution has cleared.
12. Transfer 11 µl of the supernatant to clean tubes/plate.
13. Proceed with “Optional Protocol: Quantitative Universal PCR”, page 42. Alternatively, the samples can be stored at –30 to –15°C in a constant-temperature freezer.

    Note: If the number of cycles required for template amplification has already been determined, proceed with “Protocol: Universal PCR”, page 45.

Optional Protocol: Quantitative Universal PCR

Important points before starting

- The starting material for the quantitative universal PCR reaction is 1 µl of the product from the cleanup in "Protocol: Adapter Ligation".
- This protocol is used to determine the number of cycles required in "Protocol: Universal PCR". If the number of cycles required for universal amplification has already been determined, proceed with "Protocol: Universal PCR".
- uQuant Buffer, 5x, contains a green fluorescent dye that intercalates into double-stranded DNA molecules. Avoid to exposure to light for a prolonged period of time.
- Do not vortex the HotStarTaq DNA Polymerase or library amplification reactions.

Procedure

1. Prepare reagents required for the quantitative universal PCR reaction. Thaw 5x uQuant Buffer and required index primer pair (options: QIAseq UPX 3' Trans 12 Index or QIAseq UPX 3' Trans 48 Index). Mix by flicking the tube. Centrifuge the tubes briefly to collect residual liquid from the sides of the tubes.

   Remove the HotStarTaq DNA Polymerase from the –30 to –15°C freezer just before preparation of the Master Mix, and place on ice. Return the HotStarTaq DNA Polymerase to the freezer immediately after use.

   Note: The layout and use of QIAseq UPX 3' Trans 48 Index is described in Figure 6. During reaction setup in step 2, components are added directly to the plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	U-I1	U-I2	U-I3	U-I4	U-I5	U-I6	U-I7	U-I8	U-I9	U-I10	U-I11	U-I12
B	U-I13	U-I14	U-I15	U-I16	U-I17	U-I18	U-I19	U-I20	U-I21	U-I22	U-I23	U-I24
C	U-I25	U-I26	U-I27	U-I28	U-I29	U-I30	U-I31	U-I32	U-I33	U-I34	U-I35	U-I36
D	U-I37	U-I38	U-I39	U-I40	U-I41	U-I42	U-I43	U-I44	U-I45	U-I46	U-I47	U-I48
E												
F												
G												
H												

Figure 6. QIAseq UPX 3' Trans 48 Index. Universal primer and indexing primers are pre-dried as single-use plates. During reaction setup, components are added directly to the plate. There is no need to reconstitute and transfer indices to a separate plate.

2. On ice, prepare the library amplification reaction according to Table 19. Briefly centrifuge, mix by pipetting up and down 12 times, and centrifuge briefly again.

   Note: When using the single-use QIAseq UPX 3' Trans 48 Index plate, reaction components are added directly to the plate.

Table 19. Setup of quantitative universal PCR reactions

Component	Volume/reaction Index tubes	Volume/reaction Index plate
Product from adapter ligation cleanup	1 µl	1 µl
uQuant Buffer, 5x	5 µl	5 µl
HotStarTaq DNA Polymerase	1 µl	1 µl
QIAseq UPX 3' Trans Index*	2.5 µl	0 µl
Nuclease-free Water	15.5 µl	18 µl
Total volume	**25 µl**	**25 µl**

* QIAseq UPX 3' Trans 12 Index or QIAseq UPX 3' Trans 48 Index.

3. Incubate the reaction in a real-time PCR instrument as described in Table 20.

Table 20. Quantitative uPCR protocol

Step	Time	Temperature
Hold	15 min	95°C
2-step cycling		
Denaturation	15 s	95°C
Annealing/Extension*	2 min	65°C
Cycle number	**40 cycles**	
Hold	∞	4°C

* Perform fluorescence data collection.

4. When the run has finished, observe the amplification plot in **Log View** and define the baseline using **auto baseline**.

   Using the **Log View** of the amplification plot, determine the cycle in which the amplification curve reaches its Plateau Phase, and use 3 cycles fewer. If 50 ng of cDNA was used for the previous steps, the amplification curve should read its plateau around cycle 15. Please follow the example on page 34.
5. Proceed with "Protocol: Universal PCR", page 45.

Protocol: Universal PCR

Important points before starting

- The starting material for the universal PCR reaction is 10 µl of the product from the cDNA cleanup in "Protocol: Adapter Ligation".
- The number of cycles of universal PCR amplification is determined in "Optional Protocol: Quantitative Universal PCR".
- Do not vortex the HotStarTaq DNA Polymerase or library amplification reactions.

Procedure

1. Prepare reagents required for the quantitative universal PCR reaction. Thaw 5x UPCR Buffer and required index primer pair (options: QIAseq UPX 3' Trans 12 Index or QIAseq UPX 3' Trans 48 Index). Mix by flicking the tube. Centrifuge the tubes briefly to collect residual liquid from the sides of the tubes.

   Note: The layout and use of QIAseq UPX 3' Trans 48 Index is described in Figure 6, page 43. During reaction setup in step 2, components are added directly to the plate.

   Remove the HotStarTaq DNA Polymerase from the –30 to –15°C freezer just before the preparation of the Master Mix, and place on ice. Return the HotStarTaq DNA Polymerase to the freezer immediately after use.

2. On ice, prepare the library amplification reaction according to Table 21. Briefly centrifuge, mix by pipetting up and down 12 times, and centrifuge briefly again.

   Note: When using the single-use QIAseq UPX 3' Trans 48 Index plate, reaction components are added directly to the plate.

Table 21. Setup of quantitative universal PCR reactions

Component	Volume/reaction Index tubes	Volume/reaction Index plate
Product from Adapter Ligation Cleanup	10 µl	10 µl
UPCR Buffer, 5x	5 µl	5 µl
HotStarTaq DNA Polymerase	1 µl	1 µl
QIAseq UPX 3' Trans Index*	2.5 µl	0 µl
Nuclease-free Water	6.5 µl	9 µl
Total volume	**25 µl**	**25 µl**

* QIAseq UPX 3' Trans 12 Index or QIAseq UPX 3' Trans 48 Index.

3. Incubate the reaction in a thermal cycler as described in Table 22.

Table 22. Universal PCR protocol

Step	Time	Temperature
Hold	15 min	95°C
2-step cycling		
Denaturation	15 s	95°C
Annealing/Extension	2 min	65°C
Cycle number	**Based on results from "Optional Protocol: Quantitative Universal PCR"**	
Hold	∞	4°C

4. Add 75 µl Nuclease-free Water to bring each sample to 100 µl.
5. Add 60 µl RQ Beads. Mix well by pipetting up and down 12 times.
6. Incubate for 10 min at room temperature.

7. Place the tubes/plate on a magnetic rack for 5 min. After the solution has cleared, carefully remove and discard the supernatant.

   Note: Keep the beads on the magnetic stand while handling the supernatant and during the washing steps.

   Important: Do not discard the beads, because they contain the DNA of interest.
8. Add 200 µl 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 ethanol after this second wash. To do this, briefly centrifuge and return the tubes or plate to the magnetic stand. Remove the ethanol first with a 200 µl pipette, and then a 10 µl pipette to remove any residual ethanol.
10. With the tubes (caps opened) still on the magnetic stand, air-dry at room temperature for 10 min.

    Note: Visually inspect that the pellet is completely dry. Ethanol carryover will negatively affect reaction efficiency.
11. Remove the beads from the magnetic stand and elute the DNA from the beads by adding 13 µl Nuclease-free Water. Mix well by pipetting.
12. Return the tube/plate to the magnetic rack until the solution has cleared.
13. Transfer 11 µl of the supernatant to clean tubes/plate.
14. Proceed with "Protocol: Library QC and Quantification", page 48. Alternatively, the completed library amplification product can be stored at –30 to –15°C in a constant-temperature freezer.

Protocol: Library QC and Quantification

Important points before starting

- A portion of the 11 µl sequencing library is the starting material for the library QC and quantification. When not in use, the sequencing library should be stored on ice.
- Library QC involves use of an Agilent Bioanalyzer.
- Library quantification involves the use of the QIAseq Library Quant System: the QIAseq Library Quant Array Kit or the QIAseq Library Quant Assay Kit.

Library QC (Agilent Bioanalyzer 2100)

1. Analyze 1 µl of the sequencing library on an Agilent Bioanalyzer using a High Sensitivity DNA chip according to the manufacturer's instructions. A typical appropriate-sized library is shown in Figure 7.

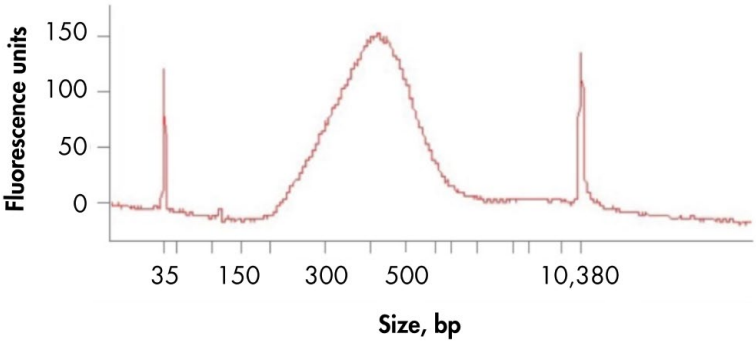

Figure 7. Bioanalyzer trace of library prepared with the QIAseq UPX 3' Transcriptome Kit. Library peaks can differ but usually fall into the range between 500 and 600 bp.

2. Proceed with library quantification (next section).

Library quantification

1. 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 with or without 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.
2. Proceed with "Protocol: Sequencing Setup on Illumina Instruments", page 50.

Section 4: QIAseq UPX 3′ Transcriptome Kit Sequencing Recommendations

Protocol: Sequencing Setup on Illumina Instruments

Important points before starting

- **Important**: Recommendations for library dilution concentrations and library loading concentrations are based on QIAseq Library Quant System.
- **Important**: The read lengths shown below are recommendations and can be adjusted if necessary; however, the read structure needs to be taken into account when changing the read length. Read 2 should not be shorter than 50.
- Guidance is provided for the MiSeq, NextSeq 500, and NovaSeq 6000 instruments. Instrument-specific imagery is included to aid in sequencing preparations. If using a different Illumina sequencing instrument, contact QIAGEN Technical Support.
  Note: A custom sequencing primer (QIAseq D Read 2 Primer I) is included with the QIAseq UPX 3′ Transcriptome Indexing Kits and may be used to shorten Read 2 from 50 to 27 bases and may be helpful under certain applications or with certain flow cells. For more information, consult Appendix C or contact QIAGEN Technical Support or a Genomics FAS Team member.

Protocol for Illumina MiSeq instruments setup

1. Sample sheet setup: Set up sample sheet using Illumina Experiment Manager, v1.2, or later (Figure 8).

Figure 8. Sample sheet wizard using Illumina Experiment Manager.

Select Category: **Other**

Select Application: **FASTQ Only**

Sample Prep Kit: **TruSeq Nano DNA**

Index Adapters: **TruSeq DNA single indexes (A, B)**

Index Reads: **1**

Read Type: **Paired End Read**

Cycles for both Read 1: **100**

Cycles for both Read 2: **50**

Important: Check **Use Adapter Trimming**

Sample dilution and pooling: Dilute libraries to 1 nM for MiSeq; then, combine libraries with different sample indexes in equimolar amounts if similar sequencing depth is needed for each library.

Library preparation and loading: Prepare and load the library on a MiSeq instrument according to the *MiSeq System Denature and Dilute Libraries Guide* (illumina Document # 15039740 v10). The final library concentration is 3 pM on MiSeq (V3 chemistry). In addition, it is highly recommended to include 10% PhiX.

2. Upon completion of the sequencing run, proceed with "Protocol: Data Analysis using QIAGEN GeneGlobe".

Protocol for Illumina NextSeq instruments:

Setup custom library prep kit in BaseSpace® Sequence Hub

The steps outlined here are intended for users generating sequencing data on a NextSeq and using the BaseSpace Sequence Hub or a BaseSpace Onsite Sequence Hub system for data analysis, which requires the use of the **Prep** tab for setup. To ensure proper sample index demultiplexing, a custom library prep kit must be created and uploaded through the **Prep** tab. To add a custom library prep kit for the QIAseq UPX 3' Transcriptome RNA Library, perform the following steps:

1. Go to **www.qiagen.com/shop/sequencing/QIAseq-UPX-3-Transcriptome-Kits**. In the **Product Resources** tab, under **Template Files**, download *Template: Setup custom library prep kit in BaseSpace Sequence Hub (NextSeq)*.
2. Log in to BaseSpace or BaseSpace Onsite and go to the **Prep** tab screen.
3. Select **Biological Samples** from the Prep tab start page.
4. Choose the samples and click **Prep Libraries**.
5. From the **Library Prep Kit** drop-down menu, select **+Custom Library Prep Kit**.
6. In the screen that appears (Figure 9), name the custom kit **UPXtranscriptome** and specify any other options such as read types (**Paired End**), indexing strategies (**Single Index**), and default read cycles (Read1 cycles **100** and Read2 cycles **50**). Click **Choose .csv File** and select **libraryprep_template_UPX_trans.csv**.

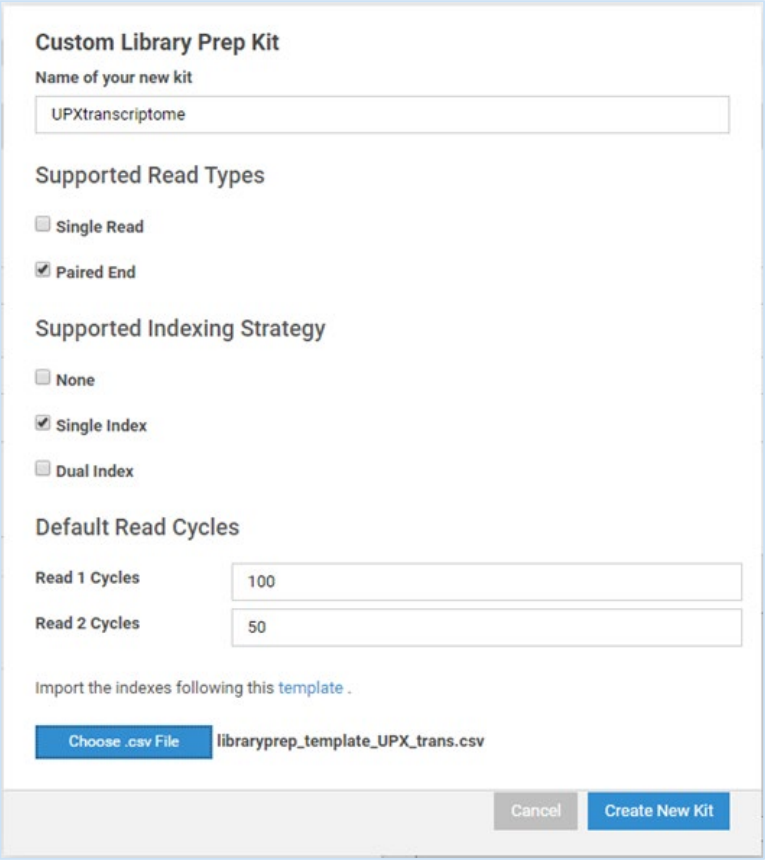

Figure 9. Custom library prep kit setup for the QIAseq UPX 3' Transcriptome Kit.

7. Click **Create New Kit** to generate library prep kit **UPXtranscriptome**. This new kit now appears in the drop-down menu and is ready for any future runs.

NextSeq: Run planning and sequencing preparations

1. From the drop-down menu on the **Libraries** tab (Figure 10), select library prep kit **UPXtranscriptome**, check the individual sample, and drag it into the corresponding well to assign **Index 1**.

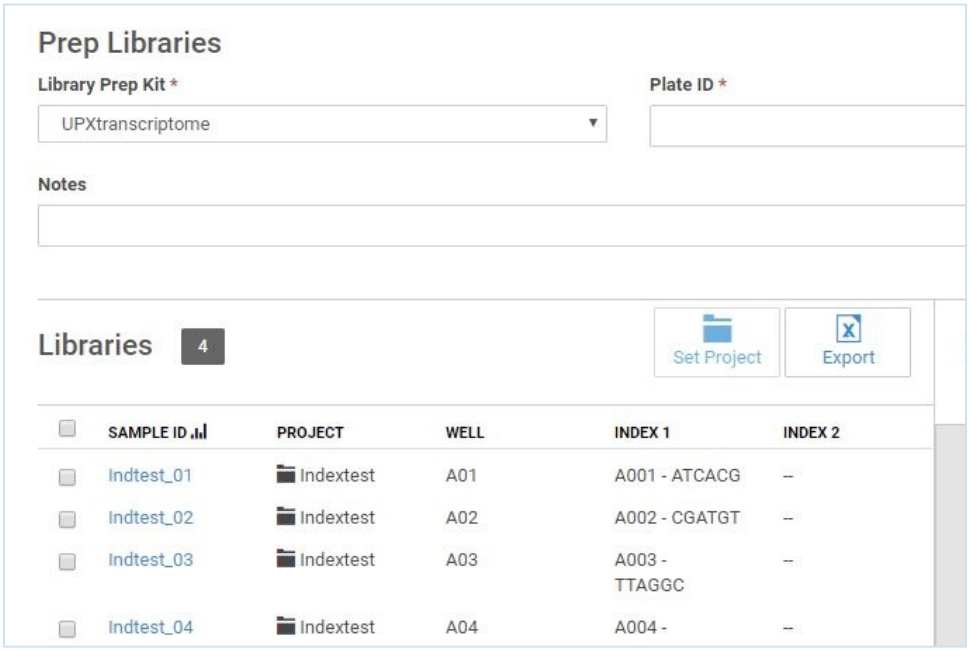

Figure 10. Assigning sample indices in the Libraries tab.

2. Once indices are assigned, select pool on the **Pools** tab and then click **Plan Run**.

   Under **Plan Run** (Figure 11):

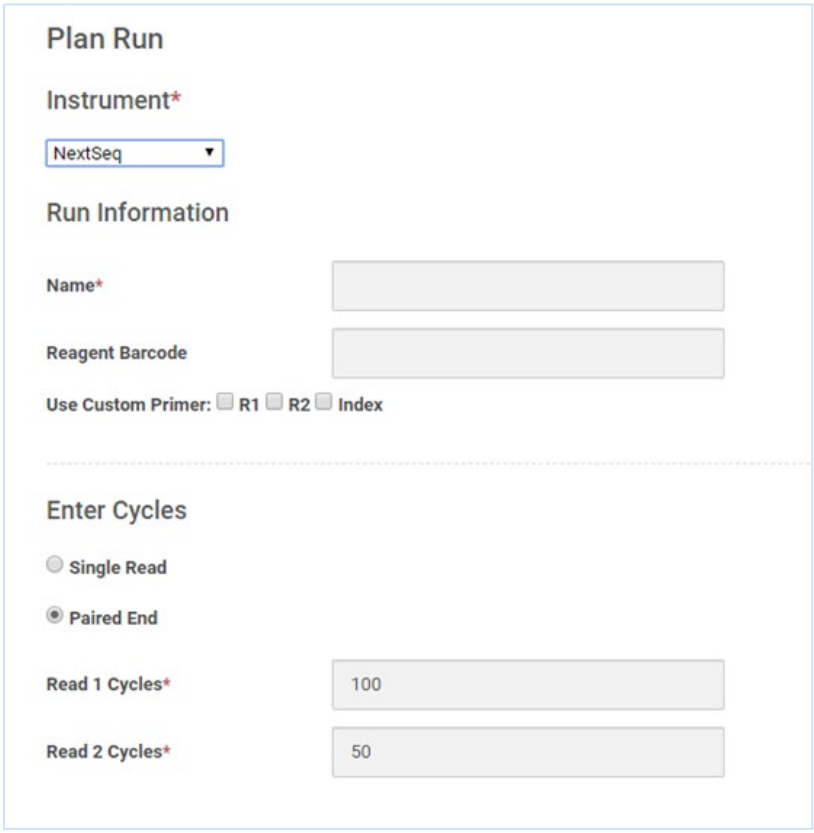

Figure 11. NextSeq run parameters.

From the **Instrument** drop-down menu, select instrument (**NextSeq**).

Under **Enter Cycles**, check **Paired End**.

For **Read 1 Cycles**, input **100**.

For **Read 2 Cycles**, input **50**.

Check **Single Index**.

For **Index 1 Cycles**, input **6**.

Sample dilution and pooling: Dilute libraries to 1 nM for NextSeq; then, combine libraries with different sample indexes in equimolar amounts if similar sequencing depth is needed for each library.

Library preparation and loading: Prepare and load library to load on a NextSeq according to the *NextSeq System Denature and Dilute Libraries Guide* (Illumina document # 15048776 v09). The final library concentration is 1.2 pM on NextSeq. Use 15% PhiX to ensure a successful sequencing run.

3. Upon completion of the sequencing run, proceed with "Protocol: Data Analysis using QIAGEN GeneGlobe", page 57.

Table 23. Summary of Illumina Sequencing recommendations for each NGS instrument tested with QIAseq UPX 3' Transcriptome Kit

Illumina Instrument	Flow cell	Recommended sequencing setup	Recommended R1	Recommended R2	Recommended index read	% PhiX
MiSeq	V3 150 cycle	Paired end	100	50	6	10%
NextSeq 500	Mid/high-output 150 cycle	Paired end	100	50	6	15%
NovaSeq 6000	200 cycle SP	Paired end	100	50	6	5%

For additional details for loading a NovaSeq run, please communicate with QIAGEN Technical Service.

For additional options for run settings and the use of the Custom R2 Primer, please refer to Appendix C in this handbook.

Section 5: QIAseq UPX 3′ Data Analysis Using QIAGEN GeneGlobe or QIAGEN CLC Genomics Workbench

Protocol: Data Analysis using QIAGEN GeneGlobe

Important point before starting

- Data analysis is available at **www.qiagen.com/GeneGlobe**.

Primary data analysis procedure

1. Click on Analyze Data and log in.
2. Under **Choose format**, select **NGS** and then select either **QIAseq UPX 3' Transcriptome/Targeted** for sample demultiplexing, read alignment, UMI, and raw transcript count or **QIAseq UPX 3' Transcriptome/Targeted – Secondary Analysis** for gene expression of transcript counts from read alignment analysis.
3. Click **Start Analysis**.
4. Under **Read Files** > **Uploaded**, choose **Upload New Files** to upload FASTQ files.
   4a. Alternatively, select **BaseSpace** to select FASTQ files directly from the run on BaseSpace. This will route you directly to **Analysis Job** (step 6)
5. Select the FASTQ files you would like to map and click **Select For Analysis**.
6. Click **Analysis Jobs**:
   6a. Click Create New Jobs.
   6b. Check **Read Files** and enter a job title for the analysis.
   6c. Select **Protocol** from the drop-down menu**: WTS** for QIAseq UPX 3' Transcriptome Kits, **Target** for QIAseq 3′ UPX Targeted RNA Panel Kits.
   6d. Select **Species** from the drop-down menu.
   6e. Select the number of files generated per lane to indicate if you are uploading concatenated lane files. Select 1-lane for a MiSeq/HiSeq/NextSeq/NovaSeq

concatenated file or 2-lane, 3-lane, and 4-lane for NextSeq/NovaSeq individual lane files.

6f. Select the correct **Cell Index Set** (for cat. nos. 333088 and 333089, select **96**; for cat. no. 333090, select **384**).

6g. Optional: Click 96 Cell Selected or 384 Cell Selected to select the cell IDs used in the experiment. Selecting which cell IDs were used can cut down on the time to demultiplex data.

Note: The blue background means the cell ID is selected.

6h. Select **Cell Index Mismatch**: **1** is default. Choose **0** for higher stringency at the risk of increased read loss.

6i. Under **Analysis Mode**, choose between **Low Input Primary Analysis** or **Single Cell Analysis**.

6j. Click **Analyze**.

Note: Periodically refresh the page by clicking the **UPX 3' Analysis Job** tab. Job status will change from "queued" to "in progress" and ultimately to "done successfully".

7. When the job is finished, you can click **Download Report** to receive the primary analysis output. If you selected **Single Cell Analysis** in step 6j, you will also receive a secondary 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 UPX 3′ Transcriptome 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 cells multiplexed per sample index	Minimally, 8 cells must be multiplexed per sample index.
b) Using the multi-use Cell ID RT Plate, primers were not properly reconstituted	Prior to use, add 2.5 µl 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	Ensure reactions are thoroughly mixed (12 times), prepared, and incubated at recommended temperatures. Do not vortex.
d) QIAseq Beads have not been rebuffered	Rebuffer QIAseq Beads using "Appendix A: Protocol: Rebuffering of QIAseq Beads (RQ Beads)", page 61.
e) 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 µl pipette, and then a 10 µl pipette to remove any residual ethanol. In addition, allow beads to dry for the appropriate amount of time.
f) Insufficient template amplification cycles	Determine optimal template amplification cycles using "Optional Protocol: Quantitative Determination of Template Amplification", page 32.
g) Insufficient universal PCR amplification cycles	Determine optimal universal PCR amplification cycles using "Optional Protocol: Quantitative Universal PCR", page 42.
Sequencing issues	
a) Too low or too high cluster density	Accurate library quantification is the key for optimal cluster density on any sequencing instrument. PCR-based quantification method is recommended. Other methods may lead to the 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: Protocol: Rebuffering of QIAseq Beads (RQ Beads)

Important points before starting

- QIAseq Beads must be rebuffered with QIAseq NGS Bead Binding Buffer to create RQ Beads. This protocol prepares rebuffered QIAseq Beads (hereafter referred to as RQ Beads).
- **Important**: QIAseq Beads and the subsequently prepared RQ Beads must be homogenous. Thoroughly resuspend the beads immediately before use and process the beads quickly. If a delay in the protocol occurs, simply vortex the beads again.
- **Important**: After preparation, store the RQ Beads at 2–8°C for up to one week.
  Note: Both the bead storage and Bead Binding Buffers are viscous. Pipette carefully to ensure the correct volumes are transferred.

Procedure

1. Thoroughly vortex QIAseq Beads and QIAseq NGS Bead Binding Buffer to mix.
   Important: Ensure the QIAseq Beads are homogenous.
2. Add QIAseq Beads to a 2 ml microcentrifuge (refer to Table 24 below for the volume of QIAseq Beads to be added depending on the number of pooled cells/samples). Then, briefly centrifuge and immediately separate the beads on a magnet rack.

Table 24. Addition of QIAseq Beads

Pooled cells/samples	QIAseq Bead volume
8	450 µl
24	450 µl
96	800 µl

Note: If beads for multiple consolidated cell/sample sets are processed together, simply scale up the amounts of QIAseq Beads and QIAseq NGS Bead Binding Buffer added below.

3. When beads have fully migrated, carefully remove and discard the supernatant.

   Note: At this step, it is acceptable to leave a small amount of supernatant in the tube.

4. Remove the tube from the magnet stand and carefully pipette 150 µl (for 8 cells/samples or 24 cells/samples) or 300 µl (for 96 cells/samples) QIAseq NGS Bead Binding Buffer onto the beads. Thoroughly vortex to completely resuspend the bead pellet. Briefly centrifuge before separating the beads on a magnet stand.
5. When beads have fully migrated, carefully remove and discard the supernatant.

   Note: Without disturbing the beads, ensure that as much supernatant as possible has been removed.

6. Remove the tube from the magnet stand and carefully pipette 450 µl (for 8 cells/samples or 24 cells/samples) or 800 µl (for 96 cells/samples) QIAseq NGS Bead Binding Buffer onto the beads (the buffer is viscous). Thoroughly vortex to completely resuspend the bead pellet.
7. Preparation of the RQ Beads is now complete. The beads can be used immediately or stored at 2–8°C for up to one week.
8. Proceed with "Protocol: Reverse Transcription of Lysed Cells" on page 21 or "Protocol: Reverse Transcription of Purified RNA" on page 27.

Appendix B: General Remarks on Handling RNA

Handling RNA

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. In order to create and maintain an RNase-free environment, the following precautions must be taken during 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. Hand 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.

RNase contamination can alternatively be removed using general laboratory reagents. To decontaminate plasticware, rinse with 0.1 M NaOH, 1 mM EDTA*, followed by RNase-free water (see "Solutions", page 64); 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.

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.

* 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.

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 h 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

Note: QIAGEN solutions, such as the components found in the QIAseq UPX 3' Transcriptome Kit, are guaranteed RNase-free without using DEPC treatment and are therefore free of any DEPC contamination.

Solutions (water and other solutions) should be treated with 0.1% DEPC. DEPC is a strong but not absolute inhibitor of RNases. DEPC 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 h at 37°C. Autoclave for 15 min 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 min.

* 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.

Appendix C: Sequencing Recommendations When Using QIAseq D Read 2 Primer I

The QIAseq UPX 3’ Transcriptome Kit can also be sequenced using a custom sequencing primer. The custom sequencing primer saves 23 bases of common sequence in the Read 2 adapter allowing for more efficient read allocation, which may be beneficial for certain sequencing runs. Under these conditions, PhiX will not be detected in Read 2. Recommended sequencing setups using the custom sequencing primer are show in Table 25.

Table 25. Sequencing setups using the custom sequencing primer

Illumina instrument	Flow cell	Recommended sequencing setup	Recommended R1	Recommended R2	Recommended index read	% PhiX
MiSeq	V2/V3 150 cycle	Paired end	100	27	6	10%
NextSeq 500	Mid/high-output 150 cycle	Paired end	100	27	6	10%
NovaSeq 6000	100 cycle SP	Paired end	80	27	6	2%

For detailed instructions on the dilution and loading of the custom R2 primer, please refer to the information provided for your respective sequencing instrument.

Appendix D: Supplementary Protocol: Reverse Transcription of Purified RNA with Integrated rRNA and/or Globin Depletion

Important points before starting:

This protocol is used instead of "Protocol: Reverse Transcription of Purified RNA".

- This protocol can be used with low amounts of purified RNA (10 pg – 10 ng).
- When working with low amounts of purified RNA, two options are described:
  CID-96S Plate: 96-well single-use Cell ID RT Plate
  CID-384 Plate: 384-well single-use Cell ID RT Plate
- Ensure all reactions are mixed thoroughly and handled at the temperatures recommended in the protocol.
- QIAseq FastSelect rRNA, diluted to 0.08x, can be used to remove residual cytoplasmic and mitochondrial rRNA.
- QIAseq FastSelect Globin mRNA, diluted to 0.08X, can be used to remove residual globin mRNA.
  Important: Prepare beads prior to starting the reverse transcription or during the incubation steps of the protocol.
- Do not vortex reactions or reagents unless instructed.

Procedure

1. Thaw the tube(s) from the QIAseq FastSelect rRNA Removal Kit. Mix by vortexing and then briefly centrifuge to collect residual liquid from the sides of the tubes.
2. Dilute an aliquot for each FastSelect tube (FastSelect rRNA and/or FastSelect Globin mRNA) to 0.08X using 2 µl FastSelect tube + 23 µl Nuclease-free Water. Mix by vortexing and then briefly centrifuge to collect residual liquid from the sides of the tubes.

3. Prepare reagents required for the reverse transcription reactions. Thaw Cell Lysis Buffer, 3' Trans RT Buffer, and Nuclease-free Water at room temperature (15–25°C). Mix by flicking the tube. Centrifuge the tubes briefly to collect residual liquid from the sides of the tubes and keep at room temperature.
   EZ Reverse Transcriptase and RNase Inhibitor should be removed from the –20°C freezer just before preparation of the master mix and placed on ice. Both enzymes should be returned to the freezer immediately after use.
4. Prepare the reaction on ice as described in Table 26. Briefly centrifuge, mix by pipetting up and down 10 times, and briefly centrifuge again.

Table 26. Preparation of RT premix for single-use Cell ID RT Plates (CID-96S Plate or CID-384 Plate)

Component	Each well*
Cell Lysis Buffer	1 µl
3' Trans RT Buffer	1 µl
QIAseq FastSelect –rRNA HMR (0.08X)	0.5 µl
QIAseq FastSelect –Globin (0.08X)	0.5 µl
RNA	2 µl†
Total volume	**5 µl**

* A master mix can also be prepared. For this, prepare 1.2X for each component. Add 3 µl to each well. Then, add 2 µl of each RNA sample separately to each well.

† The volume of RNA must be 2 µl or less. If the volume of RNA is less than 2 µl, add Nuclease-free Water to make the volume 2 µl.

Note: If using only QIAseq FastSelect –Globin or QIAseq FastSelect –rRNA HMR, replace the missing volume with Nuclease-free Water

5. Incubate as described in Table 27 using a thermal cycler with a heated lid.

Table 27. QIAseq FastSelect hybridization protocol

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	Hold at 4°C

6. Add 0.25 µl RNase Inhibitor and 0.25 µl EZ Reverse Transcriptase to each well.

   Incubate as described in Table 28. Briefly centrifuge, mix by pipetting up and down 10 times, and briefly centrifuge again.

Table 28. Reverse transcription incubation

Step	Time	Temperature
1	10 min	25°C
2	90 min	42°C
3	15 min	70°C
4	∞	4°C

7. Upon completion of the reverse transcription reactions, combine the synthesized cDNA from the different wells into one 2 ml microcentrifuge tube. Up to 96 wells can be combined in one tube.

   Note: The cDNA generated from each well of a Cell ID RT Plate contains a specific cell ID that enables tracking of that particular sample.

   Note: Minimally, the volume of the combined sample must be 100 µl. If the combined sample is not 100 µl, add Nuclease-free Water to bring the volume to 100 µl (indicated in Table 29 for 8 combined wells).

Table 29. Addition of RQ Beads for cDNA cleanup

Number of wells combined	Nuclease-free Water	QIAseq Bead volume
8	60 µl	90 µl
24	0 µl	108 µl
96*	0 µl	432 µl

* When working with 384 wells, perform the cleanup as four sets of 96 wells. The supernatants will be combined after step 15, prior to the second cleanup.

8. Add 0.9X (volume) of RQ Beads to the combined cDNA synthesis reactions from step 5 (e.g., 90 µl beads to 100 µl synthesis reactions). Mix well by pipetting up and down 12 times.
9. Incubate for 10 min at room temperature.
   Place the tubes/plate on a magnetic rack for 5 min. After the solution has cleared, carefully remove and discard the supernatant.
   Note: Keep the beads on the magnetic stand while handling the supernatant and during the washing steps.
   Important: Do not discard the beads as they contain the DNA of interest.
10. Add 200 µl 80% ethanol. Rotate the tube (three times) to wash the beads. Carefully remove and discard the wash.
11. Repeat the ethanol wash.
    Important: Completely remove all traces of the ethanol wash after this second wash. To do this, briefly centrifuge and return the tubes or plate to the magnetic stand. Remove the ethanol first with a 200 µl pipette, and then a 10 µl pipette to remove any residual ethanol.
12. With the tubes (caps opened) still on the magnetic stand, air-dry at room temperature for 10 min.
    Note: Visually inspect that the pellet is completely dry.
13. Remove the beads from the magnetic stand and elute the DNA from the beads by adding 25 µl Nuclease-free Water. Mix well by pipetting.
14. Return the tube/plate to the magnetic rack until the solution has cleared.
15. Transfer 23 µl of the supernatant to clean tubes.
    Important: When working with 384 wells, combine all four eluates to give 92 µl.

16. Adjust the supernatant volume to 100 µl using Nuclease-free Water.
17. Add 0.9X (volume) of RQ Beads. Mix well by pipetting up and down 12 times.
18. Incubate for 10 min at room temperature.
    Place the tubes/plate on a magnetic rack for 5 min. After the solution has cleared, carefully remove and discard the supernatant.
    Note: Keep the beads on the magnetic stand while handling the supernatant and during the washing steps.
    Important: Do not discard the beads as they contain the DNA of interest.
19. Add 200 µl 80% ethanol. Rotate the tube (three times) to wash the beads. Carefully remove and discard the wash.
20. Repeat the ethanol wash.
    Important: Completely remove all traces of the ethanol wash after this second wash. To do this, briefly centrifuge and return the tubes or plate to the magnetic stand. Remove the ethanol first with a 200 µl pipette and then with a 10 µl pipette to remove any residual ethanol.
21. With the tubes (caps opened) still on the magnetic stand, air-dry at room temperature for 10 min.
    Note: Visually inspect that the pellet is completely dry. Ethanol carryover to the next step will affect reaction efficiency.
22. Remove the beads from the magnetic stand and elute the DNA from the beads by adding 13 µl Nuclease-free Water. Mix well by pipetting.
23. Return the tube/plate to the magnetic rack until the solution has cleared.
24. Transfer 11 µl of the supernatant to clean tubes.
25. From this point forward in the protocol, the procedures assume that all cDNA wells (either 8, 24, 96, or 384) have been combined into a single tube.
26. Proceed with "Optional Protocol: Quantitative Determination of Template Amplification". Alternatively, the samples can be stored at –20°C in a constant-temperature freezer.
    Note: If the number of cycles required for template amplification has already been determined, proceed with "Protocol: Template Amplification". Alternatively, the samples can be stored at –20°C in a constant-temperature freezer.

Ordering Information

Product	Contents	Cat. no.
QIAseq UPX 3' Transcriptome Kit (96)	For 3' transcriptome library prep of 96 cells, cell pellets, or ultra-low input RNA samples	333088
QIAseq UPX 3' Transcriptome Kit (96-M)	For 3' transcriptome library prep of 4 x 96 cells, cell pellets, or ultralow input RNA samples	333089
QIAseq UPX 3' Transcriptome Kit (384)	For 3' transcriptome library prep of 384 cells, cell pellets, or ultralow input RNA samples	333090
QIAseq UPX 3' Trans. 12-Index (48)	Indexes and custom-read primers compatible with Illumina platforms	333074
QIAseq UPX 3' Trans. 48-Index (192)	High-throughput sample index plates and custom read primers compatible with Illumina platforms	333075
Related products		
QIAseq UPX Cell Lysis Kit (6 ml)	Reagents for Cell lysis, RNase inhibition, and Nuclease-free Water for dilutions. Enough for 6 ml of cell lysis solution	333076
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

Product	Contents	Cat. no.
Human XpressRef Universal Total RNA	2 tubes, each with 100 ug 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 ug 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 ug of total RNA prepared from several male and female rats (SD Wistar strain), whole bodies without fur	338116
QIAseq FastSelect – Globin Kit (24)	Includes tubes of QIAseq FastSelect reagent for globin mRNA removal; from human, mouse, rat, and related samples	334376
QIAseq FastSelect – rRNA HMR (24)	Includes tubes of QIAseq FastSelect reagent for rRNA removal; human, mouse, rat, and related samples	334386
QIAseq FastSelect – rRNA/Globin Kit (24)	Includes tubes of QIAseq FastSelect reagent for rRNA removal and tubes of QIAseq FastSelect reagent for globin mRNA removal; from human, mouse, rat, and related samples	335376
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

Product	Contents	Cat. no.
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 into a typical NGS workflow. CLC Genomics Workbench is available for Windows, Mac OS X, and Linux platforms	832021

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

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at **www.qiagen.com** or can be requested from QIAGEN Technical Services or your local distributor.

Document Revision History

Date	Changes
08/2019	In “Kit Contents”, volumes of Fragmentation Buffer, 10x, Fragmentation Enzyme Mix and FERA Solution for cat. no. 333089 were changed. Editorial changes.
03/2020	Change in the volume of QIAseq Beads in Box 2 from 7 ml to 10 ml.
01/2021	Updated "Shipping and Storage". Increased the number of cells and RNA that can be processed with the kit. Inserted new Figure 5 to show typical amplification curve. Specified expected C_T value and required no. of amplification cycles for universal PCR of 50 ng DNA. Added description on usual range for library peaks in Figure 7. Modified instructions for working with software user interfaces. Increased vol. of PhiX recommended for library preparation and loading in setup sequencing protocol for Illumina instruments. Removed recommendation for custom sequencing primer for Read 2 preparation and loading. Removed recommendation for RNaseKiller (5 PRIME) as method of decontamination. Corrected volumes of UPX AMP Primer and UPCR Buffer, 5x, in cat. nos. 333088 and 333090. Deleted quick-start protocol from “Kit Contents”. Inserted Appendices A, C, and D. Inserted new tables. Table 9 has been split to two parts. Replaced the artwork in Figure 2. Updated "Protocol: Data Analysis using QIAGEN GeneGlobe" by adding the "Data Analysis using QIAGEN CLC Genomics Workbench" subsection. Editorial changes and layout changes: inserted section headers and revised tables containing temperature profile.

Notes

Limited License Agreement for QIAseq UPX 3' Transcriptome Kit

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

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

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

HB-2485-007 01/2021