March 2021

QIAseq® Single Cell RNA Library Kits with Unique Dual Indexes

For RNA-seq library construction from single cells and low-input RNA samples for Next-generation sequencing using Illumina® NGS instruments

Sample to Insight

QIAGEN

Contents

Kit Contents

QIAseq Single Cell RNA Lib Kit UDI	**(24)**	**(96)**	**(96)**	**(96)**	**(96)**	**(384)**
Catalog no.	**180703**	**180705**	**180725**	**180765**	**180785**	**180707**
No. of preps	**24**	**96**	**96**	**96**	**96**	**384**
QIAseq Single Cell RNA Enzyme Kit	1124560	1124561	1124561	1124561	1124561	4 x 1124561
QIAseq UDI Y-Adapter Kit (24) In plate format	1 plate 180310	–	–	–	–	–
QIAseq UDI Y-Adapter Kit A, B, C, or D In plate format		1 plate 180312	1 plate 180314	1 plate 180316	1 plate 180318	4 plates 180312 180314 180316 180318
QIAseq Beads	1107149 10 ml	1124693 1 x 25 ml	1124693 1 x 25 ml	1124693 1 x 25 ml	1124693 1 x 25 ml	1124696 2 x 50 ml

QIAseq Single Cell RNA Enzyme Kit	(24)	(96)	4 x (96)
Catalog no.	1124560	1124561	1124561
No. of preps	24	96	384
Sample preparation			
Lysis Buffer	1 tube	4 tubes	16 tubes
Denaturation Buffer	1 tube	4 tubes	16 tubes
Enzymatic template preparation			
gDNA Wipeout Buffer, WTA	1 tube	4 tubes	16 tubes
RT/Polymerase Buffer	1 tube	4 tubes	16 tubes
Random Primer	1 tube	4 tubes	16 tubes
Oligo-dT Primer	1 tube	4 tubes	16 tubes
Quantiscript® RT Enzyme Mix	1 tube	4 tubes	16 tubes
Ligase Mix	1 tube	4 tubes	16 tubes
Ligase Buffer	1 tube	4 tubes	16 tubes
Amplification of cDNA			
REPLI-g® SensiPhi DNA Pol.	1 tube	4 tubes	16 tubes
REPLI-g SC Dilution Buffer	1 tube	5 tubes	20 tubes
REPLI-g SC universal oligo	1 tube	3 tubes	12 tubes
REPLI-g SC advanced oligo	1 tube	3 tubes	12 tubes
H_2O sc	3 tubes	8 tubes	32 tubes
Library preparation			
HiFi PCR Master Mix, 2x	2 tubes	2 tubes	8 tubes
Illumina Library Ampl. Primer Mix	2 tube	1 tube	4 tubes
FX Enzyme Mix	1 tube	1 tube	4 tubes
FX Buffer, 10x	1 tube	1 tube	4 tubes
FX Enhancer	1 tube	1 tube	4 tubes
DNA Ligase	1 tube	1 tube	4 tubes
5x DNA Ligase Buffer	1 tube	2 tubes	8 tubes

Shipping and Storage

The QIAseq Single Cell RNA Library Kit is shipped in several boxes.

- The QIAseq Single Cell RNA Enzyme Kit and the QIAseq UDI Y-Adapter Kit should be stored immediately upon receipt at –30 to –15°C in a constant-temperature freezer.
- QIAseq Beads should be stored immediately at 2–8°C in a refrigerator. QIAseq Beads should never be frozen.

Intended Use

The QIAseq Single Cell RNA Library Kit is intended for molecular biology applications. This product is not intended for the diagnosis, prevention, or treatment of a disease.

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

Safety Information

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

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of the QIAseq Single Cell RNA Library Kit is tested against predetermined specifications to ensure consistent product quality.

Introduction

Single cell analysis enables researchers to gain novel insights across a diverse range of research areas, including developmental biology, tumor heterogeneity, and disease pathogenesis and progression. Using single cell transcriptomics, researchers are able to identify subpopulations within tissues that may have specific functions or could respond differently to drug treatments and small molecule inhibitors. When using RNA-seq library kits that provide for full transcript coverage, additional information about exon usage and critical sequencing information can lead to new discoveries on a per cell basis, which is not possible with bulk sequencing methods.

The QIAseq Single Cell RNA Library Kit has been designed to allow researchers to interrogate the full transcriptome from diverse types of single cells, while also being flexible enough to accommodate low input RNA samples. The workflow has been designed to allow for unbiased amplification and unbiased NGS library construction. This allows for the reliable investigation of the transcriptome from single cells, low input RNA samples, and even small viral RNA genomes.

An important feature of the QIAseq Single Cell RNA Library Kit is the utilization of multiple-displacement amplification (MDA) to uniformly amplify cDNA and PCR-free NGS library construction. The MDA reaction utilizes a unique HiFi polymerase, which minimizes the incorporation of amplification errors. The kit provides a time-saving, one-tube library preparation protocol that eliminates sample cleanup between steps – minimizing starting material loss and cross-contamination risk. Co-optimization of MDA and NGS library construction processes enables a highly streamlined and efficient protocol that can be easily automated. The highly optimized enzyme, buffers, and workflow ensure the generation of high-diversity, NGS-ready libraries in just one working day (Figure 1, *Workflow chart*).

Principle and procedure

Regulation of transcription is driven by a variety of influences, including stress, cellular environment, the presence of a particular disease, and somatic genomic variation (e.g., point mutations, copy number variations, or structural variations). Additionally, transcriptional post-processing – such as alternative splicing – results in a differential transcription pattern and, ultimately, physiology. Because of the composite structure of tissues, investigating transcription regulation in single cells – rather than analyzing a larger number of cells and basing the resulting interpretation on average cell behavior – has been becoming of increasing scientific interest.

The QIAseq Single Cell RNA Library Kit is designed to reliably investigate gene expression, transcript regulation, and small RNA genomes at the single-cell level or from low inputs of RNA. The kit provides everything required to (1) uniformly amplify all transcripts from single cells or enrich specific RNA, (2) fragment the amplified cDNA, and (3) generate a PCR-free library for analysis on Illumina NGS instruments. The QIAseq Single Cell RNA Library Kit utilizes HiFi and proofreading polymerases and can generate RNA-seq libraries without using PCR amplifications. This saves time and reduces PCR-induced duplication and PCR-based errors.

In the first step of the procedure, the cell sample is lysed and the gDNA is removed. Reverse transcription using Quantiscript RT Enzyme Mix is carried out for 60 minutes, followed by ligation of cDNAs (30 min). The cDNA amplification is an isothermal reaction that proceeds for 120 minutes. The amplified cDNA can then be stored long-term at –30 to –15°C with no negative effects, and enough cDNA is amplified for both NGS library preparation and follow-up experiments, for example, with qPCR.

The QIAseq Single Cell RNA Library Kit uses isothermal genome amplification – termed "multiple displacement amplification" (MDA) – which involves the binding of random hexamers to denatured cDNA. This amplification is followed by strand displacement synthesis at a constant temperature with REPLI-g SensiPhi DNA Polymerase, which has exceptionally strong

strand displacement properties. Additional priming events occur on each displaced strand that serves as a template, enabling the generation of high yields of amplified cDNA. REPLI-g SensiPhi DNA Polymerase is a DNA polymerase with 3'→5' exonuclease activity (proofreading activity) that delivers up to 1000-fold higher fidelity compared to *Taq* DNA polymerase. Supported by the unique, optimized buffer system, REPLI-g SensiPhi DNA Polymerase easily solves secondary structures such as hairpin loops – thereby preventing slipping, stoppage, and dissociation of the polymerase during amplification. This feature enables the generation of cDNA fragments of up to 100 kb without sequence bias.

For library construction, the samples consisting of long amplified cDNA strands are first enzymatically sheared into smaller fragments. The median fragment size is dependent on the experimental goals and sequencing read length, and can be adjusted by varying the enzymatic fragmentation reaction conditions. The fragmented cDNA is directly end-repaired and an 'A' is added to the 3' ends in a single tube reaction, making the DNA fragments ready for ligation. Following this step, Illumina-specific adapters are ligated to both ends of the DNA fragments. These adapters contain sequences essential for bridge amplification and sequencing. The MDA procedure normally results in high yields of DNA so that library preparation can be performed with a high amount of input DNA, and subsequent PCR-based library enrichment can be avoided. If library amplification is required, HiFi PCR amplification can also be performed with the included reagents.

Unique components of the QIAseq Single Cell RNA Library Kit

- All of the kit's enzymes and amplification components undergo a unique, controlled decontamination procedure to ensure elimination of REPLI-g amplifiable-contaminating DNA or RNA. Following this process, the kits undergo stringent quality control to ensure complete functionality.
- The innovative lysis buffer effectively stabilizes cellular RNA – ensuring that the resulting RNA accurately reflects the in vivo gene expression profile.

- All enzymatic steps have been specifically developed to enable efficient processing of RNA for accurate amplification. These steps, for example, include effective gDNA removal prior to cDNA synthesis.
- Novel REPLI-g SensiPhi DNA Polymerase is used for MDA. It is a newly developed, high-affinity enzyme that binds cDNA more efficiently, particularly, when the cDNA concentration is low in the reaction mixture. In contrast to PCR-based methods, REPLI-g SensiPhi DNA Polymerase has a 3'→5' exonuclease proofreading activity, resulting in a 1000-fold higher fidelity than *Taq* DNA Polymerase during replication. It also has strong strand-displacement activity, enabling replication of cDNA through stable hairpin structures that are resistant to *Taq*-based whole genome or whole transcriptome amplification procedures.
- Library construction enzymes and buffers are specially optimized for a convenient, single-tube protocol and for a high-efficiency adapter ligation.

The QIAseq Single Cell RNA Library Kit provides a simple and reliable method to efficiently generate RNA-seq libraries. These libraries are suitable for use on Illumina NGS instruments from either a single cell or from as little as 50 picograms of total RNA. In the enrichment workflow, as little as 5000 copies of viral RNA in 5 ng of background total RNA can be efficiently amplified. The kit provides a complete workflow for reliable reverse transcription and for highly uniform amplification across the entire transcriptome with negligible sequence bias – followed by fast, one-tube library construction (Figure 1).

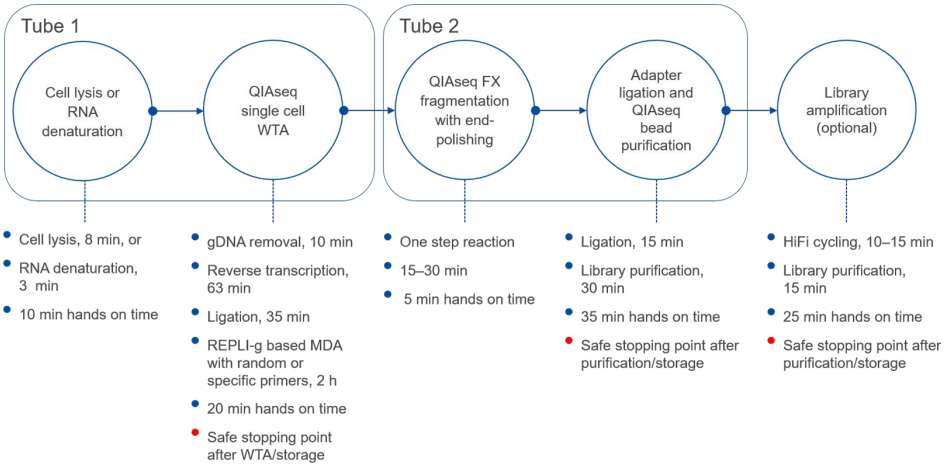

Figure 1. A time-saving, streamlined protocol delivers RNA-seq libraries – ready for use on Illumina NGS platforms. The QIAseq Single Cell RNA Library Kit provides a complete workflow for whole transcriptome amplification using random primers and NGS library preparation when starting with sorted cells or purified RNA, or enrichment of viral RNA. The workflow requires 1 hour of hands on time and can be completed in only 6 hours. In addition, specific enrichment and amplification of RNA can be performed when using target-specific primers (not included in this kit).

NGS adapter and index technologies

Sample multiplexing is one of the most important NGS tools for increasing throughput and reducing costs. It works by combining multiple samples to be processed together in a single sequencing run; as a consequence, sequencing reads need to be demultiplexed by reassigning each single read to its original source library. This is facilitated by the integration of index sequences into the individual adapter molecules.

QIAseq Unique Dual-Index (UDI) Y-Adapter Kits (24, 96 A/B/C/D, and 384) are included in the QIAseq Single Cell RNA Library Kits UDI.

The UDI adapter plates supplied as hard plastic plates sealed with foil with liquid Y-shaped indexes available in 24- and 96-plex formats. The 384 kit combines the QIAseq UDI Y-Adapter Kits A–D plates and enables multiplexing of up to 384 samples per sequencing run. For more information on QIAseq Dual-Index Y-Adapters and index sequence motives, see Appendix C and "Ordering Information".

QIAseq UDI Adapters use a fixed combination of 2 unique barcode motives per adapter molecule. Therefore, each single-index motive is only used once on any UDI adapter plate.

Usage of UDI adapters effectively mitigates the risk of read misassignment due to index hopping. This is enabled by filtering misassigned reads during the demultiplexing of individual samples, thus generating highly accurate output data.

Description of protocols

Different protocols in this handbook provide detailed instructions for using the QIAseq Single Cell RNA Library Kit for (1) cDNA amplification from single cells or purified RNA, (2) specific enrichment of RNA and cDNA amplification, and (3) construction of an NGS library.

The protocol "Amplification of Poly A+ mRNA from Single Cells" is optimized for single cell material from eukaryotic species without a cell wall – including cells from vertebrates, individual cells isolated using cell sorting automation, cells from tissue culture, protoplasts, cells isolated with laser-capture microdissection, and cells or tissue from biopsies. The protocol avoids the amplification of ribosomal RNA and enriches for mRNA and other polyadenylated RNAs by omitting Random Primers during reverse transcription.

The protocol "Amplification of Total RNA from Single Cells" is used for the amplification of the complete transcriptome, including RNAs with and without poly A+ tails. Note that ribosomal RNA is also amplified when using a combination of Random and Oligo-dT Primers, and will represent a high percentage of reads after sequencing.

The QIAseq FastSelect™ –rRNA Removal Kits can be used to block ribosomal RNA from being reverse transcribed into cDNA and subsequently amplified during the MDA reaction. "Appendix G: QIAseq FastSelect Blocking of rRNA and/or Globin mRNA" provides a protocol for the incorporation of QIAseq FastSelect into the workflow.

The protocol "Amplification of Purified RNA" is optimized for whole transcriptome amplification from total or enriched RNA templates (Poly A+ mRNA, rRNA-depleted mRNA) that do not require additional selection for poly-adenylated RNAs.

The protocol "Specific Enrichment of Purified RNA" is optimized for amplification of total RNA using target-specific primers that can be designed against the RNA genome to be analyzed. Recommendations for target-specific primers design are included in Appendix E, page 85.

Following reverse transcription and WTA, the amplified cDNA is incorporated into an NGS library using a PCR-free library preparation procedure that includes fragmentation, end-repair, A-addition, and adapter ligation. Cleanup and removal of adapters and adapter-dimers using the QIAseq beads complete the workflow, which is described in "Protocol: "Enzymatic Fragmentation and Library Preparation Using the QIAseq Single Cell RNA Amplified cDNA". The prepared library can be quantified and is optimized for use on any Illumina sequencing platform.

Depending on the protocol, the QIAseq Single Cell RNA Library Kit is suitable for transcriptome amplification for the analysis of the following.

- mRNA with poly A+ tails
- Total RNA
- Viral RNA

Note: Highly fragmented RNA, such as exosomal RNA, can lead to a high number of broken read pairs (chimeric reads). In this case, mapping paired-end reads may result in a high number of reads unable to be uniquely mapped to the reference genome or transcriptome. Switching to single end-read mapping will increase the mapping rate and the number of detected genes.

The kit is not suitable for use with small nucleic acids, such as the following.

- tRNAs, miRNAs
- Severely degraded RNA
- RNA from FFPE material or samples fixed by formaldehyde, glutaraldehyde, or other fixatives

Typical DNA yields from the WTA reaction of the QIAseq Single Cell RNA Library Kit are 10–20 µg cDNA per 60 µl reaction, depending on the quality of the cells or input RNA used. The protocol for RNA enrichment will deliver from 1–10 µg per 60 µl reaction depending on the input. For best amplification results, a cell sample that has been properly collected should be used directly, since storage and collection conditions can alter transcription profiles as well as RNA quality. The resulting amplified cDNA is stable during long-term storage (up to several years) with no structural changes or degradation effects, enabling biobanking of the sample for follow-up experiments.

Sample specifications

The sample specifications below provide guidance on input samples that have been tested with this kit. If your samples fall outside of these guidelines, consider optimizing the RNA purification workflow and maintaining an RNase-free environment to ensure higher quality samples.

Recommended sample input

Eukaryotic single cells

- Viability: >90%

Isolated RNA

- Minimum amount: 2 pg viral RNA or 50 pg total eukaryotic RNA
- Maximum amount: 100 ng eukaryotic total RNA and for enrichment of viral RNA: 5 ng total RNA

Sample purity

- 260/280 ratio minimum: 1.9
- 260/230 ratio minimum: 1.9

Integrity

- RIN score minimum: 7.5
- RIS score minimum: 7.6
- DV 200: 80% minimum

Compatible sequencing platforms:

- iSeq®
- MiniSeq®
- Illumina HiSeq®
- Illumina MiSeq®

- Illumina NextSeq®
- Illumina NovaSeq®

Equipment and Reagents to Be Supplied by User

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

- Microcentrifuge tubes or PCR strips
- PCR tubes or plates
- LoBind tubes (e.g., from Axygen or Eppendorf)
- Thermocycler or heating block
- Microcentrifuge
- Vortexer
- Pipettes and pipette tips
- Magnetic racks for magnetic beads separation (e.g., Thermo Fisher Scientific/Life Technologies, DynaMag™-2 Magnet, cat. no. 12321D)
- Ice
- Nuclease-free water or 10 mM Tris·Cl (pH 8.0)
- Microcentrifuge tubes
- 100% ethanol (ACS grade)
- QIAseq FastSelect –rRNA Kit (optional)
- QIAxcel, Agilent 2100 Bioanalyzer®, or similar, to evaluate the DNA fragmentation profile (optional); or a comparable capillary electrophoresis device or method to assess the quality of DNA library
- QIAseq Library Quant Assay Kit (cat. no. 333314)
- Quant-iT™ PicoGreen® dsDNA Assay Kit (optional)

Important Notes

RNA sample, DNA preparation, and quality control

Single Cells and high-quality RNA, free of inhibitors of enzymatic reactions, are critical for obtaining good amplification and sequencing results. Therefore, cell handling and RNA isolation procedures are critical to the success of the experiment.

Cells that are dead or have been damaged during isolation will contain highly fragmented RNAs. Highly fragmented RNAs decrease the efficiency of amplification and the quality of the generated libraries. Residual traces of proteins, salts, or other contaminants will degrade the RNA or decrease the efficiency of the enzymatic activities necessary for amplification and library preparation.

If determination of amplified cDNA is required, we recommend using fluorometric methods such as Qubit®, PicoGreen, or another fluorometric method specific for double stranded DNA. Spectroscopy instruments like the QIAxpert® (cat. no. 9002340) may also be used following cleanup of the amplified cDNA by QIAseq beads (See "Appendix F: Purification of Amplified cDNA After Whole Transcriptome Amplification").

General precautions

- Use good laboratory practices to minimize cross-contamination of nucleic acid products.
- Always use PCR tubes, microcentrifuge tubes, and pipette tips that are certified sterile, DNase-free, and RNase-free.
- Before starting, wipe down work area and pipettes with an RNase and DNA cleaning product such as RNase Away® (Molecular Bio-Products, Inc.) or LookOut® DNA Erase (Sigma-Aldrich).

- For consistent genome amplification, library construction, and amplification, ensure that the thermal cycler used in this protocol is in good working order and has been calibrated according to the manufacturer's specifications.
- Read the entire protocol before beginning. Take note of stopping points where samples can be frozen at –30 to –15°C, and plan your workflow accordingly.
- Enzyme-based DNA fragmentation is sensitive to many factors, such as salt concentration, reaction temperature, time and setup conditions – as well as the quality of the input DNA.

Recommended library quantification method

QIAGEN's QIAseq Library Quant Assay Kit (cat. no. 333314) uses quantitative PCR for accurate quantification of the NGS library. This kit uses laboratory-verified forward and reverse primers together with a DNA standard for accurate quantification of the prepared library. Failure to accurately quantify the NGS library will result in sub-optimal pooling and clustering during the NGS run, thus resulting in lower quality data.

Protocol: Amplification of Poly A+ mRNA from Single Cells

This protocol is for the amplification of polyadenylated mRNA starting with single cells.

For amplification of total RNA from single cells, use the protocol "Amplification of Total RNA from Single Cells". For whole transcriptome amplification of purified total RNA or enriched mRNA, use the protocol "Amplification of Purified RNA".

Important points before starting

- This protocol is optimized for cells (1–1000 cells) from all vertebrate species (e.g., human, mouse, rat, sorted cells, cells without cell wall, tissue culture cells, cells picked under the microscope, or microdissected cells from frozen tissue (>100 cells).
- The protocol cannot be used for bacterial cells, plant cells, or other cells and organisms that contain cell walls. For these starting materials, purify the RNA first and perform WTA using the protocol "Amplification of Purified RNA".
- The protocol cannot be used for fixed cells that are treated with formalin or other cross-linking agents (e.g., single cell samples obtained by laser microdissection from formalin-fixed, paraffin-embedded tissues).
- Samples of 1–1000 intact cells (e.g., human or cell culture cells) are optimal for whole transcriptome amplification reactions using the QIAseq Single Cell RNA Library Kit. Avoid using more than 1000 cells in the reaction, because samples containing too many cells may not be lysed effectively.
- Avoid any DNA or RNA contamination of reagents by using separate laboratory equipment (e.g., pipettes, filter pipette tips, reaction vials, etc.). Set up the single cell reactions in a location free of nucleic acids.
- The high-molecular-weight DNA that may be generated by random extension of primers (primer-multimer formation) in no-template controls (NTC) does not contain genetic

information and will not affect the quality of downstream applications. In reactions containing viable cells and thus sufficient cDNA, these products are not formed.

- Because the QIAseq Single Cell RNA Library Kit is intended to generate amplified cDNA from a minimal amount of starting RNA, be sure to take appropriate measures to avoid inadvertently introducing RNase contamination. Create and maintain an RNase-free environment when working with RNA by following proper microbiological and aseptic technique. The use of disposable plastic tubes and pipette tips from freshly opened boxes or bags is strongly recommended.
- The reagents for whole transcriptome amplification are not suitable for the amplification of small RNA molecules, such as tRNAs or miRNAs.
- Note that the final reaction volume is 59 µl.
- Although all sequences are well represented, the amplified cDNA does not contain full-length cDNAs. The amplification process is started by random-primed cDNA synthesis. Consequently, transcript sequences are amplified in pieces. Due to the nature of the ligation reaction, DNA fragments might not be assembled in the order in which they originally existed in the organism. The QIAseq Single Cell RNA Library Kit is designed to make these events rare, and thus, the detection and quantification of nucleic acid sequences are not affected (e.g., sequence polymorphisms, differential expression analysis). However, analysis of novel gene fusions should be subsequently verified using alternative methods.

Things to do before starting

- The Quantiscript RT mix, ligation mix, and REPLI-g SensiPhi amplification mix described in the protocol must always be prepared fresh. They cannot be stored for later use.
- All buffers and reagents should be vortexed before use to ensure thorough mixing.
- Quantiscript RT Enzyme Mix, Ligase Mix, and REPLI-g SensiPhi DNA Polymerase should be thawed on ice. All other components can be thawed at room temperature (15–25°C).
- For increased speed and convenience, all incubation steps of the protocol can be preprogrammed on a thermal cycler (Table 1).

Table 1. Thermal cycling parameters

Step	Time	Temperature
Set the heating lid to 50°C for all steps		
Cell lysis	5 min	24°C
	3 min	95°C
	∞	4°C
gDNA removal	10 min	42°C
	∞	4°C
Reverse transcription	60 min	42°C
	3 min	95°C
	∞	4°C
Ligation	30 min	24°C
	5 min	95°C
	∞	4°C
Whole transcriptome amplification	2 h	30°C
	5 min	65°C
	∞	4°C

Procedure

1. Place 7 µl cell material (supplied with PBS) into a microcentrifuge tube. If using less than 7 µl of cell material, add H_20 sc to bring the volume up to 7 µl.

   Note: Proceed immediately with step 2.

2. Add 4 µl Lysis Buffer. Mix carefully by gently flicking the tube, and centrifuge briefly.

   Note: Ensure that the cell material does not stick to the tube wall above the meniscus and that mixing of the lysis buffer with the cell material is complete.

3. Incubate at 24°C for 5 min, followed by 95°C for 3 min. Cool to 4°C.
4. Add 2 µl gDNA Wipeout Buffer, mix by vortexing, and centrifuge briefly.
5. Incubate at 42°C for 10 min. If more time is needed to prepare the next step, place on ice.

6. Prepare the Quantiscript RT mix (Table 2). Add 6 µl Quantiscript RT Mix to the lysed cell sample, mix by vortexing, and centrifuge briefly.

   Note: The Quantiscript RT mix must be prepared fresh.

Table 2. Preparation of Quantiscript RT mix*

Component	Volume/reaction
RT/Polymerase Buffer	4 µl
Oligo-dT Primer	1 µl
Quantiscript RT Enzyme Mix	1 µl
Total volume†	**6 µl**

* To prepare Quantiscript RT mix for multiple reactions, scale up according to the number of reactions.
† Mix by vortexing and centrifuge briefly.

7. Incubate at 42°C for 60 min. Stop the reaction by incubating at 95°C for 3 min; then, cool on ice.
8. Prepare the ligation mix (Table 3). Add 10 µl ligation mix to the RT reaction from step 7. Mix by vortexing and centrifuge briefly.

   Important: When preparing the ligation mix, add the components in the order shown in Table 3.

   Note: The ligation mix must be prepared fresh.

Table 3. Preparation of the ligation mix*

Component	Volume/reaction
Ligase Buffer	8 µl
Ligase Mix	2 µl
Total volume†	**10 µl**

* To prepare ligation mix for multiple reactions, scale up according to the number of reactions.
† Mix by vortexing and centrifuge briefly.

9. Incubate at 24°C for 30 min. Stop the reaction by incubating at 95°C for 5 min; then, cool on ice.

10. Prepare the REPLI-g SensiPhi amplification mix (Table 4). Add 30 µl REPLI-g SensiPhi amplification mix to the ligation reaction from step 9. Mix by vortexing and centrifuge briefly.

    Note: REPLI-g SensiPhi amplification mix must be prepared fresh.

Table 4. Preparation of REPLI-g SensiPhi amplification mix*

Component	Volume/reaction
REPLI-g SC Dilution Buffer	14.5 µl
REPLI-g SC Universal Oligo	14.5 µl
REPLI-g SensiPhi DNA Polymerase	1 µl
Total volume†	**30 µl**

* To prepare REPLI-g SensiPhi amplification mix for multiple reactions, scale up according to the number of reactions. We recommend to use the REPLI-g universal oligo for most applications and when transitioning from REPLI-g WTA Single Cell kits. The REPLI-g WTA advanced oligo may offer improved uniformity when working with human primary samples. Researchers should experimentally confirm which REPLI-g oligo is best for their specific application.

† Mix by vortexing and centrifuge briefly.

11. Incubate at 30°C for 2 h.
12. Stop the reaction by incubating at 65°C for 5 min; then, cool on ice.
13. If not being used directly, store the amplified cDNA at –30 to –15°C until required for downstream applications. We recommend storage of the amplified DNA at a minimum concentration of 100 ng/µl.

    Note: The high-molecular-weight DNA generated by random extension of primers (primer-multimer formation) in NTC does not contain genetic information and will not affect the quality of downstream applications. These products are outcompeted by cDNA of viable cells present during WTA.
14. Amplified cDNA can be directly used for the library construction or for target-directed amplification and library construction (see "Protocol: Enzymatic Fragmentation and Library Preparation Using the QIAseq Single Cell RNA Amplified cDNA"). Amplified cDNA behaves like purified genomic DNA and has an approximate length of 2,000–70,000 bp.

Note: If quantification of the amplified cDNA is required, follow the instructions in Appendix B. Optical density (OD) measurements may overestimate the amplified cDNA from step 12 and the amplified cDNA must be purified before spectroscopy measurement.

Protocol: Amplification of Total RNA from Single Cells

This protocol is for amplification of total RNA from single cell material and amplification of full length transcripts regardless of their poly-A status. Note that rRNA is also amplified using this protocol and may represent 80% or more of all cDNAs after amplification, and thus, a high percentage of reads will not align with genes in the resulting dataset. To enrich for polyadenylated RNAs including mRNAs, we recommend using the protocol "Amplification of Poly A+ mRNA from Single Cells", which avoids amplification of rRNA and generates cDNA perfectly suitable for NGS.

In the case that total RNA needs to be amplified, the kit can be used in combination with the QIAseq FastSelect –rRNA HMR Kit (sold separately) to block transcription and amplification of ribosomal RNA. For whole transcriptome amplification of purified RNA, refer to the protocol "Amplification of Purified RNA".

Important points before starting

- This protocol is optimized for cells (1–1000 cells) from all vertebrate species (e.g., human, mouse, rat, sorted cells, cells without cell wall, tissue culture cells, cells picked under the microscope, or microdissected cells from frozen tissue (>100 cells).
- The protocol cannot be used for bacterial cells, plant cells, or other cells and organisms that contain cell walls. For these starting materials, purify the RNA first and perform WTA using the protocol "Amplification of Purified RNA".
- The protocol cannot be used for fixed cells that are treated with formalin or other cross-linking agents (e.g., single cell samples obtained by laser microdissection from formalin-fixed, paraffin-embedded tissues).
- Samples of 1–1000 intact cells (e.g., human or cell culture cells) are optimal for whole transcriptome amplification reactions using the QIAseq Single Cell RNA Library Kit.

Avoid using more than 1000 cells in the reaction, because samples containing too many cells may not be lysed effectively.

- Avoid any DNA or RNA contamination of reagents by using separate laboratory equipment (e.g., pipettes, filter pipette tips, reaction vials, etc.). Set up the single cell reactions in a location free of nucleic acids.
- The high-molecular-weight DNA that may be generated by random extension of primers (primer-multimer formation) in no-template controls (NTC) does not contain genetic information and will not affect the quality of downstream applications. In reactions containing viable cells and thus sufficient cDNA, these products are not formed.
- Because the QIAseq Single Cell RNA Library Kit is intended to generate amplified cDNA from a minimal amount of starting RNA, be sure to take appropriate measures to avoid inadvertently introducing RNase contamination. Create and maintain an RNase-free environment when working with RNA by following proper microbiological and aseptic technique. The use of disposable plastic tubes and pipette tips from freshly opened boxes or bags is strongly recommended.
- The reagents for whole transcriptome amplification are not suitable for the amplification of small RNA molecules, such as tRNAs or miRNAs.
- Note that the final reaction volume is 60 µl.

Things to do before starting

- The Quantiscript RT mix, ligation mix, and REPLI-g SensiPhi amplification mix described in the protocol must always be prepared fresh. They cannot be stored for later use.
- All buffers and reagents should be vortexed before use to ensure thorough mixing.
- Quantiscript RT Enzyme Mix, Ligase Mix, and REPLI-g SensiPhi DNA Polymerase should be thawed on ice. All other components can be thawed at room temperature (15–25°C).
- For increased speed and convenience, all incubation steps of the protocol can be preprogrammed on a thermal cycler (Table 5).

Table 5. Thermal cycling parameters

Step	Time	Temperature
Set the heating lid to 50°C for all steps		
Cell lysis	5 min	24°C
	3 min	95°C
	∞	4°C
gDNA removal	10 min	42°C
	∞	4°C
Reverse transcription	60 min	42°C
	3 min	95°C
	∞	4°C
Ligation	30 min	24°C
	5 min	95°C
	∞	4°C
Whole transcriptome amplification	2 h	30°C
	5 min	65°C
	∞	4°C

Procedure

1. Place 7 µl cell material (supplied with PBS) into a microcentrifuge tube. If using less than 7 µl of cell material, add H_20 sc to bring the volume up to 7 µl.
2. Add 4 µl Lysis Buffer. Mix carefully by gently flicking the tube, and centrifuge briefly.

   Note: Ensure that the cell material does not stick to the wall of the tube above the meniscus.
3. Incubate at 24°C for 5 min, followed by 95°C for 3 min. Cool to 4°C.
4. Add 2 µl gDNA Wipeout Buffer, mix by vortexing, and centrifuge briefly.

5. Incubate at 42°C for 10 min. If more time is needed to prepare the next step, place on ice.

   Note: If rRNA and/or globin mRNA need to be depleted, QIAseq FastSelect can be added to the sample prior to addition of Quantiscript RT mix, to block rRNA and/or globin mRNA during reverse-transcription and amplification. Follow the instructions in "Appendix G: QIAseq FastSelect Blocking of rRNA and/or Globin mRNA".

6. Prepare Quantiscript RT mix (Table 6).

   Add 7 µl Quantiscript RT mix to the lysed cell sample, mix by vortexing, and centrifuge briefly.

   Note: Quantiscript RT mix must be prepared fresh.

Table 6. Preparation of Quantiscript RT mix*

Component	Volume/reaction
RT/Polymerase Buffer	4 µl
Random Primer	1 µl
Oligo-dT Primer	1 µl
Quantiscript RT Enzyme Mix	1 µl
Total volume†	**7 µl**

* To prepare Quantiscript RT mix for multiple reactions, scale up according to the number of reactions.

† Mix by vortexing and centrifuge briefly.

7. Incubate at 42°C for 60 min. Stop the reaction by incubating at 95°C for 3 min; then, cool on ice.

8. Prepare the ligation mix (Table 7). Add 10 µl ligation mix to the RT reaction from step 7. Mix by vortexing and centrifuge briefly.

   Important: When preparing the ligation mix, add the components in the order shown in Table 7.

   Note: The ligation mix must be prepared fresh.

Table 7. Preparation of the ligation mix*

Component	Volume/reaction
Ligase Buffer	8 µl
Ligase Mix	2 µl
Total volume†	**10 µl**

* To prepare ligation mix for multiple reactions, scale up according to the number of reactions.

† Mix by vortexing and centrifuge briefly.

9. Incubate at 24°C for 30 min. Stop the reaction by incubating at 95°C for 5 min; then, cool on ice.
10. Prepare REPLI-g SensiPhi amplification mix (Table 8). Add 30 µl REPLI-g SensiPhi amplification mix to the ligation reaction from step 9. Mix by vortexing and centrifuge briefly.

    Note: REPLI-g SensiPhi amplification mix must be prepared fresh.

Table 8. Preparation of REPLI-g SensiPhi amplification mix*

Component	Volume/reaction
REPLI-g SC Dilution Buffer	14.5 µl
REPLI-g SC Universal Oligo	14.5 µl
REPLI-g SensiPhi DNA Polymerase	1 µl
Total volume†	**30 µl**

* To prepare REPLI-g SensiPhi amplification mix for multiple reactions, scale up according to the number of reactions. We recommend to use the REPLI-g universal oligo for most applications and when transitioning from REPLI-g WTA Single Cell kits. The REPLI-g advanced oligo may offer improved uniformity when working with human primary samples. Researchers should experimentally confirm which REPLI-g oligo is best for their specific application.

† Mix by vortexing and centrifuge briefly.

11. Incubate at 30°C for 2 h.
12. Stop the reaction by incubating at 65°C for 5 min; then, cool on ice.
13. If not being used directly, store the amplified cDNA at –30 to –15°C until required for downstream applications. We recommend storage of the amplified cDNA at a minimum concentration of 100 ng/µl.

Note: The high-molecular-weight DNA generated by random extension of primers (primer-multimer formation) in NTC does not contain genetic information and will not affect the quality of downstream applications. These products are outcompeted by cDNA generated from viable cells present during MDA.

14. Amplified cDNA can be directly used for the library construction or for target-directed amplification and library construction (see "Protocol: Enzymatic Fragmentation and Library Preparation Using the QIAseq Single Cell RNA Amplified cDNA"). Amplified cDNA behaves like purified genomic DNA and has an approximate length of 2000–70,000 bp.

    Note: If quantification of the amplified cDNA is required, follow the instructions in Appendix B. Optical density (OD) measurements overestimate the amplified DNA from step 12 and should not be used.

Protocol: Amplification of Purified RNA

This protocol is for whole transcriptome amplification of purified RNA. Different types of purified RNA can be used (see "Important points before starting").

Important points before starting

- The protocol can be applied to any type of purified RNA, such as total RNA, poly A+ RNA (from using the RNeasy® Pure mRNA Bead Kit) or ribosomal RNA-depleted RNA. It is not suited for degraded RNA, such as that derived from FFPE tissues.
- QIAseq FastSelect RNA removal technology can be incorporated into the protocol to remove unwanted ribosomal or other RNAs. See Appendix G for protocol modifications.
- The specific protocol used for WTA of purified RNA depends on the starting material and the downstream application.
- Use 50 pg – 100 ng of purified RNA for the WTA protocol. Starting from 500pg will maximize the number of detected genes.
- Avoid any DNA or RNA contamination of reagents by using separate laboratory equipment (e.g., pipettes, filter pipette tips, reaction vials, etc.). Set up the REPLI-g Single Cell reaction in a location free of nucleic acids.
- The high-molecular-weight DNA generated by random extension of primers (primer-multimer formation) in NTC does not contain genetic information and will not affect the quality of downstream applications. These products are outcompeted by DNA of viable cells present during WTA.
- Because the QIAseq Single Cell RNA Library Kit is intended to generate amplified cDNA from a minimal amount of starting RNA, take appropriate measures to avoid inadvertently introducing RNase contamination. Create and maintain an RNase-free environment when working with RNA by following proper microbiological and aseptic technique. The use of disposable plastic tubes and pipette tips from freshly opened boxes or bags is strongly recommended.

- The reagents for whole transcriptome amplification are not suitable for the amplification of small RNA molecules, such as tRNAs or miRNAs.
- Although all sequences are well represented, the amplified cDNA does not contain full-length cDNAs. The amplification process is started by random-primed and Oligo-dT – primed cDNA synthesis. Consequently, transcript sequences are amplified in pieces. Due to the nature of the ligation reaction, DNA fragments might not be assembled in the order in which they originally existed in the organism. However, kit chemistry is designed to make these events rare, and thus, detection of nucleic acid sequences is not affected (e.g., polymorphisms) in downstream NGS applications.

Things to do before starting

- The Quantiscript RT mix, ligation mix, and REPLI-g SensiPhi amplification mix described in the protocol must always be prepared fresh. They cannot be stored for later use.
- All buffers and reagents should be vortexed before use to ensure thorough mixing.
- Quantiscript RT Enzyme Mix, Ligase Mix, and REPLI-g SensiPhi DNA Polymerase should be thawed on ice. All other components can be thawed at room temperature (15–25°C).
- For increased speed and convenience, all incubation steps of the protocol can be preprogrammed on a thermal cycler. Use the cycling parameters listed below in Table 9.

Table 9. Thermal cycling parameters

Step	Time	Temperature
Set the heating lid to 50°C for all steps		
RNA denaturation	3 min	95°C
	∞	4°C
gDNA removal	10 min	42°C
	∞	4°C
Reverse transcription	60 min	42°C
	3 min	95°C
	∞	4°C
Ligation	30 min	24°C
	5 min	95°C
	∞	4°C
Whole transcriptome amplification	2 h	30°C
	5 min	65°C
	∞	4°C

Procedure

1. Place 8 µl purified RNA (>50 pg) into a microcentrifuge tube. If using less than 8 µl of purified RNA, add H_20 sc to bring the volume up to 8 µl.
2. Add 3 µl NA Denaturation Buffer, mix by vortexing, and centrifuge briefly.
3. Incubate at 95°C for 3 min; then, cool to 4°C. Immediately place on ice.
4. Add 2 µl gDNA Wipeout Buffer, mix by vortexing, and centrifuge briefly.

5. Incubate at 42°C for 10 min. If more time is needed to prepare the next step, place on ice.

   Note: Due to self-priming events, rRNA might be amplified despite the use of Oligo-dT only.

   Note: If rRNA is needed to be depleted, the QIAseq FastSelect –rRNA HMR Kit can be used prior to addition of Quantiscript RT mix to block rRNA transcription and amplification. To block rRNA, and/or globin mRNA, follow the instructions for the QIAseq FastSelect –rRNA HMR and –Globin Kits in Appendix G.

6. Proceed as described in step 6 of the protocol "Amplification of Poly A+ mRNA from Single Cells" or "Amplification of Total RNA from Single Cells".

Protocol: Specific Enrichment of Purified RNA

This protocol is for enrichment of total viral RNA from total RNA preparations. Different types of purified RNA can be used (see "Important points before starting").

Important points before starting

- The protocol can be applied to any type of purified total RNA. It is not suited for degraded RNA, such as that derived from FFPE tissues.
- Use 500 pg – 10 ng of purified total RNA for the WTA protocol.
- Avoid any DNA or RNA contamination of reagents by using separate laboratory equipment (e.g., pipettes, filter pipette tips, reaction vials, etc.). Set up the REPLI-g Single Cell reaction in a location free of nucleic acids.
- Because the QIAseq Single Cell RNA Library Kit is intended to generate amplified cDNA from a minimal amount of starting RNA, take appropriate measures to avoid inadvertently introducing RNase contamination. Create and maintain an RNase-free environment when working with RNA by following proper microbiological and aseptic technique. The use of disposable plastic tubes and pipette tips from freshly opened boxes or bags is strongly recommended.
- The reagents for whole transcriptome amplification are not suitable for the amplification of small RNA molecules, such as tRNAs or miRNAs.
- The protocol replaces the random oligos and Oligo-dT by target-specific primers in the RT and MDA amplification to specifically enrich viral RNA in host RNA background.
  It is optimized to enrich RNA from as little as 1 pg in the presence of up to 5 ng contaminating RNA. Recommendations for primer design are applied in Appendix E, page 85.
  Note: In cases of a very low number of copies (< 5000 copies) of viral RNA in high total RNA background (>1 ng total RNA), enrichment will be enhanced by rRNA depletion, using the QIAseq FastSelect –rRNA HMR Kit. This can be used prior to addition of the Quantiscript RT mix to block rRNA transcription and amplification. To block rRNA

and/or globin mRNA, follow the instructions for the QIAseq FastSelect rRNA –HMR and –Globin Kits in Appendix G.

Things to do before starting

- The Quantiscript RT mix, ligation mix, and REPLI-g SensiPhi amplification mix described in the protocol must always be prepared fresh. They cannot be stored for later use.
- All buffers and reagents should be vortexed before use to ensure thorough mixing.
- Quantiscript RT Enzyme Mix, Ligase Mix, and REPLI-g SensiPhi DNA Polymerase should be thawed on ice. All other components can be thawed at room temperature (15–25°C).
- For increased speed and convenience, all incubation steps of the protocol can be preprogrammed on a thermal cycler. Use the cycling parameters listed in Table 10.

Table 10. Thermal cycling parameters

Step	Time	Temperature
Set the heating lid to 50°C for all steps		
RNA denaturation	3 min	95°C
	∞	4°C
gDNA removal	10 min	42°C
	∞	4°C
Reverse transcription	60 min	42°C
	3 min	95°C
	∞	4°C
Ligation	30 min	24°C
	5 min	95°C
	∞	4°C
Whole transcriptome amplification	3 h	30°C
	5 min	65°C
	∞	4°C

Procedure

1. Place 8 µl purified RNA (>500 pg) into a microcentrifuge tube. If using less than 8 µl of purified RNA, add H_20 sc to bring the volume up to 8 µl.
2. Add 3 µl NA Denaturation Buffer, mix by vortexing, and centrifuge briefly.
3. Incubate at 95°C for 3 min; then, cool to 4°C.
4. Add 2 µl gDNA Wipeout Buffer, mix by vortexing, and centrifuge briefly.
5. Incubate at 42°C for 10 min. If more time is needed to prepare the next step, place on ice.
6. Prepare Quantiscript RT mix (Table 11). Add 6 µl Quantiscript RT mix to the lysed cell sample, mix by vortexing, and centrifuge briefly.

   Note: Quantiscript RT mix must be prepared fresh.

Table 11. Preparation of Quantiscript RT mix*

Component	Volume/reaction
RT/Polymerase Buffer	4 µl
Target-specific RT Primer mix (25 µM each)	1 µl
Quantiscript RT Enzyme Mix	1 µl
Total volume†	**6 µl**

* To prepare Quantiscript RT mix for multiple reactions, scale up according to the number of reactions.
† Mix by vortexing and centrifuge briefly.

7. Incubate at 42°C for 60 min. Stop the reaction by incubating at 95°C for 3 min; then, cool on ice.
8. Prepare the ligation mix (Table 12). Add 10 µl ligation mix to the RT reaction from step 7. Mix by vortexing and centrifuge briefly.

   Important: When preparing the ligation mix, add the components in the order shown in Table 12.

   Note: The ligation mix must be prepared fresh.

Table 12. Preparation of the ligation mix*

Component	Volume/reaction
Ligase Buffer	8 µl
Ligase Mix	2 µl
Total volume†	**10 µl**

* To prepare ligation mix for multiple reactions, scale up according to the number of reactions.
† Mix by vortexing and centrifuge briefly.

9. Incubate at 24°C for 30 min. Stop the reaction by incubating at 95°C for 5 min; then, cool on ice.
10. Prepare REPLI-g SensiPhi amplification mix (Table 13). Add 30 µl REPLI-g SensiPhi amplification mix to the ligation reaction from step 9. Mix by vortexing and centrifuge briefly.

    Note: REPLI-g SensiPhi amplification mix must be prepared fresh.

Table 13. Preparation of REPLI-g SensiPhi amplification mix*

Component	Volume/reaction
REPLI-g SC Dilution Buffer	28 µl
Target-specific primer mix (10 µM each)	1 µl
REPLI-g SensiPhi DNA Polymerase	1 µl
Total volume†	**30 µl**

* To prepare REPLI-g SensiPhi amplification mix for multiple reactions, scale up according to the number of reactions.
† Mix by vortexing and centrifuge briefly.

11. Incubate at 30°C for 3 h.
12. Stop the reaction by incubating at 65°C for 5 min; then, cool on ice.
13. If not being used directly, store the amplified cDNA at –30 to –15°C until required for downstream applications. Amplified cDNA behaves like purified genomic DNA and has an approximate length of 2000–70,000 bp. We recommend storage of the amplified cDNA at a minimum concentration of 100 ng/µl.

Amplified cDNA can be directly used for the NGS library construction or for targeted NGS panels (see "Protocol: Enzymatic Fragmentation and Library Preparation Using the QIAseq Single Cell RNA Amplified cDNA").

Note: To proceed with library preparation, quantify the amplified DNA following the instructions in Appendix B. Optical density (OD) measurements overestimate REPLI-g amplified DNA and should not be used without purifying the amplified cDNA (see Appendix F).

The input amount of amplified cDNA used in the FX reaction will indicate if library amplification is required.

Protocol: Enzymatic Fragmentation and Library Preparation Using the QIAseq Single Cell RNA Amplified cDNA

This protocol describes end-repair, A-addition, adapter ligation, and cleanup and size selection of amplified cDNA, for the preparation of high-diversity, PCR-free libraries that are ready for quantification and sequencing on Illumina instruments.

Important points before starting

- This protocol is for constructing sequencing libraries for Illumina NGS platforms using QIAseq Single Cell RNA Library Kit.
- The amplified cDNA should be diluted in H_2O before starting.

Things to do before starting

- Program cycles. For increased speed and convenience, all incubation steps of the protocol can be preprogrammed and saved on a thermal cycler in advance (Table 14). Refer to Table 14 to determine the time and protocol required to fragment input DNA to the desired size.
- Prepare fresh 80% ethanol.
- Prepare Buffer 10 mM Tris·Cl, pH 8.0.
- Program thermal cyclers. For increased speed and convenience, all incubation steps of the protocol can be preprogrammed and saved in advance.

Procedure: Enzymatic fragmentation and library preparation

FX single-tube fragmentation, end-repair, and A-addition

1. Thaw all kit components on ice. Once reagents are thawed, mix buffers thoroughly by quickly vortexing to avoid any localized concentrations. Briefly spin down vortexed reagents before use.

   Program a thermocycler according to Table 14 and start the program. If possible, set the temperature of the heated lid to ~70°C.

2. When the thermocycler block reaches 4°C, pause the program.

Table 14. Amplified cDNA fragmentation reaction conditions to produce ~450 bp fragments

Step	Temperature	Incubation time (Transcriptome analysis)	Incubation time (Enriched RNA analysis)
1	4°C	1 min	1 min
2	32°C	30 min*	15 min
3	65°C	30 min	30 min
4	4°C	Hold	Hold

* The insert size of the completed libraries is determined by the duration of step 2. For example, when using 200–500 ng input of WTA-cDNA, 30 minutes of fragmentation time produces a library fragment distribution of ~450 bp if the FX enhancer is used. The fragment size can be adjusted by varying the duration of step 2. Please refer to Table 15 for more information. Use a thermocycler with a heated lid set at 70°C.

Table 15. Fragment size versus fragmentation time

Incubation time	Fragment size after library amplification	Fragment size without library amplification
15 min	511	543
30 min	474	496
45 min	474	431
60 min	506	385

Note: Nonamplified libraries do not accurately migrate in capillary electrophoresis because they consist of ligated and non-ligated fragments. Moreover, a fragment may form higher size fragments by hybridization of the Y-shape adapters. Amplification of the library removes this type of fragments, which leads to migration artifacts and allows accurate fragment size evaluation.

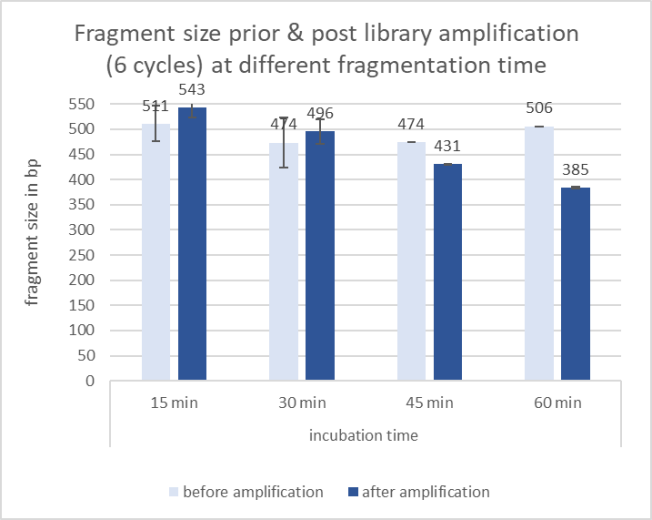

Figure 2. Fragment size distribution versus fragmentation time before and after amplification of the library.

3. Mix 3 µl amplified cDNA and 7 µl H_2O sc. This should give 500–1000 ng total amplified DNA in 10 µl H_2O sc (50–100 ng/µl). Pipette 10 µl of the diluted DNA in PCR tubes or PCR-strip tubes and place them on ice or a cooling block.

   Note: If you have quantified the cDNA to control the input in fragmentation, do not exceed 5 µl undiluted cDNA input in the FX reaction.

Note: If WTA has been previously purified (see Appendix F) to remove reaction carry over and to concentrate the amplified cDNA, then higher input volumes are possible. Adjust WTA input volume and H_2O sc accordingly in Table 16. Total volume of the reaction is 50 µl

4. Prepare the FX Reaction Mix on ice according to Table 16. Add the components of the FX Reaction Mix in the same order as stated in the table. Before adding the FX Enzyme Mix, pipette up and down the Buffer Mix. You can scale up the FX Reaction Mix according to the number of samples processed.

   Note: WTA-cDNA is high molecular weight DNA and, if not purified, includes a high content of salt and additives from previous reaction steps. Addition of FX Enhancer is essential and increases reproducibility.

Table 16. FX reaction setup for insert fragment size of 300 bp

Component	Volume/reaction*
FX Buffer, 10x	5 µl
H_2O sc	20 µl
FX Enhancer	5 µl
FX Enzyme Mix	10 µl
Total reaction volume	**40 µl**

* Mix by pipetting, and keep on ice.

5. Add 40 µl FX Reaction Mix to each 10 µl diluted amplified cDNA sample on ice and gently vortex to mix. The final volume of the reaction is 50 µl.
6. Briefly spin down the PCR plate/tubes, immediately transfer to the pre-chilled thermocycler (4°C) and resume the program. Once the fragmentation program is complete, transfer samples to ice.
7. Immediately proceed with adapter ligation as described in the next protocol.

Adapter ligation

8. Equilibrate QIAseq Beads at room temperature for 20–30 min before use.
9. Vortex and spin down the adapter plate. Remove the protective adapter plate lid, carefully pierce the foil seal, and transfer 5 µl from one DNA adapter well to each 50 µl sample from the previous protocol. Track the barcodes used for each sample.
10. Replace the adapter plate lid and freeze unused adapters. The adapter plate is stable for a minimum of 10 freeze-thaw cycles.

    Important: Only 1 single adapter should be used per ligation reaction. If adapters from another supplier are used, follow the manufacturer's instructions.
11. Prepare the ligation master mix (per DNA sample) in a separate tube on ice according to Table 17. Mix well by gently vortexing at low rpm.

Table 17. Ligation master mix (per sample)

Component	Volume/reaction*
DNA Ligase Buffer, 5x	20 µl
H_2O sc	15 µl
DNA Ligase	10 µl
Total reaction volume	**45 µl**

* Mix by pipetting, and keep on ice.

12. Add 45 µl of the ligation master mix to each sample. Mix well and incubate at 20°C for 15 min.

    Important: Do not use a thermocycler with a heated lid.
13. Proceed immediately to adapter ligation cleanup (steps 14–23) using 0.6x (60 µl) QIAseq beads.
14. Add 60 µl resuspended QIAseq Beads slurry to each ligated sample, and mix well by pipetting or gently vortexing.

15. Incubate the mixture for 5 min at room temperature. Short spin down to collect liquid on the bottom of the tube.
16. Pellet the beads on a magnetic stand for 2 min, visually confirm that the supernatant is clear, and carefully discard the supernatant.
17. Wash the beads by adding 200 µl fresh 80% ethanol to each pellet. Pellet the beads on the magnetic stand for 2 min and then carefully discard the supernatant.
18. Repeat the wash (step 17) once (for a total of 2 ethanol washes).
19. Incubate on the magnetic stand for 5–10 min or until the beads are dry. Avoid overdrying, which may result in lower DNA recovery. Remove from the magnetic stand.
20. Elute by resuspending in 52.5 µl 10 mM Tris·Cl, pH 8.0, or H_2O sc. Pellet beads on the magnetic stand. Carefully transfer 50 µl supernatant to a new PCR plate.
21. Perform a second purification. Add 55 µl of resuspended QIAseq Beads to each sample and mix.
22. Repeat steps 15–19.
23. Elute by resuspending in 26 µl 10 mM Tris·Cl, pH 8.0, or H_2O sc. Pellet the beads on the magnetic stand. Carefully transfer 23.5 µl of supernatant into a new PCR plate. Store purified libraries at –30 to –15°C until ready for sequencing.
24. Assess the quality of the library using a capillary electrophoresis device or other comparable method. Check for the correct size distribution (Figure 3) of library fragments and for the absence of adapters or adapter-dimers.

    Note: The median size of the DNA insert should be shifted by 120 bp, the size of the adapters that were ligated to the library fragments.

    Note: The median fragment size can be used for subsequent qPCR-based quantification methods. Non-amplified libraries generate artifacts when analyzed over capillary electrophoresis, due to several reasons as follows. (1) This is a mix of ligated and nonligated fragments; thus, they may generate multiple peaks or shoulders differing by the size added by the ligated adapters. (2) The end of non-amplified libraries have y-shaped single stranded ends. Due to complementarity, they may form concatemeric

fragments, which will not be resolved in capillary electrophoresis and will lead to higher peaks and shoulders.

Amplification of the library for a few cycles (e.g., 4 cycles) will lead to a homogenous library, which will give electropherogram traces with one main peak. However, multiple peaks of non-amplified libraries will not affect the sequencing quality, but will need quantification over qPCR.

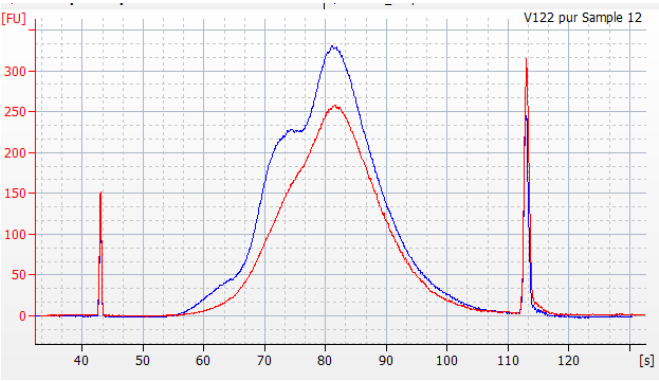

Figure 3. Capillary electrophoresis device trace of generated libraries. Capillary electrophoresis device trace data showing the size distribution of a non-amplified (blue) and an amplified library (red) and the absence of adapters or adapter-dimers.

25. Quantify the library using the QIAseq Library Quant Assay Kit (sold separately) or another comparable method.

    Note: Library quantitation via qPCR is strongly recommended to ensure accurate library dilution and clustering, maximizing pass-filter reads. Capillary electrophoresis or Qubit measurements can overestimate library quantity since these cannot distinguish sequenceable library fragments from inserts containing only one adapter.

    With 200 ng – 1 µg WTA-cDNA input, sufficient library should be generated for sequencing on Illumina platforms without further PCR amplification (Figure 4).

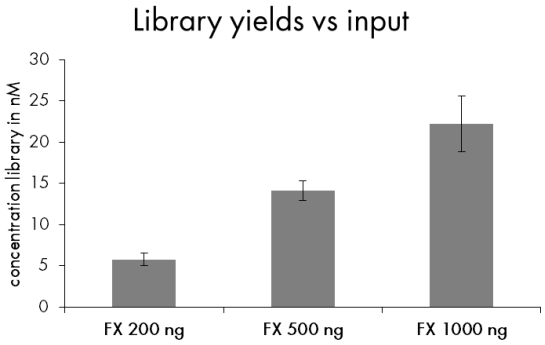

Figure 4. Library yields vs input of WTA-cDNA. Plotted data are means of triplicate reactions with SD.

26. The purified library can be safely stored at –30 to –15°C until needed.
27. Pool and dilute qPCR-quantified libraries to 2 nM. For optimal clustering on Illumina flow cells, follow the library dilution recommendations as provided by Illumina for each instrument. The table below provides sequencing recommendations depending on the sequencing instrument and type analysis.

Table 18. Sequencing recommendations for Transcriptome analysis and viral RNA sequencing

Instrument	Sequencing recommendation for viral RNA sequencing	Sequencing recommendation for mRNA/ total RNA sequencing
iSeq 100	PE 2 x 150 bp	
MiniSeq	PE 2 x 150 bp	
MiSeq	PE 2 x 150 bp	
NextSeq 500		PE 2 x 75 bp
HiSeq 2500		PE 2 x 50 bp SE 1 x 100 bp PE 2 x 100 bp PE 2 x 125 bp
NovaSeq 6000		SE 1 x 35 bp PE 2 x 50 bp SE 1 x 100 bp PE 2 x 100 bp

Protocol: Amplification of Library DNA and Final Cleanup

PCR-based library amplification is not normally required, but can be used if insufficient WTA product was generated and if samples are irreplaceable. This protocol is for the high-fidelity amplification of completed libraries using the HiFi polymerase included in the PCR Master Mix with this kit.

Thing to do before starting

- Thaw all reagents on ice. Once reagents are thawed, mix them thoroughly by vortexing to avoid any localized concentrations.

Procedure

1. Prepare a reaction mix according to Table 19.

Table 19. Reaction mix for library amplification

Component	Volume/reaction (µl)
HiFi PCR Master Mix, 2x	25
Primer Mix (10 µM each)	1.5
Library DNA (from step 23, page 47)	Variable
RNase-free water	Variable
Total reaction volume	**50**

2. Program a thermocycler according to Table 20. Place the amplification mixes in the cycler and start the program.

Table 20. Thermal cycling parameters

Time	Temperature	Number of cycles per input 100–200 ng	Number of cycles per input 40–100 ng	Number of cycles per input 10–40 ng	Number of cycles per input <10 ng
2 min	98°C	1	1	1	1
20 s 30 s 30 s	98°C 60°C 72°C	4	6	8	10
1 min	72°C	1	1	1	1
∞	4°C	Hold	Hold	Hold	Hold

3. Proceed with library cleanup using 1x (50 µl) QIAseq Beads.

   Note: library can be also stored at –30 to –15°C before cleanup.

4. Add 50 µl resuspended QIAseq Beads slurry to each amplified sample and mix well by pipetting or gently vortexing.
5. Incubate the mixture for 5 min at room temperature.
6. Pellet the beads on a magnetic stand for 2 min and carefully discard the supernatant.
7. Wash the beads by adding 200 µl fresh 80% ethanol to each pellet. Pellet the beads on the magnetic stand for 2 min and then carefully discard the supernatant.
8. Repeat the wash step (step 7) once for a total of 2 ethanol washes.
9. Incubate on the magnetic stand for 5–10 min or until the beads are dry. Avoid overdrying, which may result in lower DNA recovery. Remove from the magnetic stand.
10. Elute by resuspending in 26 µl 10 mM Tris·Cl, pH 8.0, or H_2O sc. Pellet beads on the magnetic stand. Carefully transfer 23.5 µl supernatant to a new Lo-bind tube.
11. Store purified libraries at –30 to –15°C until ready for sequencing or other further applications.
12. Quantify the library using the QIAseq Library Quant Assay Kit (sold separately) or other comparable methods.

Troubleshooting Guide

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

		Comments and suggestions
Little or no amplified cDNA		
a)	Lysed cells sample not immediately used in WTA reaction	Use the lysed cell sample immediately, without any storage prior to performing WTA reaction.
b)	Cell sample collected or stored improperly	Use cells stored under the correct conditions for WTA analysis. RNA may degrade quickly in cells that are stored incorrectly. When working with single or small numbers of cells, ensure that they do not stick to the tube wall.
c)	Inefficient lysis due to an excess of cells in the sample	Use 1–1000 cells. If cells are clumpy the cell counting may not be accurate.
d)	Incorrect reaction temperature	Make sure to carry out reverse transcription, ligation, and amplification reactions at the temperatures specified in the protocol. If necessary, check the temperature of your thermal cycler, heating block, or water bath.
e)	Pipetting error or missing reaction component	Use pipettes with care and make sure all reaction components are included.
f)	Incorrect incubation time	Make sure to use the incubation times specified in the protocol for reverse transcription, ligation, and amplification reactions.
g)	RT mix, ligation mix, and amplification mix not freshly prepared	Quantiscript RT mix, ligation mix, and REPLI-g SensiPhi amplification mix should be freshly prepared before use. Storage of these mixes prior to use may affect whole transcriptome amplification.
h)	Choice of primer used during reverse transcription step	Using the Oligo-dT Primer instead of a mixture of Oligo-dT Primer and Random Primer results in less cDNA amplified during whole transcriptome amplification.

		Comments and suggestions
i)	Possible RNase contamination	Take appropriate measures to avoid inadvertent RNase contamination. Create and maintain an RNase-free environment by following proper microbiological and aseptic technique. The use of disposable plastic tubes and pipette tips from freshly opened boxes or bases in strongly recommended.
j)	Low yields with viral RNA enrichment protocol	For low input or strong fragmented DNA, increase RT primers to 30 µM each and Sensi-phi amplification primers to 15 µM.

cDNA yields of approximately 10 µg in negative (no template) controls, but no mappable reads in these samples

DNA is generated during the QIAseq Single Cell RNA Library Kit WTA reaction by random extension of primer-dimers	High-molecular-weight product can be generated by random extension of primer-dimers. This DNA will not affect the amplification quality of actual samples. This non-specific amplification is out competed by cDNA amplification in cDNA presence.

cDNA yields of approximately 10 µg in negative (no template) controls and reads mapping to either the correct annotated reference or other sequences

DNA is generated during the QIAseq Single Cell RNA Library Kit WTA reaction by contaminating RNA or DNA templates	Decontaminate all laboratory equipment and take all necessary precautions to avoid contamination of reagents and samples with extraneous DNA. If possible, work in a laminar-flow hood. Use sterile equipment and barrier pipette tips only, and keep amplification chemistry and DNA templates in separate storage locations.

Protocols using cells as a starting material

Little or no transcripts are detected, but cDNA yield is approximately 20 µg

a)	Sample does not contain a cell	Dilutions of cells down to 1 cell/volume often contain less than a single cell due to Poisson distribution.
b)	Cells are not intact	Use viable cells for QIAseq Single Cell RNA Library Kit reactions. Best results are obtained with samples containing >90% viable cells. Although according to cell staining, the number of dead cells in such samples is very low, it has been found that the number of damaged cells that still have an intact membrane is much higher.
c)	Cells have cell walls	Cells with cell walls cannot be lysed efficiently. Do not use cells with cell walls (e.g. cells from plants, bacteria, or fungi).
d)	Cells have been fixed	Cells that have been fixed (e.g., formaldehyde) cannot be used for WTA.
e)	Low-abundance transcript analyzed	Due to the Poisson distribution, the QIAseq Single Cell RNA Library Kit may provide variable amplification of low-abundance transcripts.
f)	Small transcripts analyzed	Small transcripts, such as tRNA or miRNAs, cannot be amplified by the QIAseq Single Cell RNA Library Kit. Only RNA transcripts longer than 500 nt can be amplified efficiently.

	Comments and suggestions
Protocols using purified RNA as a starting material	
Little or no transcripts are detected but DNA yield is approximately 20 µg	
a) Incorrect amount of RNA template	Do not use less than 10 pg total RNA as template. A single human cell contains approximately 10 pg of total RNA. Due to the Poisson distribution, not all transcripts of low-copy mRNAs are present in a volume containing 10 pg RNA.
b) RNA template degraded	Use nondegraded RNA or larger amounts of RNA, if possible. Only RNA transcripts longer than 500 nucleotides can be amplified.
c) Low-abundance transcript analyzed	The QIAseq Single Cell RNA Library Kit amplifies low-abundance transcripts to a variable extent due to the Poisson distribution.
d) Small transcripts analyzed	Only RNA transcripts longer than 500 nt can be amplified.
e) 5' regions analyzed when using the protocol "Amplification of the Poly A+ mRNA from Single Cells"	In the protocol "Amplification of the Poly A+ mRNA from Single Cells", 3' regions of polyadenylated transcripts are amplified. 5' regions are underrepresented.
f) RNA template contains carrier RNA	Use RNA template that was purified without using carrier RNA.
Library preparation protocol	
Low library yields	
a) WTA yields were lower as expected	Quantify the yield of WTA using PicoGreen Reagent. Typically, 100 ng of WTA-cDNA generates enough Illumina-compatible library to use directly for sequencing without amplification. If the final library yield is not sufficient, a library amplification step can be performed following the adapter ligation step.
Unexpected signal peaks in capillary electrophoresis device traces	
a) Presence of shorter peaks between 60 and 120 bp	These peaks represent library adapters and adapter-dimers that occur when there is no, or insufficient, adapter depletion after library preparation. As adapter-dimers can form clusters on the flow cell and will be sequenced, this will reduce the capacity of the flow cell for the library fragments, even though a low ratio of adapter-dimers versus library will not be a problem. Ensure to equilibrate and mix well the QIAseq Beads prior the purification protocol.
b) Presence of larger library fragments after library enrichment	In case of performing library enrichment, if the fragment population shifts higher than expected after adapter ligation and PCR enrichment (e.g., more than the expected 120 bp shift), this can be a PCR artifact due to over-amplification of the DNA library. Make sure to use as few amplification cycles as possible (8–10) to avoid this effect.

		Comments and suggestions
c)	Incorrect library fragment size after adapter ligation	During library preparation, adapters of approximately 60 bp are ligated to both ends of the inserts. This should be reflected on a capillary electrophoresis device by a shift in size of all library fragments of 120 bp. If using adapters from other suppliers, please refer to the size information given in the respective documentation. The absence of a clear size shift may indicate no, or only low, adapter ligation efficiency. Make sure to use the parameters and incubation times described in the handbook for end-repair, A-addition, and ligation – as well as the correct amount of starting cDNA.

Appendix A: Determination of Concentration and Quality of Amplified cDNA

Quantification of cDNA yield

A 60 µl QIAseq WTA reaction typically yields approximately 20 µg of cDNA, allowing direct use of the amplified cDNA in library preparation. Depending on the quality of the input material, the resulting amount of cDNA may be less (due to cells not freshly prepared or different input materials). For a more accurate quantification of the amplified cDNA, it is important to utilize a cDNA quantification method that is specific for double-stranded DNA, since amplification products contain unused reaction primers. Quant-iT PicoGreen dsDNA reagent displays enhanced binding to double-stranded DNA and may be used, in conjunction with a fluorometer, to quantify the double-stranded DNA product. A protocol for the quantification of QIAseq Single Cell RNA amplified cDNA can be found in Appendix B.

Quantification of transcript abundance

As downstream NGS is often expensive, especially with larger numbers of cells, we recommend controlling the quality of the WTA samples using qPCR and probes and primer sets designed towards commonly expressed transcripts or transcripts of interest.

Each qPCR reaction should contain 5–10 ng of the cDNA amplified via WTA. Alternative for easy handling, WTA amplified cDNA can be diluted 1:100 and 3 µl can be used in the PCR assay. Real-time PCR assays that recognize exons, as well as exon-intron or intron regions, are recommended. For example, QIAGEN's QuantiTect® Assays – which detect exon region of the genes – in combination with QuantiFast® or QuantiTect SYBR Green PCR Master Mixes are recommended for such quality control assays.

Appendix B: PicoGreen Quantification of QIAseq Single Cell RNA Amplified cDNA (WTA-cDNA)

This protocol is designed for quantification of double stranded QIAseq Single Cell RNA amplified cDNA using Quant-iT PicoGreen dsDNA reagent.

Alternatively, Qubit quantification might be also performed according to manufacturer's protocol. We recommend diluting the QIAseq Single Cell RNA amplified cDNA 1:100 when using the Qubit dsDNA HS Assay Kit (**https://www.lifetechnologies.com/de/de/home/life-science/laboratory-instruments/fluorometers/qubit/qubit-assays.html#ion**).

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

Equipment and reagents to be supplied by user

- Quant-iT PicoGreen dsDNA Reagent (Life Technologies, cat. no. P7581)
- TE buffer (10 mM Tris·Cl; 1mM EDTA, pH 8.0)
- Human genomic DNA (e.g., Promega, cat. no. G3041)
- 2 ml microcentrifuge tube, or 15 ml Falcon tubes
- 96-well plates (suitable for use in a fluorescence microplate reader)
- Fluorescence microplate reader (e.g., TECAN® Ultra)

Procedure

1. Make a 1:200 dilution of PicoGreen stock solution in TE buffer. Each quantification reaction requires 50 µl. Depending on the final volume, use a 2 ml microcentrifuge tube

or a 15 ml Falcon tube. Cover the tube in aluminum foil or place it in the dark to avoid photodegradation of the PicoGreen reagent.

For example, to prepare enough PicoGreen working solution for 100 samples, add 25 µl PicoGreen to 4975 µl TE buffer.

Important: Prepare the PicoGreen/TE solution in a plastic container as the PicoGreen reagent may adsorb to glass surfaces.

2. Prepare a 16 µg/ml stock solution of control genomic DNA in TE buffer.
3. Make 200 µl of 1.6, 0.8, 0.4, 0.2, and 0.1 µg/ml DNA standards by further diluting the 16 µg/ml control genomic DNA with TE buffer.
4. Transfer 50 µl of each DNA standard in duplicate into a 96-well plate labeled A (Figure 5).

   Note: The 96-well plate must be suitable for use in a fluorescent microplate reader.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H	blank	blank	1.6	0.8	0.4	0.2	0.1	1.6	0.8	0.4	0.2	0.1

Figure 5. 96-well plate. Gray squares: genomic DNA standards (µg/µl).

5. Place 2 µl of each QIAseq Single Cell RNA amplified cDNA sample for quantification into a new 96-well plate and add 198 µl TE buffer to make a 1:100 dilution. Store the remaining QIAseq Single Cell RNA amplified cDNA at –30 to –15°C.

6. Place 5 µl diluted QIAseq Single Cell RNA amplified cDNA (from step B5) into an unused well of 96-well plate A and add 45 µl TE buffer to make a 1:1000 dilution.

   The 1:100 dilutions from step B5 can be stored at –30 to –15°C and used for future downstream sample analysis.

7. For Blanc measurements, pipet 50 µl TE Buffer in 2 empty wells of plate A.
8. Add 50 µl PicoGreen working solution (from step 1) to each sample (amplified cDNA and control DNA standards) in 96-well plate A. Seal and gently shake the plate on the bench top or pipet up and down to mix the samples and reagent.
9. Centrifuge the 96-well plate briefly to collect residual liquid from the walls of the wells and incubate in the dark for 5 min at room temperature.
10. Measure the sample fluorescence using a fluorescence microplate reader and standard fluorescence filters (excitation: ~480 nm; emission: ~520 nm).

    To ensure that the sample readings remain in the detection range of the microplate reader, adjust the instrument's gain so that the sample with the highest DNA concentration yields fluorescence intensity near the fluorometer's maximum.

Calculation of DNA concentration and yield

11. Generate a standard curve by plotting the concentration (µg/ml) of DNA standards (x-axis) against the fluorescence reading generated by the microplate reader (y-axis). Plot an average of the fluorescence recorded for each DNA standard of the same concentration.
12. Use the standard curve to determine the concentration (µg/ml) of the diluted QIAseq Single Cell RNA amplified cDNA sample. This is achieved by plotting the fluorescence reading of the sample against the standard curve and reading the DNA concentration on the x-axis.

    Note: The calculation of cDNA concentration depends on the standard curve and the determination of the slope. For accurate results, the standard curve should be a straight line. Any deviation from this may cause inaccuracies in the measurement of QIAseq Single Cell RNA amplified cDNA concentrations.

13. Multiply the value determined in step 11 by 1000 to show the concentration of undiluted sample cDNA (since the sample DNA measured by PicoGreen fluorescence had been diluted 1:1000).
14. To determine the total amount of cDNA in your sample, multiply the concentration (µg/ml) of undiluted sample DNA (determined in step 12) by the reaction volume in milliliters (i.e., for a 60 µl reaction, multiply by 0.06).

Appendix C: QIAseq Dual-Index Y-Adapters

Generation of sample sheets for Illumina instruments

Index sequences for QIAseq Unique and Combinatorial Dual-Index Y-Adapters are available for download at **www.qiagen.com**. Library prep definition files allow you to use the Illumina Experiment Manager Software to create sample sheets according to your needs. Alternatively, ready-to-use sample sheets containing all UDI Y-Adapter barcode sequences are available for MiSeq, NextSeq, MiniSeq, HiSeq, and NovaSeq instruments. These can be edited using the Illumina Experiment Manager Software or any text editor.

The following guide describes how to set up custom library prep kits within the Illumina Experiment Manager Software (version 1.18.1). Alternatively, refer to the *Illumina Experiment Manager User Guide* (**support.illumina.com/downloads/illumina-experiment-manager-user-guide-15031335.html**).

1. Download custom library prep definition files from **www.qiagen.com**.
2. Locate the installation directory of the Illumina Experiment Manager. (Typically, it would be in **Program Files\Illumina\Illumina Experiment Manager**)
3. Place copies of the downloaded files **QIAseq UDI Y.txt** in the "SamplePrepKits" folder.
4. Navigate to the "Applications" folder and locate the files:
   - GenerateFASTQ.txt
   - NextSeqGenerateFASTQ.txt
   - HiSeqGenerateFASTQ.txt
   - NovaSeqGenerateFASTQ.txt.

   Apply the actions in the next steps to each file.
5. Open each file in a text editor and locate the text block **[Compatible Sample Prep Kits]**.
6. Generate 2 new lines underneath the header, and then add this entry:

- QIAseq UDI-Y

7. Save and close the file when complete.
8. Restart the Illumina Experiment Manager and select **Create Sample Sheet**.
9. After selecting the instrument, navigate to the respective "FASTQ Only" workflow.
10. In the run settings for "Library Prep Workflow", select **QIAseq UDI-Y** to generate a sample sheet for QIAseq Y-adapters.

Unique Dual-Index Y-Adapters

The layout of the 24-plex and 96-plex (A/B/C/D) single-use UDI adapter plate is shown in Figure 6 to Figure 10. The index motives used in the QIAseq Unique Dual-Index Kits are listed in Table 21. To make sequencing preparation more convenient, you can download Illumina-compatible sample sheets for different sequencing instruments at **www.qiagen.com**.

	1	2	3	4	5	6	7	8	9	10	11	12
A	UDI 001	UDI 009	UDI 017	empty	empty	empty	empty	empty	empty	empty	empty	empty
B	UDI 002	UDI 010	UDI 018	empty	empty	empty	empty	empty	empty	empty	empty	empty
C	UDI 003	UDI 011	UDI 019	empty	empty	empty	empty	empty	empty	empty	empty	empty
D	UDI 004	UDI 012	UDI 020	empty	empty	empty	empty	empty	empty	empty	empty	empty
E	UDI 005	UDI 013	UDI 021	empty	empty	empty	empty	empty	empty	empty	empty	empty
F	UDI 006	UDI 014	UDI 022	empty	empty	empty	empty	empty	empty	empty	empty	empty
G	UDI 007	UDI 015	UDI 023	empty	empty	empty	empty	empty	empty	empty	empty	empty
H	UDI 008	UDI 016	UDI 024	empty	empty	empty	empty	empty	empty	empty	empty	empty

Figure 6. QIAseq UDI Y-Adapter Plate (24) layout (UDI 1–24).

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

Figure 7. QIAseq UDI Y-Adapter Plate A (96) layout (UDI 1–96).

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

Figure 8. QIAseq UDI Y-Adapter Plate B (96) layout (UDI 97–192).

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

Figure 9. QIAseq UDI Y-Adapter Plate C (96) layout (UDI 193–288).

	1	2	3	4	5	6	7	8	9	10	11	12
A	UDI 289	UDI 297	UDI 305	UDI 313	UDI 321	UDI 329	UDI 337	UDI 345	UDI 353	UDI 361	UDI 369	UDI 377
B	UDI 290	UDI 298	UDI 306	UDI 314	UDI 322	UDI 330	UDI 338	UDI 346	UDI 354	UDI 362	UDI 370	UDI 378
C	UDI 291	UDI 299	UDI 307	UDI 315	UDI 323	UDI 331	UDI 339	UDI 347	UDI 355	UDI 363	UDI 371	UDI 379
D	UDI 292	UDI 300	UDI 308	UDI 316	UDI 324	UDI 332	UDI 340	UDI 348	UDI 356	UDI 364	UDI 372	UDI 380
E	UDI 293	UDI 301	UDI 309	UDI 317	UDI 325	UDI 333	UDI 341	UDI 349	UDI 357	UDI 365	UDI 373	UDI 381
F	UDI 294	UDI 302	UDI 310	UDI 318	UDI 326	UDI 334	UDI 342	UDI 350	UDI 358	UDI 366	UDI 374	UDI 382
G	UDI 295	UDI 303	UDI 311	UDI 319	UDI 327	UDI 335	UDI 343	UDI 351	UDI 359	UDI 367	UDI 375	UDI 383
H	UDI 296	UDI 304	UDI 312	UDI 320	UDI 328	UDI 336	UDI 344	UDI 352	UDI 360	UDI 368	UDI 376	UDI 384

Figure 10. QIAseq UDI Y-Adapter Plate D (96) layout (UDI 289–384).

Table 21. UDI motives used in the QIAseq UDI Y-Adapter Kits (24 and 96 A/B/C/D)

Unique Dual-Index adapters 1–24 are identical on the adapter plates of the QIAseq UDI Y-Adapter Kit (24) and QIAseq UDI Y-Adapter Kit A (96).

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

Indices for entry on sample sheet			
Unique dual-index number	**i5 bases for entry on sample sheet (MiSeq, HiSeq 2000/2500, NovaSeq 6000)**	**i5 bases for entry on sample sheet (iSeq100, MiniSeq, NextSeq, HiSeqX, HiSeq 3000/4000)**	**i7 bases for entry on sample sheet (all instruments)**
UDI 001	ATGGCCGACT	AGTCGGCCAT	TGAACGTTGT
UDI 002	CGATGAGCAC	GTGCTCATCG	ACCAGACTTG
UDI 003	GATAAGTCGA	TCGACTTATC	ACTGGCGAAC
UDI 004	TCACGCCTTG	CAAGGCGTGA	GCGTTAGGCA
UDI 005	AGGAACACAA	TTGTGTTCCT	TTATCGGCCT
UDI 006	CTCAGTAGGC	GCCTACTGAG	GAGGTATAAG
UDI 007	GAAGTGCCTG	CAGGCACTTC	TCAAGGATTC
UDI 008	TCTCTCGCCT	AGGCGAGAGA	CGAACCGAGA
UDI 009	AGGCACCTTC	GAAGGTGCCT	GAGCCAAGTT
UDI 010	CTGTTGGTAA	TTACCAACAG	AAGGCCGTAG
UDI 011	GCTGGTACCT	AGGTACCAGC	TTAGAGAAGC
UDI 012	TAAGGAGCGG	CCGCTCCTTA	TCTAAGACCA
UDI 013	AATCGCTCCA	TGGAGCGATT	TGTAACCACT
UDI 014	CTCCTAATTG	CAATTAGGAG	CCGACACAAG
UDI 015	GCCTCATAAT	ATTATGAGGC	CTCTGATGGC
UDI 016	TGTATTGAGC	GCTCAATACA	CGGCCTGTTA
UDI 017	AGCCATAACA	TGTTATGGCT	TGCATAGCTT
UDI 018	CCACAAGTGG	CCACTTGTGG	AACCTTCTCG
UDI 019	GTTATCACAC	GTGTGATAAC	AAGAGATCAC
UDI 020	TACCGTTCTT	AAGAACGGTA	GCCTGAAGGA

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Indices for entry on sample sheet			
Unique dual-index number	**i5 bases for entry on sample sheet (MiSeq, HiSeq 2000/2500, NovaSeq 6000)**	**i5 bases for entry on sample sheet (iSeq100, MiniSeq, NextSeq, HiSeqX, HiSeq 3000/4000)**	**i7 bases for entry on sample sheet (all instruments)**
UDI 021	AGGCGTTAGG	CCTAACGCCT	ATTGTGCCTT
UDI 022	CCGTAACGTC	GACGTTACGG	TCCTCTACCG
UDI 023	GTAATAGCCA	TGGCTATTAC	TACCATGAAC
UDI 024	TAGCGCCGAT	ATCGGCGCTA	CATTGGCAGA
UDI 025	CATTCTTGGA	TCCAAGAATG	CACTGCTATT
UDI 026	ATGCAAGGTT	AACCTTGCAT	AATGGTAGGT
UDI 027	CGCCAGACAA	TTGTCTGGCG	GATACCTATG
UDI 028	GAAGGTTGGC	GCCAACCTTC	CACTAGGTAC
UDI 029	TCGCATCACG	CGTGATGCGA	AGCTCGTTCA
UDI 030	CCGGTCATGA	TCATGACCGG	TGTCAGTCTT
UDI 031	ATTCACAAGC	GCTTGTGAAT	GATGAACAGT
UDI 032	CAACCTGTAA	TTACAGGTTG	ACAATCGGCG
UDI 033	GCCAGTCGTT	AACGACTGGC	GATTGAGTTC
UDI 034	TGCCTTGTCG	CGACAAGGCA	GTAATGCCAA
UDI 035	CTATCCGCTG	CAGCGGATAG	TCGTTGCGCT
UDI 036	AATGCCGGAA	TTCCGGCATT	AGGTGAGTAT
UDI 037	CGGTTATCCG	CGGATAACCG	TCGATAATGG
UDI 038	GCGGAAGAGT	ACTCTTCCGC	GCGTCTCTTC
UDI 039	TTGGTTAGTC	GACTAACCAA	GTCTCCTGCA
UDI 040	TTCAGTGTGA	TCACACTGAA	GAGCTTCATT

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Indices for entry on sample sheet			
Unique dual-index number	**i5 bases for entry on sample sheet (MiSeq, HiSeq 2000/2500, NovaSeq 6000)**	**i5 bases for entry on sample sheet (iSeq100, MiniSeq, NextSeq, HiSeqX, HiSeq 3000/4000)**	**i7 bases for entry on sample sheet (all instruments)**
UDI 041	AGAATTCTGG	CCAGAATTCT	AGGCCTACAT
UDI 042	CATTGACTCT	AGAGTCAATG	TGTGGAACCG
UDI 043	GCGGCTTCAA	TTGAAGCCGC	CGTATTAAGC
UDI 044	TTATGGTCTC	GAGACCATAA	CCAGTGGTTA
UDI 045	CGTAACCAGG	CCTGGTTACG	GCGTTCGAGT
UDI 046	AGCTCAGATA	TATCTGAGCT	CCTTCCGGTT
UDI 047	CCGGTGTTAC	GTAACACCGG	CACAAGACGG
UDI 048	GACCTAACCT	AGGTTAGGTC	GCTTACACAC
UDI 049	TTGTAGAAGG	CCTTCTACAA	AGGATGTCCA
UDI 050	CCTAGCACTA	TAGTGCTAGG	CACCTTATGT
UDI 051	ATCGTGTTCT	AGAACACGAT	AAGCGGCTGT
UDI 052	CCAACTTATC	GATAAGTTGG	TTCCTGTGAG
UDI 053	GAAGCCAAGG	CCTTGGCTTC	AGTACAGTTC
UDI 054	TGGAGTTCAA	TTGAACTCCA	TACAGCCTCA
UDI 055	CTTCAATCCT	AGGATTGAAG	GTTCTATTGG
UDI 056	ATCTTGCGTG	CACGCAAGAT	ATATACCGGT
UDI 057	CGTCTAAGGT	ACCTTAGACG	CCTCGGAATG
UDI 058	GAGGTGAACA	TGTTCACCTC	GTTCTGGAAC
UDI 059	TCAGAACTAC	GTAGTTCTGA	AGATTCACCA
UDI 060	CGGATATTGA	TCAATATCCG	TCGGTCAGAT

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Indices for entry on sample sheet			
Unique dual-index number	**i5 bases for entry on sample sheet (MiSeq, HiSeq 2000/2500, NovaSeq 6000)**	**i5 bases for entry on sample sheet (iSeq100, MiniSeq, NextSeq, HiSeqX, HiSeq 3000/4000)**	**i7 bases for entry on sample sheet (all instruments)**
UDI 061	AGGAGTAGAT	ATCTACTCCT	CACTCTCGCT
UDI 062	CCGCCGAATA	TATTCGGCGG	GTTGGTCCAG
UDI 063	GAGTCTATAC	GTATAGACTC	AGCTCGAAGC
UDI 064	TTATTACCGG	CCGGTAATAA	AGAGGTTCTA
UDI 065	CGCTCGTTAG	CTAACGAGCG	ATGACTCGAA
UDI 066	AACAACGCTG	CAGCGTTGTT	GAACAATCCT
UDI 067	CGCGGCTATT	AATAGCCGCG	TGGCAAGGAG
UDI 068	GCTCGACACA	TGTGTCGAGC	GAATATTGGC
UDI 069	TTCTTCCAAC	GTTGGAAGAA	CCGGAACCTA
UDI 070	TTGGCGGTTG	CAACCGCCAA	ACTTGTTCGG
UDI 071	AACAGGCAAT	ATTGCCTGTT	CAAGTCCAAT
UDI 072	CAGAATGGCG	CGCCATTCTG	AACCGCAAGG
UDI 073	GTTGAGATTC	GAATCTCAAC	ACGTTGACTC
UDI 074	TGTGTGCGGA	TCCGCACACA	CCACTTAACA
UDI 075	GTTCGGCGAA	TTCGCCGAAC	AGCAGTTCCT
UDI 076	AGCTGTATTG	CAATACAGCT	TCGCCTTCGT
UDI 077	CAGCGGATGA	TCATCCGCTG	TAGGACTGCG
UDI 078	GTCCTTGGAT	ATCCAAGGAC	TCCGAGCGAA
UDI 079	TCTAGATGCT	AGCATCTAGA	TTCGGTTGTT
UDI 080	CGAGCCACAT	ATGTGGCTCG	ACAGGAGGAA

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Indices for entry on sample sheet			
Unique dual-index number	**i5 bases for entry on sample sheet (MiSeq, HiSeq 2000/2500, NovaSeq 6000)**	**i5 bases for entry on sample sheet (iSeq100, MiniSeq, NextSeq, HiSeqX, HiSeq 3000/4000)**	**i7 bases for entry on sample sheet (all instruments)**
UDI 081	ATGGAATGGA	TCCATTCCAT	CCTCCATTAA
UDI 082	CATTCCTCAC	GTGAGGAATG	AGTCGCGGTT
UDI 083	GCATAGGAAG	CTTCCTATGC	CTCATCCAGG
UDI 084	TGTTCGTGTT	AACACGAACA	TGTGGTTGAA
UDI 085	TAAGACCGTT	AACGGTCTTA	TTATGCGTGG
UDI 086	ATGGTACCAG	CTGGTACCAT	GCGAATGTAT
UDI 087	CCGACAGCTT	AAGCTGTCGG	GTCAAGCTCG
UDI 088	GACGATATGA	TCATATCGTC	TAGAGTTGGA
UDI 089	TTGTACTCCA	TGGAGTACAA	CTGATGATCT
UDI 090	GTGCACATAA	TTATGTGCAC	ACTAGGTGTT
UDI 091	AGGACAAGTA	TACTTGTCCT	CTGTTAGCGG
UDI 092	CCGATTCGAG	CTCGAATCGG	ATCGCACCAA
UDI 093	GTAGGAACTT	AAGTTCCTAC	CTTACTTGGT
UDI 094	TACACTACGA	TCGTAGTGTA	CCTTAATGCG
UDI 095	ATGACCTTGA	TCAAGGTCAT	TCTCGCCTAG
UDI 096	CTACGTGACG	CGTCACGTAG	TCTTCAGAGA
UDI 097	AACAATCAGG	CCTGATTGTT	TACCGGTGGT
UDI 098	CTGGTGTGCA	TGCACACCAG	AGGTGTTACG
UDI 099	GCATATCCTT	AAGGATATGC	ACAGACCGAC
UDI 100	TGTCCTGTAC	GTACAGGACA	CGAATACGTA

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Indices for entry on sample sheet			
Unique dual-index number	**i5 bases for entry on sample sheet (MiSeq, HiSeq 2000/2500, NovaSeq 6000)**	**i5 bases for entry on sample sheet (iSeq100, MiniSeq, NextSeq, HiSeqX, HiSeq 3000/4000)**	**i7 bases for entry on sample sheet (all instruments)**
UDI 101	AGAACGTCGC	GCGACGTTCT	TAGCATCGAT
UDI 102	CACGGACTAG	CTAGTCCGTG	CCATGAGTCG
UDI 103	GTTGAACACT	AGTGTTCAAC	ACTAACATGC
UDI 104	TCGCGTGGTA	TACCACGCGA	ACACTCTCTA
UDI 105	AGCCACTATG	CATAGTGGCT	GCTCTTGCCT
UDI 106	CCACCTACCA	TGGTAGGTGG	AATCTTGAGG
UDI 107	GTTCCGGTGT	ACACCGGAAC	CTTAACGGTC
UDI 108	TAGGTCTGAC	GTCAGACCTA	TTGTGACCAA
UDI 109	AGGAAGCATT	AATGCTTCCT	TCACACACCT
UDI 110	CCTTAGTTGG	CCAACTAAGG	CTGCAATTAG
UDI 111	GTCCTATTCA	TGAATAGGAC	CTCCTTACTC
UDI 112	TAAGATGGAC	GTCCATCTTA	GCAACGCAGA
UDI 113	AGGCCATGGT	ACCATGGCCT	CCTTACCAAT
UDI 114	CATTGGCCAA	TTGGCCAATG	TTAATCCTCG
UDI 115	GCTATGAATC	GATTCATAGC	TTCCGAGTTC
UDI 116	TTGGTCCTCG	CGAGGACCAA	CTCGAGAGGA
UDI 117	AGCGACATAC	GTATGTCGCT	TGTTGGCTGT
UDI 118	CAAGTAGTCT	AGACTACTTG	CGTATCTGCG
UDI 119	GTCAAGAAGA	TCTTCTTGAC	CCATAGTATC
UDI 120	TCCTGTTATG	CATAACAGGA	TGGACAGTAA

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Indices for entry on sample sheet			
Unique dual-index number	**i5 bases for entry on sample sheet (MiSeq, HiSeq 2000/2500, NovaSeq 6000)**	**i5 bases for entry on sample sheet (iSeq100, MiniSeq, NextSeq, HiSeqX, HiSeq 3000/4000)**	**i7 bases for entry on sample sheet (all instruments)**
UDI 121	AAGTGCGATA	TATCGCACTT	GTACCTTGTT
UDI 122	AGGCTACACG	CGTGTAGCCT	GAGTGCCTCT
UDI 123	CTATATCGGC	GCCGATATAG	TAAGTAGCGG
UDI 124	GCTAAGGTAA	TTACCTTAGC	CGTGGTGTTC
UDI 125	TAACCTGGTT	AACCAGGTTA	CATTCCTGAA
UDI 126	AGTTGGTCTA	TAGACCAACT	AAGATGCATG
UDI 127	ATGCAGCTGG	CCAGCTGCAT	CCTTGGAGCT
UDI 128	CGTTGCCTTC	GAAGGCAACG	ACCGGAACAG
UDI 129	GCGTGGAGAA	TTCTCCACGC	GAATGGAAGC
UDI 130	TACGCCTCCT	AGGAGGCGTA	GTTCTCCATA
UDI 131	AATTCGGTAG	CTACCGAATT	GTCACTATGT
UDI 132	ATTGTCGAAC	GTTCGACAAT	TGGTAGAACT
UDI 133	CAACCTTGCG	CGCAAGGTTG	ACGCCTATGG
UDI 134	GCACTGCGTA	TACGCAGTGC	AATCCGTTAC
UDI 135	TGCTAGTAGT	ACTACTAGCA	GTTGAGGCTA
UDI 136	AAGTCACGGA	TCCGTGACTT	TATCAACTGG
UDI 137	AGCGATTGAA	TTCAATCGCT	AAGAGGAGAT
UDI 138	CTACCTCTCT	AGAGAGGTAG	GTCTTCTCGG
UDI 139	GACAACTGTC	GACAGTTGTC	GAAGCCACTC
UDI 140	TCCATTGCGG	CCGCAATGGA	GTAGGACACA

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Table continued from previous page

Indices for entry on sample sheet			
Unique dual-index number	**i5 bases for entry on sample sheet (MiSeq, HiSeq 2000/2500, NovaSeq 6000)**	**i5 bases for entry on sample sheet (iSeq100, MiniSeq, NextSeq, HiSeqX, HiSeq 3000/4000)**	**i7 bases for entry on sample sheet (all instruments)**
UDI 141	AGCCTCGCAA	TTGCGAGGCT	CTCCTCGTAT
UDI 142	AATACAGGCT	AGCCTGTATT	CCACATGATT
UDI 143	CGGACCGTTA	TAACGGTCCG	AGACGGTTGG
UDI 144	GCGCTTATGC	GCATAAGCGC	CTAGGTTGAC
UDI 145	TTAACACGAG	CTCGTGTTAA	AAGCGTACCA
UDI 146	CGCCTCTAGA	TCTAGAGGCG	TCATGTTGGT
UDI 147	AATCGACCTT	AAGGTCGATT	TTGGAATGGT
UDI 148	CCGCAATAAC	GTTATTGCGG	GTGTATGTTG
UDI 149	GTTCCAACGA	TCGTTGGAAC	TCCTGTCAAC
UDI 150	TGTTAGACCG	CGGTCTAACA	TAATCAGGCA
UDI 151	AACCTCATAG	CTATGAGGTT	GTAGTGGATT
UDI 152	ATGAATCCAC	GTGGATTCAT	AATTGCGCAT
UDI 153	CGGCTTAATT	AATTAAGCCG	GACAATAACG
UDI 154	GAGTTGCAGG	CCTGCAACTC	ACAGTTAAGC
UDI 155	TCCACGAACA	TGTTCGTGGA	AGCCACACTA
UDI 156	TGACGGAGGA	TCCTCCGTCA	CAATCGTCTT
UDI 157	AATGAGTACG	CGTACTCATT	AGGAGCTTGT
UDI 158	CGTCTTCCGA	TCGGAAGACG	TTGAGCGGAG
UDI 159	GACAGAGATT	AATCTCTGTC	AGTAGCTCTC
UDI 160	TTACGCTAAC	GTTAGCGTAA	CACGCTGTCA

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Indices for entry on sample sheet			
Unique dual-index number	**i5 bases for entry on sample sheet (MiSeq, HiSeq 2000/2500, NovaSeq 6000)**	**i5 bases for entry on sample sheet (iSeq100, MiniSeq, NextSeq, HiSeqX, HiSeq 3000/4000)**	**i7 bases for entry on sample sheet (all instruments)**
UDI 161	CTCCTCGAAG	CTTCGAGGAG	AAGACCTCTT
UDI 162	ATACCGCAGA	TCTGCGGTAT	GACCTCTTCT
UDI 163	CCTATCTGAT	ATCAGATAGG	TACTTCCTTG
UDI 164	GATCGGTTAC	GTAACCGATC	TGCGATACGC
UDI 165	TGGTGAGGTG	CACCTCACCA	GCAGGCTTAA
UDI 166	AACCGGCGTA	TACGCCGGTT	TAAGCTTGTG
UDI 167	AATACCGATC	GATCGGTATT	ATGGTCCGCT
UDI 168	CGATACTCAA	TTGAGTATCG	ATGTCAGAAG
UDI 169	GTAAGGCGGT	ACCGCCTTAC	GACGAAGGTC
UDI 170	TTCAAGGTCG	CGACCTTGAA	ATCACCGTGA
UDI 171	TATCCGAGTA	TACTCGGATA	GCTACAGTGT
UDI 172	AGCGCGCTTA	TAAGCGCGCT	CGTCGAATAT
UDI 173	CCGGAGACAT	ATGTCTCCGG	CAACCATCGG
UDI 174	GAGATAACTG	CAGTTATCTC	CGGTCCATTC
UDI 175	TTGTAAGCGC	GCGCTTACAA	AGAAGAGCCA
UDI 176	CAAGAGGAGG	CCTCCTCTTG	CTATGCAATG
UDI 177	AACCTTAGGA	TCCTAAGGTT	CACTGAACCG
UDI 178	CTGGCAACTC	GAGTTGCCAG	TACTGTGTGA
UDI 179	GAACTTGTTG	CAACAAGTTC	GCATTCTGTT
UDI 180	TGTGCAAGAT	ATCTTGCACA	CTCCGCTAAG

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Indices for entry on sample sheet			
Unique dual-index number	**i5 bases for entry on sample sheet (MiSeq, HiSeq 2000/2500, NovaSeq 6000)**	**i5 bases for entry on sample sheet (iSeq100, MiniSeq, NextSeq, HiSeqX, HiSeq 3000/4000)**	**i7 bases for entry on sample sheet (all instruments)**
UDI 181	AATCGAGAGA	TCTCTCGATT	TCGCTTGAGA
UDI 182	AGCGTGTCAG	CTGACACGCT	AACTAGCCTT
UDI 183	CTTGGTGATT	AATCACCAAG	TTCGCTCAGG
UDI 184	GAAGCAGCAA	TTGCTGCTTC	CTCTACAACA
UDI 185	TTCCGTCGAC	GTCGACGGAA	TGAGTGTGTT
UDI 186	CGAGATGCCA	TGGCATCTCG	TAGTTAGTCG
UDI 187	AAGTTCGTGC	GCACGAACTT	GCCTGATCCT
UDI 188	CGTCCATAAG	CTTATGGACG	CGAGTACAGG
UDI 189	TTGTGGCATA	TATGCCACAA	GCCTAGATTA
UDI 190	AGATCGGAAT	ATTCCGATCT	TCGGCACTGT
UDI 191	CATTCTACTG	CAGTAGAATG	CCGTGCAAGA
UDI 192	ATCGCCGTAG	CTACGGCGAT	CTGGCTGGTT
UDI 193	ATCCTTACAC	GTGTAAGGAT	CGTTAGGATT
UDI 194	CGCAAGGACT	AGTCCTTGCG	TTCCATTACG
UDI 195	GCTGGCGTTA	TAACGCCAGC	TAGCGGTAAC
UDI 196	TACTTAGAGG	CCTCTAAGTA	GTAGCCAGGA
UDI 197	ATGGCGATGC	GCATCGCCAT	AGGATACTCT
UDI 198	CATTGGTGCG	CGCACCAATG	TATCCTCCAG
UDI 199	GCGAGATATA	TATATCTCGC	TAAGTCGTTC
UDI 200	TGACTGCTAT	ATAGCAGTCA	TCCGGATTGA

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Indices for entry on sample sheet			
Unique dual-index number	**i5 bases for entry on sample sheet (MiSeq, HiSeq 2000/2500, NovaSeq 6000)**	**i5 bases for entry on sample sheet (iSeq100, MiniSeq, NextSeq, HiSeqX, HiSeq 3000/4000)**	**i7 bases for entry on sample sheet (all instruments)**
UDI 201	AACGTCCGCT	AGCGGACGTT	ACGTCTTGTT
UDI 202	CGCACATGTC	GACATGTGCG	ATGAAGTGCG
UDI 203	GCACACCTGA	TCAGGTGTGC	CGATCACTGC
UDI 204	TTGTCCAGAG	CTCTGGACAA	CCTATCGGAA
UDI 205	AGCCTTCCTG	CAGGAAGGCT	CAGAGAGCTT
UDI 206	CCTTACGCCA	TGGCGTAAGG	GCAACTTGCG
UDI 207	GAATACGTAC	GTACGTATTC	TATGGAGGAC
UDI 208	TTGGCACCGT	ACGGTGCCAA	TGAGATCAGA
UDI 209	ATTAGGTGGC	GCCACCTAAT	TCAGCCTATT
UDI 210	CGATCAAGAA	TTCTTGATCG	GTTGTGAGCG
UDI 211	GCTGTCTTCT	AGAAGACAGC	TCAGTAACAC
UDI 212	TACATGTCTG	CAGACATGTA	AAGGCTCAGA
UDI 213	AACCAGTTGA	TCAACTGGTT	GTGTGGTGGT
UDI 214	CCGGTAAGCT	AGCTTACCGG	CCGAGCTTAG
UDI 215	GTTCGAATAG	CTATTCGAAC	ATCACGCTTC
UDI 216	TGTCAGGCTC	GAGCCTGACA	TAGCTATGCA
UDI 217	CAACAGTGTT	AACACTGTTG	TGTTCCTCAT
UDI 218	AAGAGAGGAA	TTCCTCTCTT	CATACCTTCT
UDI 219	CGGTTGTAGC	GCTACAACCG	GCCTTCAATG
UDI 220	GCCTGAAGTG	CACTTCAGGC	CTTGACCAGC

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Indices for entry on sample sheet			
Unique dual-index number	**i5 bases for entry on sample sheet (MiSeq, HiSeq 2000/2500, NovaSeq 6000)**	**i5 bases for entry on sample sheet (iSeq100, MiniSeq, NextSeq, HiSeqX, HiSeq 3000/4000)**	**i7 bases for entry on sample sheet (all instruments)**
UDI 221	TTACGACACT	AGTGTCGTAA	CTACACACAA
UDI 222	CGCCTAGATC	GATCTAGGCG	TAGGCTGAAT
UDI 223	AATCTGGATG	CATCCAGATT	TCGGAGTCCT
UDI 224	CGACGGTACA	TGTACCGTCG	AACATCGCGG
UDI 225	GTAGTATTGC	GCAATACTAC	GTTGTCTTAC
UDI 226	TCCAGCGGAT	ATCCGCTGGA	GTGGCAACTA
UDI 227	CAACCACCTC	GAGGTGGTTG	GAGCAGGCAT
UDI 228	AGCTTAGGCG	CGCCTAAGCT	AACGGCACCT
UDI 229	CCGGTTCCTT	AAGGAACCGG	AGTAACCTTG
UDI 230	GACATTGAAC	GTTCAATGTC	TCTCATAAGC
UDI 231	TTAGAGGCGA	TCGCCTCTAA	TGCTTGCCAA
UDI 232	CAAGCCGAAC	GTTCGGCTTG	CGGTTCCTGT
UDI 233	AGGAGAACGG	CCGTTCTCCT	CCAAGTAGAT
UDI 234	CCTGTTAGAC	GTCTAACAGG	AAGGTTGGCG
UDI 235	GTTCTACGTT	AACGTAGAAC	TGCTCTGGTC
UDI 236	TAAGTCCACA	TGTGGACTTA	ACTGTAACGA
UDI 237	CAAGAACCAT	ATGGTTCTTG	GATTCCAGGT
UDI 238	AGTTGATGAC	GTCATCAACT	TTCACCAGAT
UDI 239	CCTACTCTTG	CAAGAGTAGG	ACTTCCAAGG
UDI 240	GAACAATCCA	TGGATTGTTC	CCGAATATTC

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Indices for entry on sample sheet			
Unique dual-index number	**i5 bases for entry on sample sheet (MiSeq, HiSeq 2000/2500, NovaSeq 6000)**	**i5 bases for entry on sample sheet (iSeq100, MiniSeq, NextSeq, HiSeqX, HiSeq 3000/4000)**	**i7 bases for entry on sample sheet (all instruments)**
UDI 241	TTCTGTTGGT	ACCAACAGAA	CTCTTATCCA
UDI 242	CATCGTCAGG	CCTGACGATG	TCACAGCGGT
UDI 243	ATGCATGAAG	CTTCATGCAT	CCTCTGTCGT
UDI 244	CGTGAATCGC	GCGATTCACG	TCTGTTCTCG
UDI 245	GAGCAGCCTT	AAGGCTGCTC	GATACTTCAC
UDI 246	TCGATTACCA	TGGTAATCGA	AGTGCTGATA
UDI 247	CAGTCCAATT	AATTGGACTG	ATCCTTCGGT
UDI 248	AGAGGCTTGG	CCAAGCCTCT	GACAACGATT
UDI 249	CAGGCTCTCA	TGAGAGCCTG	GAACCGGTAG
UDI 250	GTTCGCTCTC	GAGAGCGAAC	AGCAATGAGC
UDI 251	TCGGACTAAT	ATTAGTCCGA	CAAGACTCCA
UDI 252	CGAGATCTTC	GAAGATCTCG	ACCGTGTAGG
UDI 253	ATAACCGGAC	GTCCGGTTAT	AGGCACAGGT
UDI 254	CGTGTAGTTA	TAACTACACG	CGACAGATCG
UDI 255	GAACATAGGT	ACCTATGTTC	ACGCGACAAC
UDI 256	TCTAACATCG	CGATGTTAGA	ACTTGCGTTA
UDI 257	AACGGTGGCA	TGCCACCGTT	CACCACTCAT
UDI 258	AGGACGGTGT	ACACCGTCCT	CTTCGTAACT
UDI 259	CTGTGACCTG	CAGGTCACAG	CAGTATTCGG
UDI 260	GCTGTAACAA	TTGTTACAGC	CAGTCTGGAC

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Indices for entry on sample sheet			
Unique dual-index number	**i5 bases for entry on sample sheet (MiSeq, HiSeq 2000/2500, NovaSeq 6000)**	**i5 bases for entry on sample sheet (iSeq100, MiniSeq, NextSeq, HiSeqX, HiSeq 3000/4000)**	**i7 bases for entry on sample sheet (all instruments)**
UDI 261	TACGGACGTC	GACGTCCGTA	TACCGTTCTA
UDI 262	CCTAAGGAGC	GCTCCTTAGG	GTGTCCACAG
UDI 263	ATAAGGCCAG	CTGGCCTTAT	TTACGACTGT
UDI 264	CTCATCTGTA	TACAGATGAG	GACGCGAATG
UDI 265	GAAGGCATCT	AGATGCCTTC	CAACGTACGC
UDI 266	TCTCTACTGC	GCAGTAGAGA	AGCTCAGGAA
UDI 267	AACCGAACAA	TTGTTCGGTT	GATAGGCGGT
UDI 268	ATCTCGCCAC	GTGGCGAGAT	AGTAGGAAGT
UDI 269	CCATGCAACG	CGTTGCATGG	CATGTTGTAG
UDI 270	GAATGGTGTA	TACACCATTC	CACATTCTTC
UDI 271	TATATGCCGT	ACGGCATATA	GCAGCTCGTA
UDI 272	CTCGATAGAT	ATCTATCGAG	GTTCAGACGG
UDI 273	AACACAAGAG	CTCTTGTGTT	TCCTGGAAGT
UDI 274	CGCAATCGGT	ACCGATTGCG	GCATTGTTAG
UDI 275	GTTGCGTAGA	TCTACGCAAC	GACCTACAGC
UDI 276	TAGAGTGATC	GATCACTCTA	CACCGACGTA
UDI 277	AAGACGCAGC	GCTGCGTCTT	CTCTCACCTT
UDI 278	AACTTCTCGA	TCGAGAAGTT	CTCGTTCATT
UDI 279	CGCAACTGAG	CTCAGTTGCG	TGGTGGCAAG
UDI 280	GCTCCGCAAT	ATTGCGGAGC	GATTGCTTGA

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Indices for entry on sample sheet			
Unique dual-index number	**i5 bases for entry on sample sheet (MiSeq, HiSeq 2000/2500, NovaSeq 6000)**	**i5 bases for entry on sample sheet (iSeq100, MiniSeq, NextSeq, HiSeqX, HiSeq 3000/4000)**	**i7 bases for entry on sample sheet (all instruments)**
UDI 281	GTAACTTCCG	CGGAAGTTAC	CCGTTAAGGT
UDI 282	CTCACGACTA	TAGTCGTGAG	TGCTGAGAGG
UDI 283	AACCAACGGC	GCCGTTGGTT	TTGTCACTTG
UDI 284	CCTGCCTGTA	TACAGGCAGG	GCTGTTATGT
UDI 285	TACGCTGCAG	CTGCAGCGTA	GCAGCAGTTG
UDI 286	AATGTTGCGA	TCGCAACATT	GCAGATCAAT
UDI 287	CGACGTTCTG	CAGAACGTCG	TGGTTCACGG
UDI 288	AATAGGACAC	GTGTCCTATT	TCGACCGCAT
UDI 289	ATGTGCCTCA	TGAGGCACAT	TAACCTAGGT
UDI 290	CGACTCCGTT	AACGGAGTCG	AACTCATGCG
UDI 291	GCTGTTGTGG	CCACAACAGC	CCGGATGAAC
UDI 292	TACCAATCAC	GTGATTGGTA	CGTTGCCGTA
UDI 293	ATGTCTTACG	CGTAAGACAT	GCTCTACGGT
UDI 294	CGCAACAATA	TATTGTTGCG	TGCATTGGCG
UDI 295	GAACGAAGAC	GTCTTCGTTC	CGATTGTGAC
UDI 296	TCGAGGACGT	ACGTCCTCGA	GACTGCACTA
UDI 297	ATTATGAGCG	CGCTCATAAT	GTTAACTGCT
UDI 298	CGCGTTATAA	TTATAACGCG	TCGGACCTTG
UDI 299	GCGTGCATGT	ACATGCACGC	TGCAGCAAGC
UDI 300	TAAGCGGCTC	GAGCCGCTTA	CACATGCGAA

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Indices for entry on sample sheet			
Unique dual-index number	**i5 bases for entry on sample sheet (MiSeq, HiSeq 2000/2500, NovaSeq 6000)**	**i5 bases for entry on sample sheet (iSeq100, MiniSeq, NextSeq, HiSeqX, HiSeq 3000/4000)**	**i7 bases for entry on sample sheet (all instruments)**
UDI 301	AACATGGAGA	TCTCCATGTT	CAGACGTAAT
UDI 302	CCGAGTCTCT	AGAGACTCGG	ATTCGGTACG
UDI 303	GTACTTCTAC	GTAGAAGTAC	TTAGCACGGC
UDI 304	TGTTCACATG	CATGTGAACA	GAGGATAGTA
UDI 305	AAGGTAACGC	GCGTTACCTT	AACTGTGGTT
UDI 306	CCGCCTTACT	AGTAAGGCGG	ATTACCTCGG
UDI 307	GTTGAGGCAG	CTGCCTCAAC	CGCTGTATAC
UDI 308	TGGCGACCTA	TAGGTCGCCA	CTTGCTCACA
UDI 309	AGAAGCGACA	TGTCGCTTCT	CAACACCTGT
UDI 310	CAGGATAATC	GATTATCCTG	CAATTGCTCG
UDI 311	GCTCCTACAG	CTGTAGGAGC	CATAGACAAC
UDI 312	TTCAACAGGT	ACCTGTTGAA	TTGGTGTCTA
UDI 313	CCTCGTCCAT	ATGGACGAGG	TATGTCCTGT
UDI 314	AGCGTTGGTT	AACCAACGCT	GCCAATTCGT
UDI 315	CATTCGAACA	TGTTCGAATG	TAGGCGATCG
UDI 316	GCTTACCGAC	GTCGGTAAGC	ATGAGTGTAC
UDI 317	TTAGCTTAGG	CCTAAGCTAA	CCGAAGGATA
UDI 318	CCGACACACA	TGTGTGTCGG	AGTCCACTGT
UDI 319	ATTCGCTGAT	ATCAGCGAAT	GCGGCTAATT
UDI 320	CCAAGAGGCA	TGCCTCTTGG	TCTAACTCAG

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Indices for entry on sample sheet			
Unique dual-index number	**i5 bases for entry on sample sheet (MiSeq, HiSeq 2000/2500, NovaSeq 6000)**	**i5 bases for entry on sample sheet (iSeq100, MiniSeq, NextSeq, HiSeqX, HiSeq 3000/4000)**	**i7 bases for entry on sample sheet (all instruments)**
UDI 321	GACGCAGTTC	GAACTGCGTC	CAAGCTGAGC
UDI 322	TGGAACTCGG	CCGAGTTCCA	CCAGAGCACA
UDI 323	CCACACCAAT	ATTGGTGTGG	TGTACAAGGT
UDI 324	AGTTCTCGGC	GCCGAGAACT	TAGAATGCCT
UDI 325	CTTGACGACG	CGTCGTCAAG	TGTCTTACTG
UDI 326	GAGGTCGCTA	TAGCGACCTC	ATGACTAAGC
UDI 327	TCAGTAGCAT	ATGCTACTGA	ATGTAGGCAA
UDI 328	CTAACGTGGA	TCCACGTTAG	GCGAAGAGGT
UDI 329	ATGCCAACCG	CGGTTGGCAT	CGGTGGTTCT
UDI 330	CGGTCGATTC	GAATCGACCG	CTGTCGTTGG
UDI 331	GAAGTACAGT	ACTGTACTTC	TGATCGACAC
UDI 332	TCTGCAGTAA	TTACTGCAGA	CCACCAGCTA
UDI 333	CTATCCTAGC	GCTAGGATAG	CACGGTTCGT
UDI 334	AACACTCCTT	AAGGAGTGTT	AGTGAGAGCT
UDI 335	CCGAACCTAA	TTAGGTTCGG	TTGCATGCGG
UDI 336	GTCTAGTCGC	GCGACTAGAC	TATACGTGTC
UDI 337	TGGATGTACG	CGTACATCCA	TGACGCGTTA
UDI 338	CTACCAGCGT	ACGCTGGTAG	TACAGAACGT
UDI 339	AAGGATTCAG	CTGAATCCTT	CTTGTCAGGT
UDI 340	CGAGGTGTGT	ACACACCTCG	ATCCACAGCG

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Indices for entry on sample sheet			
Unique dual-index number	**i5 bases for entry on sample sheet (MiSeq, HiSeq 2000/2500, NovaSeq 6000)**	**i5 bases for entry on sample sheet (iSeq100, MiniSeq, NextSeq, HiSeqX, HiSeq 3000/4000)**	**i7 bases for entry on sample sheet (all instruments)**
UDI 341	GTAGACGCTC	GAGCGTCTAC	CCTATCCATC
UDI 342	TCGTCCGTCA	TGACGGACGA	ACCGCGAGTA
UDI 343	CCGTGATAGG	CCTATCACGG	AAGTTCTGGT
UDI 344	AGGATGACCT	AGGTCATCCT	ACAGGTATCG
UDI 345	CCTCGAGTAC	GTACTCGAGG	ATGACGGATT
UDI 346	GTCACTGAGG	CCTCAGTGAC	GTCTGAGTAG
UDI 347	TACGGTTAGA	TCTAACCGTA	TGCCAGATGT
UDI 348	CAACGAGAAT	ATTCTCGTTG	GCTAAGCATT
UDI 349	AATACACCGG	CCGGTGTATT	ACAGCATGGT
UDI 350	CCGATCCATC	GATGGATCGG	ATAGAGACCG
UDI 351	GAATCTCGCT	AGCGAGATTC	ATATCGCGTA
UDI 352	TGACCGGCAA	TTGCCGGTCA	TTAAGGAGGT
UDI 353	CATGATAGCA	TGCTATCATG	CTGTGCGACT
UDI 354	AACAGCTTCG	CGAAGCTGTT	TCCGTATGCT
UDI 355	CTAGTGCTTA	TAAGCACTAG	CCATCGATGT
UDI 356	TGTGATACGT	ACGTATCACA	GTGAGCCGTT
UDI 357	ATGAGCGTAT	ATACGCTCAT	TGCCGTTAAT
UDI 358	CTAGATATGG	CCATATCTAG	CGGATGTGGT
UDI 359	CGCTATGCTG	CAGCATAGCG	TCGCGTGTTG
UDI 360	TACTACGTGA	TCACGTAGTA	CCGCGATCAT

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Indices for entry on sample sheet			
Unique dual-index number	**i5 bases for entry on sample sheet (MiSeq, HiSeq 2000/2500, NovaSeq 6000)**	**i5 bases for entry on sample sheet (iSeq100, MiniSeq, NextSeq, HiSeqX, HiSeq 3000/4000)**	**i7 bases for entry on sample sheet (all instruments)**
UDI 361	ATGTGGAGGT	ACCTCCACAT	CGCGTTATCG
UDI 362	CCATGGCTCA	TGAGCCATGG	GTAGCCTCCT
UDI 363	CCAATCACGC	GCGTGATTGG	ACTAGACACT
UDI 364	TTAGATCCAG	CTGGATCTAA	CGATTCGTTG
UDI 365	AGGAATATCG	CGATATTCCT	GAAGAGATGT
UDI 366	CCTCCTATGT	ACATAGGAGG	AGATCCGACG
UDI 367	TAGAGACACG	CGTGTCTCTA	CCAGGACATT
UDI 368	CCAGCTCAGT	ACTGAGCTGG	ACGTGGCATT
UDI 369	ATGGCTCATA	TATGAGCCAT	AAGCAGGACG
UDI 370	CGGAGTGAAG	CTTCACTCCG	ACGAGTCGGT
UDI 371	TACCTATGGT	ACCATAGGTA	AGTGTACGCG
UDI 372	ATGAGACAGT	ACTGTCTCAT	ACCGACCATT
UDI 373	CTAAGAGTTG	CAACTCTTAG	TTGCTAACGT
UDI 374	TAACCGTATG	CATACGGTTA	CTTGATACTG
UDI 375	AGAGTCCATG	CATGGACTCT	CTGGATAAGT
UDI 376	CTAGACCGCA	TGCGGTCTAG	ATAGCTTACG
UDI 377	TATGGCTTGT	ACAAGCCATA	GTCCATGAGT
UDI 378	CGTTGTTCCT	AGGAACAACG	ACTCCAGTCG
UDI 379	CCGACATTAG	CTAATGTCGG	TCTCAGCACG
UDI 380	TGTGAAGGCA	TGCCTTCACA	ATCGTGATGT
UDI 381	AGCATCGTCT	AGACGATGCT	ACGCAATCCG
UDI 382	CCGACTAGGA	TCCTAGTCGG	GAGATCGGCT
UDI 383	AACATTACCG	CGGTAATGTT	CTACGTCTCG
UDI 384	CCTAATTCGT	ACGAATTAGG	CTCAGGCTGT

Appendix D: Multiplex PCR-Based Targeted Enrichment Using REPLI-g Amplified DNA and Library Construction for Sequencing on Illumina Platforms

PCR-based targeted enrichment can be performed using REPLI-g amplified DNA and the GeneRead® DNAseq Targeted Panels V2. Proceed directly with dilution of REPLI-g amplified DNA as described in the *GeneRead DNAseq Targeted Panels V2 Handbook* (see "Protocol: PCR Setup"). Follow protocol PCR Setup in the GeneRead DNAseq Targeted Panels V2 Handbook – starting from Step 1, with DNA dilution.

Appendix E: Design of Primer for Specific Amplification of Small Genomes

Target-specific primers can be designed using open-source online primer design tools, such as Primer3 (**https://primer3.ut.ee/**) or Primer-BLAST (**https://www.ncbi.nlm.nih.gov/tools/primer-blast/**). The Primer Blast allows specificity checking. With this option on, the program will search the primers against the selected database and determine whether a primer pair can generate an amplification product on any targets in the database based on their matches to the targets and their orientations. The program will return, if possible, only primers that do not generate a valid PCR product on unintended sequences and are therefore specific to the intended template.

The recommend to set T_m of RT primer to 38–42°C and MDA primer 34–40°C and length between 17–21 bp. Amplicon length 300–4000 bp. We recommend to choose alternating primer on both strands every 2000–3000 bp to capture the total length of the genome intended to be amplified as shown in Figure 11.

Figure 11. Schema of specific primer design.

Primers for MDA reaction should stabilized on 3 primer end with Phosphothioate modifications on the last 3´-bases as shown in the example 5´-NNNNNNNNNN*N*N-3´

Primers have to be diluted in H_2O sc and the concentration in the primer mix should be 20–30 µM each for the RT reaction and 10 µM each for the MDA reaction.

Appendix F: Purification of Amplified cDNA After Whole Transcriptome Amplification

If purification of the amplified cDNA is required for additional downstream applications, other than described in this handbook, the following purification protocol may be used.

Procedure

1. Dilute amplified cDNA from the WTA or enrichment MDA reaction 1:2 with H_2O sc.
2. Add 50 µl resuspended QIAseq Beads slurry to 50 µl diluted WTA sample and mix well by pipetting.
3. Incubate the mixture for 5 min at room temperature.
4. Pellet the beads on a magnetic stand for 2–5 min and carefully discard the supernatant.
5. Wash the beads by adding 200 µl fresh 80% ethanol to each pellet. Pellet the beads on the magnetic stand for 2–5 min, and then carefully discard the supernatant.
6. Repeat the wash step (step 5) once, for a total of 2 ethanol washes.
7. Incubate on the magnetic stand for 5–10 min or until the beads are dry. Avoid overdrying, which may result in lower DNA recovery. Remove from the magnetic stand.
8. Elute by resuspending in 20 µl 10 mM Tris·Cl, pH 8.0. Pellet beads on the magnetic stand. Carefully transfer 17 µl supernatant to a new PCR plate.
9. Store purified amplified cDNA at –30 to –15°C until further processing.

Appendix G: QIAseq FastSelect Blocking of rRNA and/or Globin mRNA

This protocol allows for the removal of FastSelect targeted RNAs from NGS library and subsequent sequencing results when starting with purified RNA. The FastSelect method can remove ribosomal RNA and/or globin mRNA as wells as custom RNAs. Use the appropriate QIAseq FastSelect Kit or build a Custom FastSelect Kit for your specific application.

1. The protocol starts with the 13 µl reaction following the gDNA elimination step (step 5) in "Protocol: Amplification of Purified RNA"
2. Add 1 µl rRNA HMR and/or FastSelect –Globin to 4 µl RT Polymerase Buffer. You can prepare a master mix if more samples will be processed. Mix and vortex. Add 5 µl of the mix to the RNA sample from step 5 in "Protocol: Amplification of Purified RNA" and incubate as described in the following table to allow blocking of ribosomal RNA.

Table 22. Conditions for annealing of blocking primers

Input RNA	Step	Incubation
Single cell/purified RNA	1	No fragmentation
	2	2 min at 75°C
	3	2 min at 70°C
	4	2 min at 65°C
	5	2 min at 60°C
	6	2 min at 55°C
	7	2 min at 37°C
	8	2 min at 25°C
	9	Hold at 4°C

3. Add 1 µl of each Primer mix (oligo-dT and random primer) and 1 µl Quantiscript RT Enzyme Mix to the 18 µl RNA sample from the previous step. Proceed with RT reaction and incubation step as described in the Protocols: “Amplification of Poly A+ mRNA from Single Cells” (step 7), page 21; “Amplification of Purified RNA” (step 5), page 33 ; and “Specific Enrichment of Purified RNA” (step 7) page 37.

Ordering Information

Product	Contents	Cat. no.
QIAseq Single Cell RNA Library Kit UDI (24)	For 24 reactions: Buffers and reagents for cell lysis, whole transcriptome amplification, and library preparation including DNA fragmentation, end-repair and adapter ligation. Includes QIAseq beads and a plate containing 24 UDI barcoded adapters for use with Illumina instruments.	180703
QIAseq Single Cell RNA Library Kit UDI A, B, C, or D (96)	For 96 reactions: Buffers and reagents for cell lysis, whole transcriptome amplification, and library preparation including DNA fragmentation, end-repair and adapter ligation. Includes QIAseq beads and a plate containing 96 UDI barcoded adapters (either A,B,C, or D) for use with Illumina instruments.	180705 180725 180765 180785
QIAseq Single Cell RNA Library Kit UDI (384)	For 384 reactions: Buffers and reagents for cell lysis, whole transcriptome amplification, and library preparation including DNA fragmentation, end-repair and adapter ligation. Includes QIAseq beads and 4 plates containing 96 UDI barcoded adapters (A, B, C, D) for use with Illumina instruments.	180707

Product	Contents	Cat. no.
QIAseq products for next-generation sequencing applications		
QIAseq Single Cell DNA Library Kit UDI (24)	For 24 reactions: Buffers and reagents for cell lysis, whole genome amplification, and library preparation including DNA fragmentation, end-repair and adapter ligation. Includes QIAseq Beads and a plate containing 24 UDI barcoded adapters for use with Illumina instruments.	181703
QIAseq Single Cell DNA Library Kit UDI (96) A,B,C, or D	For 96 reactions: Buffers and reagents for cell lysis, whole genome amplification and library preparation including DNA fragmentation, end-repair and adapter ligation. Includes QIAseq beads and a plate containing 96 UDI barcoded adapters for use with Illumina instruments.	181705 181725 181765 181785
QIAseq Single Cell DNA Library Kit UDI (384)	For 384 reactions: Buffers and reagents for cell lysis, whole genome amplification, and library preparation including DNA fragmentation, end-repair and adapter ligation. Includes QIAseq beads and 4 plates containing 96 UDI barcoded adapters (A, B, C, or D) for use with Illumina instruments.	181707
QIAseq Library Quant Assay Kit	1 tube of Primer Mix (500 µl), 1 bottle of Dilution Buffer (30 ml), 1 tube DNA Standard (100 µl), and 5 tubes GeneRead qPCR SYBR Green (1.35 ml) for sample library quantification prior to NGS	333314

Product	Contents	Cat. no.
QuantiTect Primer Assays — for use in real-time RT-PCR with SYBR Green detection*		
QuantiTect Primer Assays	For qPCR and qRT-PCR gene expression analysis using predesigned assays together with QuantiFast Kits	Varies†
QIAseq FastSelect Kits		
QIAseq FastSelect –rRNA HMR Kit	For rRNA depletion prior RT reaction	Varies†
QIAseq FastSelect –Globin Kit	For Globin mRNA removal	Varies†

* Search for and order assays at **www.qiagen.com/GeneGlobe**.

† Other kit sizes/formats available; see **www.qiagen.com**.

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Document Revision History

Date	Changes
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Notes

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