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QIAseq® UPX 3' Transcriptome Handbook

For high-throughput 3' transcriptome next-generation sequencing from single cells, cell pellets, and ultralow amounts of RNA

Sample to Insight

QIAGEN

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Kit Contents

QIAseq UPX 3' Transcriptome Kit **Catalog no.** **Number of reactions**	**(96)** **333088** **96**	**(96-M)** **333089** **96 x 4**	**(384)** **333090** **384**
Cell Lysis Buffer	1 ml	1 ml	1 ml
RNase Inhibitor	96 µl	150 µl	150 µl
Cell Index 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
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	85 µl	323 µl	85 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
Ligation Solution	970 µl	970 µl	970 µl
uQuant Buffer, 5x	75 µl	300 µl	75 µl
UPCR Buffer, 5x	75 µl	300 µl	75 µl
HotStarTaq® DNA Polymerase	60 µl	240 µl	60 µl
QIAseq Beads	10 ml	26 ml	10 ml
QIAseq Bead Binding Buffer	10.2 ml	34 ml	10.2 ml
Indented flat 12-cap strips	1 bag	1 bag	–
Optical adhesive film	–	–	1
384-EasyLoad Covers	–	–	1
Quick-Start Protocol	3	3	3

QIAseq UPX 3' Trans. 12-index (48) Catalog no. Number of reactions	(48) 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

Storage

The QIAseq UPX 3' Transcriptome Kit is shipped in 2 boxes. Box 1 is shipped on dry ice. Box 2 (QIAseq Beads and Cell Index RT Plate) is shipped on blue ice. Upon receipt, all components in Box 1 should be stored immediately at –30 to –15°C in a constant-temperature freezer. QIAseq Beads and the Cell Index RT Plate should be stored immediately at 2–8°C. QIAseq Index kits are shipped on dry ice and should be stored at –30 to –15°C upon arrival.

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 Sample to Insight, high-throughput next-generation sequencing (NGS) of polyadenylated RNAs from single cells on Illumina NGS instruments. The kit is intended for library construction and analysis of cell pellets (up to 100 cells) and purified RNA (10 pg – 1 ng). The QIAseq UPX 3' Transcriptome Kit presents an innovative advantage in that during reverse transcription, each cell is tagged with a unique ID (up to 384 different IDs), and each RNA molecule is tagged with a unique molecular index (UMI). Following reverse transcription with integrated template switching, all individually tagged cDNAs can be combined, which enables all subsequent library construction steps to be performed in a single tube. This prevents sample mixup, saves substantial time and dramatically reduces library prep costs. During subsequent amplification and library construction, up to 48 different Sample IDs can be assigned. Together, the combination of Cell IDs and Sample IDs enables up to 18,432 libraries to be sequenced together. Ultimately, QIAseq UPX data analysis enables primary mapping, single-cell clustering analysis and differential expression analysis. Collectively, the Sample to Insight workflow of the QIAseq UPX 3' Transcriptome Kit defines a new generation of high-throughput NGS technologies (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 is included between each step.

Direct cell lysis or purified RNA

5' TS oligo GGG AAAAAA 3'
3' CCC NVTTTTTT 5'
UMI Cell ID
Reverse transcription with template switching (TS) and UMI and Cell ID assignment

TS primer
5' AAAAAA 3'
3' TTTTTT 5'
TS primer
UMI Cell ID
TS amplification

Fragmentation, end-repair, A-addition and adapter ligation

Reverse with Index
5' AAAAAA 3'
3' TTTTTT 5'
Universal forward
UMI Cell ID
Universal amplification of 3' end
Sample index assigment

Library quantification

Sequencing preparation → Sequencing → Data analysis

Figure 2. QIAseq UPX 3' Transcriptome Kit workflow.

- **Direct cell lysis or purified total RNA**: Single cells and pellets of up to 100 cells can be lysed using the provided cell lysis buffer and RNase inhibitor. Alternatively, 10 pg – 1 ng of purified total RNA can be added to the reaction.

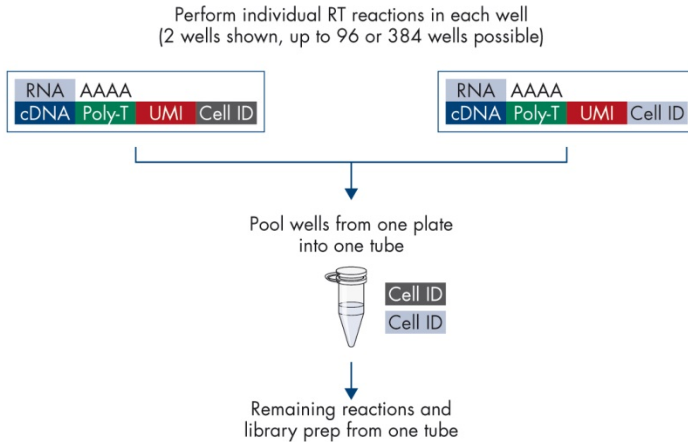

Figure 3. QIAseq UPX 3' Transcriptome Kit handling. Individual reverse transcription reactions are performed for each sample that tags the cDNA with a unique Cell ID and each molecule with a unique molecular index (UMI). Following reverse transcription, all wells for a plate (up to 96 or 384 wells, respectively) can be pooled into a single tube. The remaining reactions and library prep associated with that plate are performed in a single tube.

- **Reverse transcription:** The LNA-enhanced anchored oligo-dT reverse transcription (RT) primer contains an integrated Cell ID and UMI (Figure 4A) and is provided in single-use 96-well plates, multi-use 96-well plates or single-use 384-well plates (Figure 4B and Figure 4C). 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. The UMI is a 12-base fully random sequence. Statistically, this process provides 4^{12} possible indices per RT primer, and each cDNA molecule in the sample receives a unique UMI sequence. In addition, a template-switching oligonucleotide is included in the reaction, enabling template switching. 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.

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 96-well single-use and multi-use Cell ID RT Plates. (**C**) Layout of 384-well single-use Cell ID RT Plate.

- **Template-switching amplification**: Amplification of the template-switching reaction 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 DNA is first fragmented, end repaired and A-tailed within a single, controlled multi-enzyme reaction. The prepared DNA fragments are then ligated at their 5' ends with a sequencing-platform-specific adapter.
- **Universal PCR**: 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. For each sample index, up to 384 cell IDs can be included from the reverse transcription reaction. As a result, up to 18,432 cells can be sequenced together at one time.
- **Library quantification**: The library yield measurements of the Agilent® Bioanalyzer® or TapeStation® system using fluorescence dyes, which intercalate into DNA or RNA 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 or Assay Kit, which contains laboratory-verified forward and reverse primers together with a DNA standard, is highly recommended for accurate quantification of the prepared library.
- **NGS**: The QIAseq UPX 3' Transcriptome Kit is compatible with Illumina NGS systems (MiniSeq®, MiSeq® Personal Sequencer, NextSeq® 500, HiSeq® 1000, HiSeq 1500, HiSeq 2000, HiSeq 2500, HiSeq 3000/4000, NovaSeq™ and GAIIx). A custom sequencing primer for Read 2 (QIAseq D Read 2 Primer I) is required when using the NextSeq. Recommendations for the number of libraries that can be multiplexed are given, based on the number of multiplexed primers in Table 1.

Table 1. Read allocation recommendations per sample

RNA input	Read allocation
Single-cells	50,000
100 cells	1 M
10 pg	50,000
1 ng	1 M

- **Data analysis**: The QIAseq UPX 3' Transcriptome Kit analysis pipeline is available at **www.qiagen.com/GeneGlobe**. The pipeline automatically performs all steps necessary to perform primary mapping, single cell cluster analysis and differential expression.

Equipment and Reagents to be Supplied by User

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

- Nuclease-free pipette tips and tubes
- Microcentrifuge tubes (2 ml)
- PCR tubes (0.2 ml individual tubes or tubes strips) (VWR cat. No. 20170-012 or 93001-118)
- Ice
- Microcentrifuge
- Thermal cycler
- MagneSphere® Technology Magnetic Separation Stand (Promega, cat. no. Z5342)
- **Optional spike-in**:
  ERCC ExFold RNA Spike-In Mixes (Thermo Fisher Scientific, cat. no. 4456739)
- **Concentration readings**:

  Qubit® Fluorometer (Thermo Fisher Scientific, cat. no. varies)

  Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, cat. no. Q32854)

  Qubit Assay Tubes (Thermo Fisher Scientific, cat. no. Q32856)
- **Library QC**:

  2100 Bioanalyzer (Agilent, cat. no. varies)

  Agilent High Sensitivity DNA Kit (Agilent, cat. no. 5067-4626)
- **Preferred Library Quantification Method**: qPCR instrument and QIAseq Library Quant System: QIAseq Library Quant Array Kit (QIAGEN, cat. no. 333304) or QIAseq Library Quant Assay Kit (QIAGEN, cat. no. 333314)

Important Notes

- For direct cell lysis, single cells and pellets of up to 100 cells can be lysed using the provided cell lysis buffer and RNase inhibitor. Alternatively, 10 pg – 1 ng of purified total RNA can be used instead of cells. QIAGEN provides a range of solutions for purification of total RNA (Table 2).

Table 2. Recommended kits for purification of total RNA

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

- Ensure that total RNA samples are of high quality relative to their sample type. For additional information, please see "Appendix A: General remarks on handling RNA", page 51.
- **RNA quantification**: The concentration and purity of total RNA isolated from cells and fresh/frozen tissues should be determined by measuring the absorbance in a spectrophotometer. 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 RNA RIN should ideally be ≥8, successful library prep 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 particular sections.

Protocol: Cell Lysis

Important points before starting

- The recommended starting amount is 1–100 cells.
- When working with single cells, minimally 8 cells must be multiplexed per sample index.
- Three options are available for cell collection (described in Figure 4, page 9):

  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 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 (described in Figure 4, page 9), 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.
   RNase Inhibitor should be removed from the −20°C freezer just before preparation of the master mix and placed on ice. RNase Inhibitor should be returned 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 that required for the total number of reactions).

Table 3. 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 single-use Cell ID RT Plate (CID-96S Plate or CID-384 Plate) or empty 96-/384-well plates.

   Note: The CID-96S Plate is breakable, allowing cells to be processed in batches.
4. Capture cells into the plate containing the Cell Lysis Premix.
5. Incubate for 15 min on ice.
6. Freeze at –90 to –65°C or proceed immediately with "Protocol: Rebuffering of QIAseq Beads (RQ Beads)", page 15.

Protocol: Rebuffering of QIAseq Beads (RQ Beads)

Important points before starting

- This protocol prepares Rebuffered QIAseq Beads (hereafter referred to as RQ Beads). QIAseq Beads are rebuffered with QIAseq NGS Bead Binding Buffer to create 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, the RQ Beads should be stored on ice or at 4°C.
- **Note**: Both the bead storage and Bead Binding Buffers are viscous. Pipet 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. For 8 pooled cells/samples or 24 pooled cells/samples, add 450 µl QIAseq Beads to a 2 ml microcentrifuge tube. For 96 cells/samples, add 800 µl QIAseq Beads to a 2 ml microcentrifuge tube. Briefly centrifuge and immediately separate beads on a magnet stand.

   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 pipet 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 pipet 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 (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 on ice or at 4°C.

   Note: RQ Beads can be stored at 2–8°C for up to one week.

8. Proceed with "Protocol: Reverse Transcription of Lysed Cells" on page 17 or "Protocol: Reverse Transcription of Purified RNA" on page 22.

Protocol: Reverse Transcription of Lysed Cells

Important points before starting

- 3 µl cell lysates from "Protocol: Cell Lysis" are the starting materials for the reverse transcription reactions.
- **Important**: When cells have been collected into empty 96-well plates, RT primers must be dispensed from the CID-96M Plate, the multi-use 96-well Cell ID RT Plate (described in Figure 4, page 9) during setup of the reverse transcription reactions. Each well contains a pre-dispensed, anchored oligo-dT primer containing a UMI and Cell ID. 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 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. 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.

   EZ Reverse Transcriptase should be removed from the –20°C freezer just before preparation of the master mix and placed on ice. The enzyme should be returned to the freezer immediately after use.

2. Prepare the RT Premix on ice as described in Table 4 (when using single-use Cell ID RT Plates) or Table 5 (when using RT primers from a multi-use 96-well UPX 3' Cell ID RT Plate).

   Note: For single-cell analysis, ERCC can be added as a control. Prepare the ERCC by diluting stock by diluting the stock 1.25 x 10^{6}-fold using 0.1X TE Buffer.

Table 4. Preparation of RT premix for single-use Cell ID RT Plates (CID-96S Plate or CID-384 Plate)

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*	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 stock by diluting the stock 1.25 x 10^{6}-fold using 0.1X TE Buffer.

Table 5. Preparation of RT premix when using multi-use Cell ID RT Plates (CID-96M Plate)

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
ERCC diluted 1.25 x 10^{6}-fold*	2.5 µl	7.5 µl	30 µl	120 µl
Cell ID RT Primer†	0.5 µl added per well†	0.5 µl added per well†	0.5 µl added per well†	0.5 µl added per well†
Total volume	**15 µl**	**450 µl**	**180 µl**	**720 µl**

* For single-cell analysis, ERCC can be added as a control. Prepare the ERCC by diluting stock by diluting the stock 1.25 x 10^{6}-fold using 0.1X TE Buffer.

† Added separately from a multi-use Cell ID RT Plate in Step 3.

3. Aliquot the RT Premix and RT Primers from the multi-use Cell ID RT Plate (when applicable) as follows:

   For single-use Cell ID RT Plates (CID-96S Plate or CID-384 Plate), aliquot 2 µl of RT Premix from Table 4 into the wells of the plate.

   When using the multi-use Cell ID RT Plate (CID-96M Plate), aliquot 1.5 µl of RT Premix from Table 5 into the plate wells containing 3 µl of lysed cell, then aliquot 0.5 µl of each Cell ID RT primer from the multi-use Cell ID RT 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 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.

   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 7 for 8 combined wells).

Table 6. Reverse transcription incubation

Time	Temperature
10 min	25°C
90 min	42°C
15 min	70°C
∞	4°C

Table 7. 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.

6. 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.
7. 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.
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 the ethanol wash 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 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.
11. Remove the tube from the magnetic stand and elute the DNA from the beads by adding 25 µl Nuclease-Free Water. Mix well by pipetting.
12. Return the tube/plate to the magnetic rack until the solution has cleared.
13. Transfer 23 µl of the supernatant to clean tubes.

    Important: When working with 384 wells, combine all 4 eluates to give 92 µl.
14. Adjust the supernatant volume to 100 µl using Nuclease-Free Water.
15. Add 0.9X (volume) of RQ Beads. Mix well by pipetting up and down 12 times.
16. Incubate for 10 min at room temperature.

17. 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.

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 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.

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 27. 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", page 29.

Protocol: Reverse Transcription of Purified RNA

Important points before starting

- This protocol can be used with low amounts of purified RNA (10 pg to 1 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, the multi-use 96-well Cell ID RT Plate described in Figure 4 (page 9), during setup of the reverse transcription reactions. Each well contains a pre-dispensed, anchored oligo-dT primer containing a UMI and Cell ID. 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.
- Ensure all reactions are mixed thoroughly and handled at the temperatures recommended in the protocol.
- **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. 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.

   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.

2. Prepare the RT Premix on ice as described in Table 8 (when using single-use Cell ID RT Plates) or Table 9 (when using a multi-use 96-well Cell ID RT Plate).

Table 8. Preparation of RT premix for single-use Cell ID RT Plates (CID-96S Plate or CID-384 Plate)

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
RNA*	2.5 µl added per well*	2.5 µl added per well*	2.5 µl added per well*	2.5 µl added per well*
Total volume	**25 µl**	**75 µl**	**300 µl**	**1200 µl**

* Added separately in step 3.

Table 9. Preparation of RT premix when using multi-use Cell ID RT Plates (CID-96M Plate)

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
RNA*	2 µl added per well*	2 µl added per well*	2 µl added per well*	2 µl added per well*
Cell ID RT Primer†	0.5 µl added per well†	0.5 µl added per well†	0.5 µl added per well†	0.5 µl added per well†
Total volume	**25 µl**	**75 µl**	**300 µl**	**1200 µl**

* Added separately in step 3.

† Added separately from a multi-use Cell ID RT Plate in step 3.

3. Aliquot the RT Premix, purified RNA, and RT Primers from the multi-use Cell ID RT Plate (when applicable) as follows:

   For single-use Cell ID RT Plates (CID-96S Plate or CID-384 Plate), aliquot 2.5 µl of RT Premix from Table 8 into the wells of the plate. Subsequently add 2.5 µl of purified RNA.

   When using the multi-use Cell ID RT (CID-96M Plate), aliquot 2.5 µl of RT Premix from Table 9 per well of an empty 96-/384-well plate. Subsequently add 2 µl of purified RNA

and 0.5 µl of each Cell ID RT primer from the multi-use Cell ID RT Plate into the appropriate well.

Note: A different Cell ID 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

Time	Temperature
10 min	25°C
90 min	42°C
15 min	70°C
∞	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.

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 as they contain the DNA of interest.

9. Add 200 µl 80% ethanol. Rotate the tube (3 times) to wash the beads. Carefully remove and discard the wash.
10. 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.

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 stand while handling the supernatant and during the washing steps.

    Important: Do not discard the beads as 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 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.
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 27. 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", page 29. Alternatively, the samples can be stored at –20°C in a constant-temperature freezer.

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 29.
- 2x Quant AMP MM contains a fluorescent dye that binds 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.

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. For example, if the plateau phase is reached when the CT is 13, then 10 is the required number of universal PCR amplification cycles.
5. Proceed with “Protocol: Template Amplification”, Page 29.

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 as 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 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.

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 32. Alternatively, the completed library amplification product can be stored at –20°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.

   Fragmentation Enzyme Mix should be removed from the –20°C freezer just before preparation of the master mix and placed on ice. After use, the enzyme should be immediately returned 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 pre-chilled and paused at 4°C.

5. Transfer the tubes/plate prepared in step 2 to the pre-chilled 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 34.

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.
   DNA Ligase should be removed from the −20°C freezer just before preparation of the master mix and placed on ice. After use, the enzyme should be immediately returned 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 as 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 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.

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 36. 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: Universal PCR", page 39.

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 fluorescent dye that binds 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.

   Note: Layout and use of QIAseq UPX 3' Trans 48 Index is described in Figure 5. During reaction setup in step 2, components are added directly to the plate.

   HotStarTaq DNA Polymerase should be removed from the −20°C freezer just before preparation of the master mix and placed on ice. HotStarTaq DNA Polymerase should be returned to the freezer immediately after use.

	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 5. 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. For example, if the plateau phase is reached when the C_T is 13, then 10 is the required number of universal PCR amplification cycles.

5. Proceed with "Protocol: Universal PCR", page 39.

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: Layout and use of QIAseq UPX 3' Trans 48 Index is described in Figure 5, page 37. During reaction setup in step 2, components are added directly to the plate.

   HotStarTaq DNA Polymerase should be removed from the −20°C freezer just before preparation of the master mix and placed on ice. HotStarTaq DNA Polymerase should be returned 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 "Protocol: Quantitative uPCR"**	
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 as 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 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.

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 42. Alternatively, the completed library amplification product can be stored at –20°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 use of the QIAseq Library Quant System: QIAseq Library Quant Array Kit (QIAGEN, cat. no. 333304) or QIAseq Library Quant Assay Kit (QIAGEN, cat. no. 333314).

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 6.

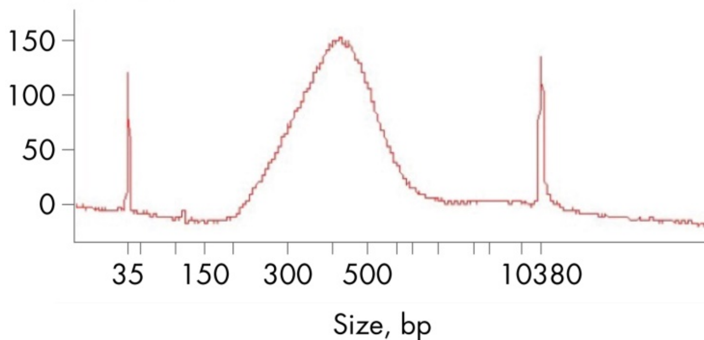

Figure 6. Bioanalyzer trace of library prepared with the QIAseq UPX 3' Transcriptome Kit.

2. Proceed with library quantification (next section).

Library quantification

1. The library yield measurements of the Bioanalyzer or TapeStation system using fluorescence dyes, which intercalate into DNA or RNA 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 or Assay Kit, which contains laboratory-verified forward and reverse primers together with a DNA standard, is highly recommended for accurate quantification of the prepared library.

   Use 1 nM RNA-seq libraries as input for the denaturation procedure to ultimately load 3 pM for the MiSeq (V3 kit) and 1.2 pM for the NextSeq.
2. Proceed with "Protocol: Sequencing Setup on Illumina MiSeq and NextSeq", page 44.

Protocol: Sequencing Setup on Illumina MiSeq and NextSeq

Important points before starting

- **Important**: Recommendations for library dilution concentrations and library loading concentrations are based on QIAseq Library Quant System.
- **Important**: QIAseq D Read 2 Primer I (Custom Read 2 Sequencing Primer) MUST be used when performing sequencing on the NextSeq. For complete instructions on how to denature sequencing libraries, prepare custom index primers and set up a sequencing run, please refer to the system-specific Illumina documents.
- Instrument-specific imagery is included to aid in sequencing preparations.

MiSeq: Sequencing preparations

1. Sample sheet setup: Set up sample sheet using Illumina Experiment Manager, v1.2 or later (Figure 7).

   Category: Select "Other"

   Select Application: Check "FASTQ Only"

   Sample Prep Kit: Select "TruSeq® LT"

   Index Reads: Select "1"

   Read Type: Select "Paired End Read"

   Cycles for both Read 1: Select "100"

   Cycles for both Read 2: Select "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 library to load on a MiSeq according to the MiSeq System Denature and Dilute Libraries Guide. The final library concentration is 3 pM on MiSeq (V3 chemistry). In addition, it is highly recommended to include 5% PhiX.

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

Figure 7. Sample sheet wizard using Illumina Experiment Manager.

NextSeq: 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 UPX 3' Targeted RNA Library, perform the following steps.

1. Download the following template from the "Resources" tab at **www.qiagen.com/shop/sequencing/QIAseq-UPX-3-Transcriptome-Kits**:

   libraryprep_template_UPX_trans.csv

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. Select “+Custom Library Prep Kit” from the “Library Prep Kit” drop-down menu.
6. In the screen that appears (Figure 8), 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 151 and Read2 Cycles 27). Click “Choose .csv File” and select “libraryprep_template_UPX_trans.csv”.
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.

Figure 8. Custom library prep kit setup for the QIAseq UPX 3' Transcriptome Kit.

NextSeq: Run planning and sequencing preparations

1. From the drop-down menu on the “Libraries” tab (Figure 9), select library prep kit “UPXtranscriptome”, check individual sample and drag it into corresponding well to assign INDEX 1.

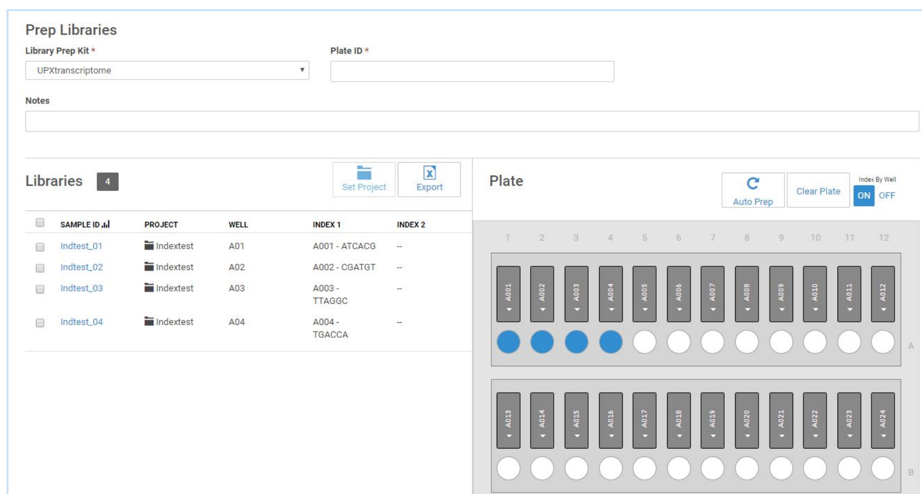

Figure 9. Assigning sample indices in the “Libraries” tab.

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

   Under “Plan Run” (Figure 10):

   Select “NextSeq” from the “Instrument” drop-down menu

   Check “R2” for “Use Custom Primer”

   Check “Paired End” and verify 100 for “Read 1 Cycles” and 27 for “Read 2 Cycles”

   Check “Single Index” and verify 6 for “Index 1 Cycles”

Plan Run

Instrument*

NextSeq

Run Information

Name*

Reagent Barcode

Use Custom Primer: R1 R2 Index

Enter Cycles

Single Read

Paired End

Read 1 Cycles* 100

Read 2 Cycles* 27

Figure 10. NextSeq run parameters.

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*. The final library concentration is 1.2 pM on NextSeq. In addition, it is highly recommended to include 10% PhiX.

Custom Sequencing Primer for Read 2 preparation and loading: Use 1994 µl HT1 (Hybridization Buffer) to dilute 6 µl of QIAseq D Read 2 Primer I (provided) to obtain a final concentration of 0.3 µM. Load 2 ml of the diluted QIAseq D Read 2 Primer I to Position #8 of the NextSeq reagent cartridge.

Note: All other steps refer to run setup workflow as described in the *NextSeq 500 System Guide* (part #15046563) or *NextSeq 550 System Guide* (part #15069765-02).

3. Upon completion of the sequencing run, proceed with “Protocol: Data Analysis”, page 49.

Protocol: Data Analysis

Important point before starting

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

Primary data analysis procedure

1. Click on "Data Analysis Center" and log in.
2. Under "Choose format", select "QIAseq UPX Single Cell Analysis" or "QIAseq UPX Primary Analysis".
3. Under "Choose Instrument", select your instrument.
4. Click "Start Analysis".
5. In the "BaseSpace Samples" tab, ".fastq" or ".fast.gz" files can be directly downloaded into the software from BaseSpace.
6. In the "File Upload" tab, ".fastq" or ".fastq.gz" files can be manually uploaded. Click to start upload.
7. In the "File Management" tab, all files that have been uploaded to the portal are listed. Here, files can be shared and deleted.
8. In the "UPX 3' Analysis Jobs" tab, select "Protocol", "Species", "Cell Index Set", "Select Reads Files", "File Lanes", "Instrument" and "Single Cell Analysis or "Low Input Primary Analysis". Once the selections are made, click "Create Job".
9. When the job has been completed, the single-cell cluster analysis can be downloaded using the "Download Single Cell Report" link, or when working with ultralow input samples, the primary mapping report can be downloaded using the "Download Report" link or the primary mapping report can be downloaded using the "Download Report" link. Alternatively, when using ultralow input samples, secondary differential expression analysis can be performed by using the "Secondary Analysis" link.

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 “Protocol: Rebuffering of QIAseq Beads (RQ Beads)”, page 15.
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 27.
g) Insufficient universal PCR amplification cycles	Determine optimal universal PCR amplification cycles using “Optional Protocol: Quantitative Universal PCR”, page 36.
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 over-amplification.
b) Very low clusters passing filter	Make sure the library is accurately quantified and that the correct amount is loaded onto the sequencing instrument. On a NextSeq, the QIAseq D Read 2 Primer I Custom Read 2 Sequencing primer MUST also be used.

Appendix A: General remarks on handling RNA

Handling RNA

Ribonucleases (RNases) are very stable and active enzymes that generally do not require cofactors to function. Since RNases are difficult to inactivate and even minute amounts are sufficient to degrade RNA, do not use any plasticware or glassware without first eliminating possible RNase contamination. Care should be taken to avoid inadvertently introducing RNases into the RNA sample during or after the purification procedure. 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. Hands and dust particles may carry bacteria and molds and are the most common sources of RNase contamination. Always wear latex or vinyl gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin or from dusty laboratory equipment. Change gloves frequently and keep tubes closed whenever possible. Keep purified RNA on ice.

To remove RNase contamination from bench surfaces, nondisposable plasticware and laboratory equipment (e.g., pipettes and electrophoresis tanks), use of RNaseKiller® (cat. no 2500080) from 5 PRIME® (**www.5prime.com**) is recommended. 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 52), or rinse with chloroform* if the plasticware is chloroform-resistant. To decontaminate electrophoresis tanks, clean with detergent* (e.g., 0.5% SDS), rinse with RNase-free water, rinse with ethanol (if the tanks are ethanol-resistant) and allow to dry.

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

Disposable plasticware

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

Glassware

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

Solutions

Note: QIAGEN solutions, such as the components found in the miScript® Single Cell qPCR 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 hours at 37°C. Autoclave for 15 minutes to remove any trace of DEPC. DEPC will react with primary amines and cannot be used directly to treat Tris buffers*. DEPC is highly unstable in the presence of Tris buffers and decomposes rapidly into ethanol and CO_2. When preparing Tris buffers, treat water with DEPC first, and then dissolve Tris to make the appropriate buffer. Trace amounts of DEPC will modify purine residues in RNA by carbethoxylation. Carbethoxylated RNA is translated with very low efficiency in cell-free systems. However, its ability to form DNA–RNA or RNA–RNA hybrids is not seriously affected unless a large fraction of the purine residues have been modified. Residual DEPC must always be eliminated from solutions or vessels by autoclaving or heating to 100°C for 15 minutes.

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

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 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.
RNeasy Micro Kit (50)	50 RNeasy MinElute® Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free DNase I, Carrier RNA, RNase-free Reagents and Buffers	74004
RNeasy Mini Kit (50)*	50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers	74104
RNeasy 96 Kit (4)*	For 4 x 96 total and cytoplasmic RNA preps: 4 RNeasy 96 Plates, Elution Microtubes CL, Caps, S-Blocks, AirPore Tape Sheets, RNase-Free Reagents and Buffers	74181

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

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Handbook Revision History

Document	Changes
05/2019	Updated Figure 10 to correct number in “Read 1 Cycles” field.
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.

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