June 2016

QIAseq FX Single Cell RNA Library Kit

For RNA library construction from single cells for Illumina® platforms

Sample to Insight

QIAGEN

Contents

Kit Contents

QIAseq FX Single Cell RNA Library Kit	(24)	(96)
Catalog no.	**180733**	**180735**
Number of preps	**24**	**96**
Sample preparation		
Lysis Buffer (clear lid)	110 µl	4 x 110 µl
NA Denaturation Buffer (clear lid)	80 µl	4 x 80 µl
Enzymatic template preparation		
gDNA Wipeout Buffer, WTA (red lid)	55 µl	4 x 55 µl
RT/Polymerase Buffer (red lid)	110 µl	4 x 110 µl
Random Primer (red lid)	26 µl	4 x 26 µl
Oligo dT Primer (red lid)	26 µl	4 x 26 µl
Quantiscript® RT Enzyme Mix (red lid)	26 µl	4 x 26 µl
Ligase Mix (blue lid)	55 µl	4 x 55 µl
Ligase Buffer (blue lid)	200 µl	4 x 200 µl
Amplification of cDNA		
REPLI-g sc Reaction Buffer (yellow lid)	700 µl	4 x 700 µl
REPLI-g SensiPhi DNA Polymerase (yellow lid)	26 µl	4 x 26 µl
H_2O sc	3 x 1500 µl	8 x 1500 µl
Library generation		
FX Enzyme Mix	1 x 24 rxn	1 x 96 rxn
FX Buffer, 10x	1 x 24 rxn	1 x 96 rxn
FX Enhancer	1 x 24 rxn	1 x 96 rxn
DNA Ligase	1 x 24 rxn	1 x 96 rxn
5x DNA Ligase Buffer	1 x 24 rxn	2 x 96 rxn
Adapter Plate Illumina*	1 x 24 plex	1 x 96 plex
Quick Start Protocol	1	1

Storage

The QIAseq FX Single Cell RNA Library Kit is shipped on dry ice. The kit, including all reagents and buffers, should be stored immediately upon receipt at –30°C to –15°C in a constant-temperature freezer.

Intended Use

The QIAseq FX 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 QIAseq FX 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 set of research areas, including developmental biology, tumor heterogeneity and disease pathogenesis and progression. The QIAseq FX Single Cell RNA Library Kit enables the reliable investigation of the transcriptome from a single cell with minimal bias. The kit combines a unique WTA chemistry and a highly optimized library construction procedure to minimize bias and maximize transcript discovery. This procedure incorporates enzymatic cDNA fragmentation and is completely PCR-free, while also minimizing GC-bias introduced during PCR and eliminating the possibility of generating PCR duplicates, thus increasing quantification accuracy. 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 developed to enable efficient processing of RNA for accurate amplification of cDNA. Amplification of cDNA is achieved with negligible sequence bias using innovative Multiple Displacement Amplification (MDA) technology. The QIAseq FX Single Cell RNA Library Kit leverages QIAGEN's unique MDA technology and QIAseq FX library construction technology to provide a complete workflow for preparing sequencing libraries without the requirement for additional kits and instruments.

With the QIAseq FX Single Cell RNA Library Kit, reaction setup is straightforward, and handling time is greatly reduced. This ease of use allows reverse transcription, WTA, cDNA fragmentation and library preparation to be completed in less than six hours, with minimal hands-on time. 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 library construction processes enables a highly streamlined and efficient protocol that can be easily automated. It reduces MDA time to only two hours and eliminates the library amplification step commonly found with other single-cell RNA-seq solutions. Optimized enzyme and buffer compositions ensure the generation of high-diversity, NGS-ready libraries in just one working day (Figure 1, *Workflow chart*).

The QIAseq FX Single Cell RNA Library Kit is optimized for whole transcriptome amplification (WTA) from just single cells – up to 1000 cells – or from equivalently small samples. Following the efficient cell lysis, the complete removal of genomic DNA (gDNA) and the sensitive reverse transcription, the kit utilizes Multiple Displacement Amplification (MDA) to uniformly amplify cDNA across the entire transcriptome with negligible sequence bias. Preparing a sequencing library using the QIAseq FX Single Cell RNA Library Kit preserves the unique gene expression profile of each individual cell.

Alternative single-cell pre-amplification methods rely heavily on PCR, introducing GC-bias, quantification bias due to stochastic effects, and length bias towards shorter transcripts that can reduce quantification accuracy and inhibit the discovery of long RNAs. Additionally, these methods can introduce sequence errors, which may make data unsuitable for certain fields of research, for example when examining sequence variation in RNA viruses. The QIAseq FX Single Cell RNA Library Kit employs the REPLI-g SensiPhi DNA Polymerase, which – together with its proprietary buffer formulation – ensures uniform amplification of cDNA regions that contain highly variable GC content. Costly false-positive or -negative results when analyzing sequence variants are minimized due to the REPLI-g SensiPhi DNA Polymerase, which has up to 1000-fold higher fidelity compared to normal PCR polymerases. The QIAseq FX Single Cell RNA Library Kit combines the advantages of the high-fidelity transcriptome amplification – resulting in high yields of accurately-amplified cDNA – with the streamlined protocol and high ligation efficiency of QIAseq FX technology. High-quality libraries ready for NGS are then delivered without the need for any library amplification, thereby avoiding any additional amplification bias. The elimination of PCR from the entire workflow results in greater sensitivity and higher library diversity, enabling the detection and quantification of a greater number of RNAs with the same sequencing depth. Maximizing transcript detection delivers a more robust dataset and ensures that transcripts that may be differentially expressed are detectable in more individual libraries, increasing the statistical robustness of the data.

The kit's ability to selectively amplify mRNA (poly A+ RNA) from total RNA preparations makes the QIAseq FX Single Cell RNA Library Kit particularly suitable for the investigation of

effects on transcription regulation at the single cell transcriptome level. Amplification of ribosomal RNA (rRNA), which makes up more than 90% of the total cellular RNA population, is virtually eliminated – allowing the generation of meaningful mRNA-Seq data. Following the QIAseq FX Single Cell RNA Library procedure, >80% of mapped reads map to annotated protein-coding genes. While many alternative cDNA amplification methods are strongly biased against long mRNAs and long non-coding RNAs, these are effectively captured in datasets generated with this kit. By effectively capturing long protein-coding transcripts and regulatory RNAs in the same mRNA-seq dataset, important information on expression regulation can be gained. Additionally, the effective analysis of long transcripts can be important in certain research areas, such as virus sequencing.

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 FX Single Cell RNA Library Kit is specifically designed to reliably investigate effects on transcription regulation at the single-cell transcriptome level. The kit provides everything required to: 1) uniformly amplify all transcripts from single cells and from very small samples, 2) fragment the amplified material and 3) generate a library for analysis on Illumina NGS instruments. The generated RNAseq library accurately represents the transcription pattern of a single cell with very limited amplification bias. The kit generates sufficient amounts of RNAseq library – eliminating the need to include an amplification step in the library preparation and thereby saving time.

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 then proceeds for 120 minutes and can be preprogrammed in a thermal cycler. The amplified cDNA can be stored long-term at –20°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 FX 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 QIAseq FX cDNA fragmentation reaction conditions. The fragmented cDNA is directly end-repaired and an 'A' is added to the 3' ends in the same tube following enzymatic shearing, making the DNA fragments ready for ligation. Following this step, platform-specific adapters are ligated to both ends of the DNA fragments. These adapters contain sequences essential for bridge amplification and sequencing on any Illumina sequencer. The WTA procedure normally results in high yields of DNA so that library preparation can be performed with a high amount of input DNA, and so that subsequent PCR-based library enrichment can be avoided. If library enrichment is required, an optional, high-fidelity amplification step using

the GeneRead DNA I Amp Kit can also be performed that provides highly accurate amplification of library DNA with low error rates and minimum bias.

The QIAseq FX Single Cell RNA Library Kit combines the benefits of highly uniform amplification across the entire transcriptome, with fast library preparation and negligible sequence bias. The need for enrichment is avoided, thereby eliminating additional amplification bias and maximizing library diversity and transcript discovery.

Unique components of the QIAseq FX 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 Multiple Displacement Amplification (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 to 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.

- Dual-barcoded, plate-format adapters are included with the QIAseq FX Single Cell DNA Library Kit (24) and with the QIAseq FX Single Cell DNA Library Kit (96). Each adapter well contains a single-use adapter consisting of a unique combination of two eight-nucleotide identification barcodes. By combining one of eight D5 barcodes and one of twelve D7 barcodes in each ready-to-use adapter, this kit supports up to 96-plexing prior to sequencing (see Appendix C for barcode IDs). For guidelines on multiplexing low numbers of samples, please refer to guidelines provided by your sequencing platform provider.

The QIAseq FX Single Cell RNA Library Kit provides a simple and reliable method to efficiently generate RNA libraries. These libraries are suitable for use on Illumina NGS instruments from either a single cell or from as little as picograms of RNA. 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).

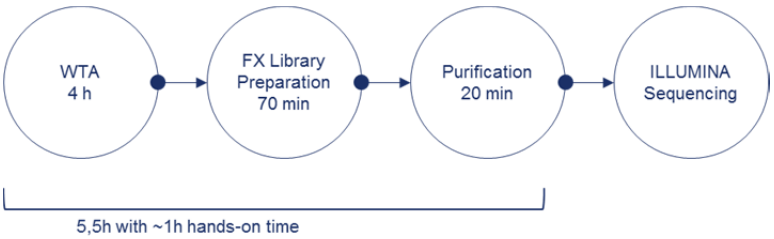

Figure 1. A time-saving, streamlined protocol delivers RNA libraries – ready for use on Illumina NGS platforms. The QIAseq FX Single Cell RNA Library Kit provides a complete WTA workflow from cell lysis, gDNA removal and cDNA synthesis to highly uniform amplification across the entire transcriptome in a one-tube protocol – with negligible sequence bias. These procedures are followed by fast, one-tube cDNA enzymatic fragmentation and library construction that does not require cleanup steps between different enzymatic reactions.

Description of protocols

Different protocols in this handbook provide detailed instructions for using the QIAseq FX Single Cell RNA Library Kit for: 1) cDNA amplification from single cells or purified RNA and 2) construction of a 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 with FACS, cells from tissue culture, cells isolated with laser-capture microdissection, and cells or tissue from biopsies. The protocol avoids the amplification of rRNA 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 rRNA is also amplified when using a combination of Random and Oligo-dT Primers, and will represent a high percentage of reads after sequencing.

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 PCR-free library preparation procedure that includes fragmentation, end-repair, A-addition, adapter ligation, cleanup and removal of adapters and adapter dimers is described in the protocol "Enzymatic fragmentation and Library Preparation using QIAseq FX SC Amplified cDNA." The prepared library can be quantified and is optimized for use on any Illumina sequencing platform.

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

- mRNA with poly A+ tails
- Total RNA

The kit is not suitable for use with small nucleic acids, such as:

- 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 FX Single Cell RNA Library Kit are approximately 20 µg cDNA per 60 µl reaction, depending on the quality of the cells or input RNA used. 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 later analysis or follow-up experiments.

Compatible sequencing platforms

- Illumina HiSeq®
- Illumina MiSeq®
- Illumina NextSeq®

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 Axygene 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)
- Agencourt AMPure XP Beads (cat. no. A63880, A63881) for bead-based size selection OR the GeneRead Size Selection Kit (QIAGEN, cat. no. 180514) for column-based size selection
- Microcentrifuge tubes
- 100% ethanol (ACS grade)
- 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 (product number 333314)
- GeneRead DNA I Amp Kit (100) (optional)

- Quant-iT™ PicoGreen® dsDNA Assay Kit (optional)

Important Notes

DNA preparation and quality control

High-quality RNA is essential for obtaining good amplification and sequencing results. The most important prerequisite for any RNA sequence analysis experiment is consistent, high-quality RNA from every experimental sample. Therefore, cell handling and RNA isolation procedures are critical to the success of the experiment. Low integrity RNA decreases the efficiency of amplification and the quality of the generated libraries. Residual traces of proteins, salts or other contaminants will degrade the RNA or decreases the efficiency of – if not completely block – the enzymatic activities necessary for amplification and library preparation. If determination of amplified cDNA is required, we recommend using Qubit®, PicoGreen® or another fluorometric method to accurately quantify dsDNA. For accurate DNA quantification, we recommend also the QIAxpert® (cat. no. 9002340).

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- and RNase-free.
- Before starting, wipe down work area and pipettes with an RNase and DNA cleaning product such as RNase Away® (Molecular BioProducts, Inc. San Diego, CA) 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 –20°C, and plan your workflow accordingly.
- Enzyme-based DNA fragmentation is sensitive to many factors, such as 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 (product number 333314), which contains laboratory-verified forward and reverse primers together with a DNA standard, is highly recommended for accurate quantification of the prepared library.

Protocol: Amplification of Poly A+ mRNA from Single Cells

This protocol is for the amplification of polyadenylated mRNA using single cell material.

For amplification of total RNA from single cell material, 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, tissue culture cells, cells picked under the microscope or microdissected cells from frozen tissue).
- The protocol cannot be used for bacterial cells. Plant cells or other cells and organisms that contain cell walls are also not suitable. 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 FX Single Cell RNA Library Kit. Avoid using more than 1000 cells in the reaction, as 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 FX 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. However, kit chemistry is designed to make these events rare and thus, the detection and quantification of nucleic acid sequences is not affected (e.g., sequence polymorphisms, differential expression analysis).

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	Additional comments
Set the heating lid to 50°C for all steps			
Cell lysis	5 min	24°C	Add Lysis Buffer (step 2)
	3 min	95°C	
	∞	4°C	Hold
gDNA removal	10 min	42°C	Add gDNA Wipeout Buffer prior to incubation (step 4)
	∞	4°C	Hold
Reverse transcription	60 min	42°C	Add Quantiscript RT mix prior to incubation (step 6)
	3 min	95°C	Stops reverse transcription
	∞	4°C	Hold
Ligation	30 min	24°C	Add ligation mix prior to incubation (step 8)
	5 min	95°C	Stops ligation
	∞	4°C	Hold
Whole transcriptome amplification	2 h	30°C	Add REPLI-g SensiPhi amplification mix prior to incubation (step 10)
	5 min	65°C	Inactivates all enzymes
	∞	4°C	Cools amplified cDNA

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 Reaction Buffer	29 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 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 –15°C to –30°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 no template controls (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. 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 overestimate the amplified cDNA from step 12 and should not be used.

Protocol: Amplification of Total RNA from Single Cells

This protocol is for amplification of total RNA from single cell material. Note that rRNA is also amplified using this protocol and will represent a high percentage of all cDNAs after amplification, and thus a high percentage of reads 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. 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, tissue culture cells, cells picked under the microscope or microdissected cells from frozen tissue).
- The protocol cannot be used for bacterial cells. Plant cells or other cells that contain cell walls are also not suitable. 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 and subsequent library preparation using the QIAseq FX Single Cell RNA Library Kit. Avoid using more than 1000 samples in the reaction, as 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 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 no template controls (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 FX 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.

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	Additional comments
Set the heating lid to 50°C for all steps			
Cell lysis	5 min	24°C	Add Lysis Buffer (step 2)
	3 min	95°C	
	∞	4°C	Hold
gDNA removal	10 min	42°C	Add gDNA Wipeout Buffer prior to incubation (step 4)
	∞	4°C	Hold
Reverse transcription	60 min	42°C	Add Quantiscript RT mix prior to incubation (step 6)
	3 min	95°C	Stops reverse transcription
	∞	4°C	Hold
Ligation	30 min	24°C	Add ligation mix prior to incubation (step 8)
	5 min	95°C	Stops ligation
	∞	4°C	Hold
Whole transcriptome amplification	2 h	30°C	Add REPLI-g SensiPhi amplification mix prior to incubation (step 10)
	5 min	65°C	Inactivates all enzymes
	∞	4°C	Hold

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.

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 Reaction Buffer	29 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 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 –15 °C to –30°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 no template controls (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. 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 GeneRead Pure mRNA Kit) or rRNA-depleted RNA (from using the GeneRead rRNA Depletion Kit). It is not suited for degraded RNA, such as that derived from FFPE tissues.
- 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.
- 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 no template controls (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 FX 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 in the protocol that corresponds to the starting material.

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.
4. Proceed with step 4 of the protocol "Amplification of Poly A+ mRNA from Single Cells" or "Amplification of Total RNA from Single Cells."

Protocol: Enzymatic Fragmentation and Library Preparation Using QIAseq FX Single Cell 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 FX Single Cell DNA Library Kit.
- The following QIAGEN products are required also for this protocol: For reaction cleanup and removal of adapter dimers following library construction, Agencourt AMPure XP Beads (cat. no. A63880, A63881) or the GeneRead Size Selection Kit (cat. no. 180514) is required and should be ordered separately.
- 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 5).

  Refer to Table 5 to determine the time and protocol required to fragment input DNA to the desired size.
- Prepare fresh 80% ethanol.
- Prepare Buffer 10 mM Tris-HCl, 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 quick vortexing to avoid any localized concentrations. Briefly spin down vortexed reagents before use.

   Program a thermocycler according to Table 9 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 9. Amplified cDNA fragmentation reaction conditions

Step	Temperature	Incubation time (Fragment size 300 bp)	Incubation time (Fragment size 500 bp)
1	4°C	1 min	1 min
2	32°C	15 min*	10 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. Using 200–1000 ng input DNA, 15 min fragmentation time produces a fragment distribution of around 300 bp if the FX enhancer is used, and 500 bp if this component is omitted. Fragment size can be adjusted by varying the duration of step 2. Use a thermocycler with a heated lid.

3. Dilute amplified cDNA 1:3 in H2O sc. This should give 500 – 1000 ng total amplified DNA in 10 µl H2O sc (50–100 ng/µl). If you have quantified the cDNA, do not exceed 5 µl undiluted cDNA input in the FX reaction. Pipette 10 µl of the diluted DNA in PCR tubes or stripes and place them on ice or a cooling block.

4. Prepare the FX Reaction Mix on ice according to Table 10 if the desired fragment size of library is 300 bp, or according to Table 11 for library fragment size of 500 bp and mix by pipetting. 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.

Table 10. FX Reaction Setup for insert fragment size of 300 bp

Component	Volume/reaction*
FX Buffer, 10x	5 µl
H2O sc	20 µl
FX Enhancer	5 µl
FX Enzyme Mix	10 µl
Total reaction volume	**40 µl**

* Mix by pipetting, and keep on ice.

Table 11. FX Reaction Setup for insert fragment size of 500 bp

Component	Volume/reaction*
FX Buffer, 10x	5 µl
H2O sc	25 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 diluted amplified cDNA sample on ice and gently vortex to mix.
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 Agencourt AMPure XP 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 one 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 12. Mix well or by gently vortexing at low rpm.

Table 12. Ligation master mix (per sample)

Component	Volume/reaction*
DNA Ligase Buffer, 5x	20 µl
H2Osc	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 0.8x (80 µl) Agencourt AMPureXP beads.
14. Add 80 µl resuspended Agencourt AMPure XP beads slurry to each ligated sample, and mix well by pipetting or gently vortexing.
15. Incubate the mixture for 5 min at room temperature.
16. Pellet the beads on a magnetic stand for 2 min 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, 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 over-drying, which may result in lower DNA recovery. Remove from the magnetic stand.

20. Elute by resuspending in 52.5 µl 10 mM Tris-HCl, pH 8.0. Pellet beads on the magnetic stand. Carefully transfer 50 µl supernatant to a new PCR plate.
21. Perform a second purification. Add 50 µl of resuspended 1x Agencourt AMPure XP beads to each sample and mix.
22. Follow steps 15–19.
23. Elute by resuspending in 26 µl 10 mM Tris-HCl, pH 8.0. Pellet the beads on the magnetic stand. Carefully transfer 23.5 µl of supernatant into a new PCR plate. Store purified libraries at –20°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 2) of library fragments and for the absence of adapters or adapter dimers.

    Note: The median size of the DNA fragment should be shifted by the size of the adapters that were ligated to the library fragments (e.g., for the GeneRead Adapter I Set 1-plex or the GeneRead Adapter I Set 12-plex, add 120 bp).

    Note: The median fragment size can be used for subsequent qPCR-based quantification methods. This median size may be shifted between amplified libraries and PCR free libraries by approx. 30 bp.

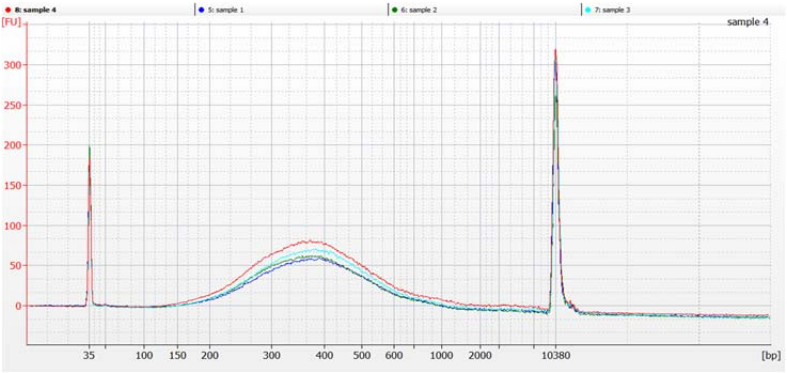

Figure 2. Capillary electrophoresis device trace of generated libraries. Capillary electrophoresis device trace data showing the correct size distribution of 4 replicate completed libraries and the absence of adapters or adapter dimers.

25. Quantify the library using the QIAseq Library Quant Assay Kit (product number 333314, 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 3).

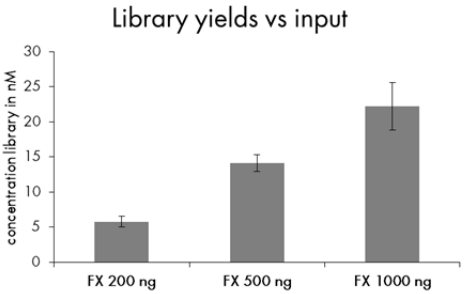

Figure 3: Library yields vs input of WTA DNA. Plotted data are means of triplicate reactions with SD.

26. The purified library can be safely stored at –20°C until needed.

Troubleshooting Guide

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

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

a)	DNA is generated during the QIAseq FX Single Cell 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 quality of actual samples or specific downstream genetic assays.

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

a)	DNA is generated during the QIAseq FX Single Cell 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 FX 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 FX 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 FX Single Cell RNA Library Kit. Only RNA transcripts longer than 500 nt can be amplified.

		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 FX 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)	Full-length transcripts analyzed	Due to random priming, amplification of full-length cDNA is not possible. We recommend analyzing smaller sequences from your target cDNA.
f)	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.
g)	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		

		Comments and suggestions
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. Agencourt AMPure Beads or the GeneRead Size Selection Kit (cat. no. 180514) efficiently removes adapter dimers as well as free adapter molecules.
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.
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 be 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 FX Single Cell 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. Real-time PCR assays that recognize exons as well as exon-intron or intron regions are recommended. For example, QIAGEN's QuantiFast® Probe Assays – which detect exon region of the genes – in combination with QuantiNova™ or QuantiFast mixes are recommended for such quality control assays.

For further information, please refer to the respective kit handbooks, which are available at **http://www.qiagen.com**, or contact QIAGEN Technical Services.

Appendix B: PicoGreen Quantification of QIAseq FX Single Cell Amplified cDNA

This protocol is designed for quantification of double stranded QIAseq FX Single Cell amplified DNA using Quant-iT™ PicoGreen® dsDNA reagent.

Alternatively, Qubit quantification might be also performed according to manufacturer's protocol. We recommend diluting the QIAseq FX Single Cell 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 TrisCl; 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

B1. 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.

B2. Prepare a 16 µg/ml stock solution of control genomic DNA in TE buffer.

B3. 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.

B4. Transfer 50 µl of each DNA standard in duplicate into a 96-well plate labeled A (see figure below).

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

96-well plate

	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

Gray squares: genomic DNA standards (µg/µl).

B5. Place 2 µl of each QIAseq FX Single Cell 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 FX Single Cell amplified cDNA at –20°C.

B6. Place 5 µl diluted QIAseq FX Single Cell 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 –20°C and used for future downstream sample analysis.

B7. For Blanc measurements pipette 50 µl TE Buffer in tow empty wells of the plate A.

B8. Add 50 µl PicoGreen working solution (from step B1) to each sample (amplified cDNA and control DNA standards) in 96-well plate A. Gently shake the plate on the bench top to mix the samples and reagent.

B9. Centrifuge the 96-well plate briefly to collect residual liquid from the walls of the wells and incubate for 5 min at room temperature in the dark.

B10. Measure the sample fluorescence using a fluorescence microplate reader and standard fluorescence filters (excitation approximately 480 nm; emission approximately 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

B11. 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.

B12. Use the standard curve to determine the concentration (µg/ml) of the diluted QIAseq FX Single Cell 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 FX Single Cell amplified cDNA concentrations.

B13. Multiply the value determined in step B11 by 1000 to show the concentration of undiluted sample cDNA (since the sample DNA measured by PicoGreen fluorescence had been diluted 1:1000).

B14. To determine the total amount of cDNA in your sample, multiply the concentration (µg/ml) of
undiluted sample DNA (determined in step B12) by the reaction volume in milliliters (i.e., for a 60 µl reaction, multiply by 0.06).

Appendix C: Adapter Barcodes for the QIAseq FX Libraries

The barcode sequences used in the QIAseq 96-plex adapter and 24-plex adapter plates are listed in Table 13. Indices 501–508 and 701–712 correspond to the respective Illumina adapter barcodes. The layout of the 96-plex and 24-plex single use adapter plate is displayed in Figure 4A and Figure 4B.

Table 13. Adapter barcodes used in the QIAseq 96-plex Adapter Plate

Codes for entry on sample sheet			
D50X barcode name	**i5 bases for entry on the sample sheet**	**D70X barcode name**	**I7 bases for entry on the sample sheet**
D501	TATAGCCT	D701	ATTACTCG
D502	ATAGAGGC	D702	TCCGGAGA
D503	CCTATCCT	D703	CGCTCATT
D504	GGCTCTGA	D704	GAGATTCC
D505	AGGCGAAG	D705	ATTCAGAA
D506	TAATCTTA	D706	GAATTCGT
D507	CAGGACGT	D707	CTGAAGCT
D508	GTACTGAC	D708	TAATGCGC
		D709	CGGCTATG
		D710	TCCGCGAA
		D711	TCTCGCGC
		D712	AGCGATAG

	1	2	3	4	5	6	7	8	9	10	11	12
A	501/701	501/702	501/703	501/704	501/705	501/706	501/707	501/708	501/709	501/710	501/711	501/712
B	502/701	502/702	502/703	502/704	502/705	502/706	502/707	502/708	502/709	502/710	502/711	502/712
C	503/701	503/702	503/703	503/704	503/705	503/706	503/707	503/708	503/709	503/710	503/711	503/712
D	504/701	504/702	504/703	504/704	504/705	504/706	504/707	504/708	504/709	504/710	504/711	504/712
E	505/701	505/702	505/703	505/704	505/705	505/706	505/707	505/708	505/709	505/710	505/711	505/712
F	506/701	506/702	506/703	506/704	506/705	506/706	506/707	506/708	506/709	506/710	506/711	506/712
G	507/701	507/702	507/703	507/704	507/705	507/706	507/707	507/708	507/709	507/710	507/711	507/712
H	508/701	508/702	508/703	508/704	508/705	508/706	508/707	508/708	508/709	508/710	508/711	508/712

Figure 4A. QIAseq 96-plex Adapter Plate layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A	501/701	501/702	501/703									
B	502/701	502/702	502/703									
C	503/701	503/702	503/703									
D	504/701	504/702	504/703									
E	505/701	505/702	505/703									
F	506/701	506/702	506/703									
G	507/701	507/702	507/703									
H	508/701	508/702	508/703									

Figure 4B. QIAseq 24-plex Adapter Plate layout.

Appendix D: Amplification of Library DNA

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 reagents provided in the GeneRead DNA I Amp Kit (cat. no. 180455). The proprietary HiFi PCR Master Mix can evenly amplify DNA regions with vastly different GC content, minimizing sequencing bias caused by PCR.

Things to do before starting

- Thaw all reagents on ice. Once reagents are thawed, mix them thoroughly by vortexing to avoid any localized concentrations.
- For PCR reaction cleanup and removal of primer-dimers following library construction, the GeneRead Size Selection Kit (cat. no. 180514) is required and should be ordered separately.

Procedure

1. Prepare a reaction mix according to Table 14.

Table 14. Reaction mix for library enrichment

Component	Volume/reaction (µl)
HiFi PCR Master Mix, 2x	25
Primer Mix (10 µM each)	1.5
Library DNA (from step 26, page 35)	Variable
RNase-free water	Variable
Total reaction volume	**50**

2. Program a thermocycler according to Table 15.

Table 15. Thermal cycling parameters

Time	Temperature	Number of cycles
2 min	98°C	1
20 s	98°C	
30 s	60°C	5–10*
30 s	72°C	
1 min	72°C	1
∞	4°C	Hold

* We recommend 5–10 amplification cycles, depending on the DNA input amount and quality. Generally, 4 amplification cycles are sufficient for >200 ng input DNA.

3. Perform size selection as described in the GeneRead Size Selection Handbook (Protocol: GeneRead Size selection of sheared DNA in common elution buffers).
4. Assess the quality of the library using a capillary electrophoresis device or other comparable method. Check for the correct size distribution (see Figure 5, blue line) of library fragments and for the absence of adapters or adapter dimers. Note: The median fragment size can be used for subsequent qPCR-based quantification methods.

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

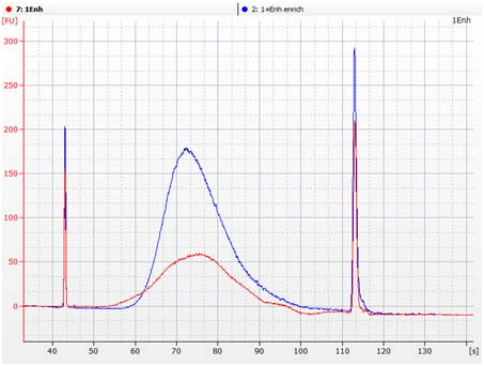

Figure 5: Capillary electrophoresis device trace of generated libraries. Capillary electrophoresis device trace data showing the correct size distribution of completed libraries and the absence of adapters or adapter dimers. Red line: library without/before enrichment. Blue line: same library after PCR enrichment.

5. Quantify the library using the QIAseq Library Quant Assay Kit (product number 333314, not provided) or other comparable method.
6. The purified library can be safely stored at –20°C until further applications.

Appendix E: 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 F: Manual 96-Well Protocol QIAseq FX Single Cell RNA Library Generation

This procedure has been adapted from the single tube protocol. It is suitable for generation of PCR-free libraries from single cells or from low amounts of purified RNA in 96-well format using the QIAseq FX Single Cell RNA Library Kit. For further recommendations, see protocol "Amplification of Poly A+ mRNA from Single Cells." If total RNA amplification is required please add random primer in the RT-reaction according to the protocol described in "Amplification of Poly A+ mRNA from Single Cells."

Equipment and reagents to be supplied by user

- Microcentrifuge tubes and 15 ml Falcon tubes (or similar)
- Microcentrifuge
- Heating block or cycler
- Vortexer
- Cooling block or ice
- Nuclease-free water
- 96-well plates
- Pipettes, Repeater Pipettes with corresponding pipette tips; multichannel pipettes are recommended for efficient sample processing.
- Recommended: reservoirs for use with multichannel pipettes
- Magnetic plate rack (e.g., GENOVISION GenoMagnet-96, G510.096)

Protocol: Amplification of Poly A+ mRNA from Single Cells

Procedure

1. Place 7 µl cell material (supplied with PBS) into each well of a 96-well PCR plate. 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 with a repeater pipette, then seal the plate using a tape sheet. Centrifuge the plate briefly at 1000 rpm to ensure that the cell material and lysis buffer are collected at the bottom of the wells.

3. Mix by a short vortexing step, then briefly centrifuge again at 1000 rpm.

   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.

4. Incubate at 24°C for 5 min, followed by 95°C for 3 min. Cool to 4°C.

5. Add 2 µl gDNA Wipeout Buffer to each well, seal and centrifuge. Then mix by vortexing and centrifuge briefly.

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

   Prepare the Quantiscript RT mix (Table 16). Add 6 µl Quantiscript RT Mix to the lysed cell sample, seal the plate using a tape sheet and centrifuge the plate briefly at 1000 rpm to ensure that RT reaction material is collected at the bottom of the wells.

   Note: The Quantiscript RT mix must be prepared fresh.

   Note: If total RNA amplification is required please add random primer in the RT-reaction according to protocol "Amplification of Poly A+ mRNA from Single Cells" step 6.

Table 16. Preparation of Quantiscript RT mix

Component	Volume/ 96 reactions*
RT/Polymerase Buffer	384 µl
Oligo dT Primer	96 µl

Component	Volume/ 96 reactions*
Quantiscript RT Enzyme Mix	96 µl
Total volume†	**576 µl**

* Add 10%.
† Mix by vortexing and centrifuge briefly.

7. Mix by vortexing and centrifuge briefly.
8. Incubate at 42°C for 60 min. Stop the reaction by incubating at 95°C for 3 min, then cool on ice.
9. Prepare the ligation mix (see Table 17). Add 10 µl ligation mix to the RT reaction from step 9, seal the plate using a tape sheet and centrifuge the plate briefly at 1000 rpm to ensure that ligation reaction material is collected at the bottom of the wells.

   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 17. Preparation of the ligation mix

Component	Volume/96 reactions*
Ligase Buffer	768 µl
Ligase Mix	192 µl
Total volume†	**960 µl**

* Add 10%.
† Mix by vortexing and centrifuge briefly.

10. Mix by vortexing and centrifuge briefly.
11. Incubate at 24°C for 30 min. Stop the reaction by incubating at 95°C for 5 min, then cool on ice.
12. Prepare the REPLI-g SensiPhi amplification mix (see Table 18). Add 30 µl REPLI-g SensiPhi amplification mix to the ligation reaction from step 12, seal the plate using a tape sheet and centrifuge the plate briefly at 1000 rpm to ensure that amplification reaction material is collected at the bottom of the wells.

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

Table 18. Preparation of REPLI-g SensiPhi amplification mix*

Component	Volume/96 reactions*
REPLI-g sc Reaction Buffer	2784 µl
REPLI-g SensiPhi DNA Polymerase	96 µl
Total volume†	2880 µl

* Add 10%.
† Mix by vortexing and centrifuge briefly.

13. Mix by vortexing and centrifuge briefly.
14. Incubate at 30°C for 2 h.
15. Stop the reaction by incubating at 65°C for 5 min, then cool on ice.
16. If not being used directly, store the amplified cDNA at –15°C to –30°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 no template controls (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.

17. Amplified cDNA can be directly used for the library construction or target-directed amplification and library construction. 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 Poly A+ mRNA from Purified RNA

Procedure

1. Place 8 µl purified RNA (>50 pg) into each well of a 96-well plate. 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 with a repeater pipette, then seal the plate using a tape sheet. Centrifuge the plate briefly at 1000 rpm to ensure that all RNA material and lysis buffer are collected at the bottom of the wells.
3. Mix by vortexing and centrifuge briefly.
4. Incubate at 95°C for 3 min, then cool to 4°C.
5. Proceed with "gDNA Wipeout" step 4 of the 96-well protocol "Amplification of Poly A+ mRNA from Single Cells."

Protocol: Enzymatic Fragmentation and Library Preparation Using QIAseq FX SC Amplified cDNA

This protocol describes the FX reaction for single-tube fragmentation, end-repair, A-addition and size selection of QIASeq FX Single Cell amplified cDNA for the preparation of 96 libraries that are ready for quantification and use in next-generation sequencing on instruments from Illumina.

Important points before starting

- The following products are required also for this protocol: For reaction cleanup and removal of adapter dimers following library construction, Agencourt AMPure XP Beads (cat. no. A63880, A63881) or the GeneRead Size Selection Kit (cat. no. 180514) is required and should be ordered separately.
- The cDNA should be diluted in H_2O before starting.

Things to do before starting

- Program thermal cyclers. For increased speed and convenience, all incubation steps of the protocol can be preprogrammed and saved on a thermal cycler in advance (Table 19).

  Refer to Table 19 to determine the time and protocol required to fragment input cDNA to the desired size.

- Prepare fresh 80% ethanol.
- Prepare Buffer 10 mM Tris-HCl, pH 8.0

FX Single-Tube fragmentation, end repair and A-addition

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

   Program a thermocycler according to Table 19 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 19. Amplified cDNA fragmentation reaction conditions

Step	Temperature	Incubation time (Fragment size 300 bp)	Incubation time (Fragment size 500 bp)
1	4°C	1 min	1 min
2	32°C	15 min*	10 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. Using 200–1000 ng input cDNA, 15 min fragmentation time produces a fragment distribution of around 300. Use a thermocycler with a heated lid.

3. Dilute amplified cDNA 1:3 in H2O sc. This should give 500–1000 ng total amplified DNA in 10 µl H2O sc (50–100 ng/µl). If you have quantified the cDNA obtained from the WTA, do not exceed 5 µl undiluted cDNA input in the FX reaction. Pipette 10 µl of the diluted cDNA into each well of the 96-well plate and place them on ice or a cooling block.
4. Prepare the FX Reaction Mix on ice according to Table 20 if the desired fragment size of library is 300 bp – or according to Table 21 for library fragment size of 500 bp – and mix by pipetting. 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 upscale the FX Reaction Mix according to the number of samples processed.

Table 20. FX reaction setup for inserting fragment sizes of 300 bp

Component	Volume/96 reactions*
FX Buffer, 10x	480 µl
H2O sc	1920 µl
FX Enhancer	480 µl
FX Enzyme Mix	960 µl
Total reaction volume†	**3840 µl**

*Add 10%.

† Mix by pipetting and keep on ice.

Table 21. FX reaction setup for inserting fragment sizes of 500 bp

Component	Volume/reaction*
FX Buffer, 10x	5 µl
H2O sc	25 µl
FX Enzyme Mix	10 µl
Total reaction volume†	**40 µl**

*Add 10%.

† Mix by pipetting and keep on ice.

5. Add 40 µl FX Reaction Mix to each diluted amplified cDNA sample on cooling block using a repeater pipette by pipetting on the upper inner walls of the wells.
6. Seal the plate with a tape sheet and centrifuge briefly at 1000 rpm.
7. Mix by short vortexing.
8. Briefly spin down the PCR plate again, immediately transfer to the pre-chilled thermocycler (4°C) and resume the program. Once the fragmentation program is complete, transfer plate to ice.
9. Immediately proceed with adapter ligation as described in the next protocol.

Adapter ligation

10. Equilibrate Agencourt AMPure XP beads to room temperature for 20–30 min before use.

11. 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 using a multichannel pipette. Ensure the right orientation of both adapter and sample plates, and track the barcodes used for each sample.

    IMPORTANT: Only one single adapter should be used per ligation reaction. If adapters from another supplier are used, follow the manufacturer's instructions.

12. Prepare the Ligation Master Mix on ice according to Table 22. Mix well by vortexing at low rpm.

Table 22. Ligation master mix

Component	Volume/96 reactions*
DNA Ligase Buffer, 5x	1920 µl
H2Osc	1440 µl
DNA Ligase	960 µl
Total reaction volume†	**4320 µl**

* Add 10%.
† Mix by vortexing and centrifuge briefly.

13. Add 45 µl of the ligation master mix to each sample by pipetting on the upper inner side of the wells to avoid any cross contamination.
14. Seal the plate with a tape sheet and centrifuge briefly at 1000 rpm.
15. Mix by short vortexing.
16. Centrifuge briefly at 1000 rpm and incubate at 20°C for 15 min.

    IMPORTANT: Do not use a thermocycler with a heated lid.

17. Proceed immediately to adapter ligation cleanup (steps 14–23) using 0.8x (80 µl) Agencourt AMPureXP beads.
18. Add 80 µl resuspended Agencourt AMPure XP beads slurry to each ligated sample using a repeater pipette and by pipetting beads on the upper side of the well, seal the plate and mix well by vortexing.

19. Incubate the mixture for 5 min at room temperature.
20. Pellet the beads on a magnetic stand for 2 min and carefully discard the supernatant.
21. Wash the beads by adding 200 µl fresh 80% ethanol to each pellet on the magnetic rack, then carefully discard the supernatant.
22. Repeat the wash step 17 once for a total of 2 ethanol washes.
23. Incubate on the magnetic rack for 5–10 min or until the beads are dry. Avoid over-drying since this may result in lower DNA recovery. Remove from the magnetic stand.
24. Elute by resuspending in 52.5 µl 10 mM Tris-HCl, pH 8.0. Pellet beads on the magnetic stand. Carefully transfer 50 µl supernatant to a new PCR plate.
25. Perform a second purification. Add 50 µl of resuspended 1x Agencourt AMPure XP beads to each sample, seal the plate and mix.
26. Follow steps 19–23.
27. Elute by resuspending in 26 µl 10 mM Tris-HCl, pH 8.0. Pellet the beads on the magnetic rack. Carefully transfer 23.5 µl of supernatant into a new PCR plate. Store purified libraries at –20°C until ready for sequencing.
28. Assess the quality of the libraries using a capillary electrophoresis device or comparable method. Check for the correct size distribution (Figure 1) of library fragments and for the absence of adapters or adapter dimers.

    Note: The median size of the DNA fragment should be shifted by the size of the adapters that were ligated to the library fragments (e.g., for the GeneRead Adapter I Set 1-plex or the GeneRead Adapter I Set 12-plex, add 120 bp).

    Note: The median fragment size can be used for subsequent qPCR-based quantification methods. This median size may be shifted between amplified libraries and PCR free libraries by approx. 30 bp.
29. Quantify the library using the QIAseq Library Quant Assay Kit (product number 333314, not provided by this kit), or other comparable method.

    Note: qPCR method is strongly recommended for the library generated with PCR-free library protocol to give accurate library quantification. Capillary electrophoresis or Qubit® methods can overestimate library quantity since they cannot distinguish DNA fragments with and without adapters ligated. However if Qubit® quantification is

required we recommend amplification of the library first (see Appendix D) prior Qubit quantification.

30. The purified library can be safely stored at –20°C until further applications or amplifications. LoBind tubes should be used to store library.

Appendix G: 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 step 13, "Protocol: Amplification of Poly A+ mRNA from Single Cells," 1:2 with H2O sc.
2. Add 50 µl resuspended Agencourt AMPure XP beads slurry to 50µl diluted WGA 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 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 over-drying which may result in lower DNA recovery. Remove from the magnetic stand.
8. Elute by resuspending in 20 µl 10 mM Tris-HCl, 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 –20°C until further processing.

Ordering Information

Product	Contents	Cat. no.
QIAseq FX Single Cell RNA Library Kit (24)	REPLI-g SensiPhi DNA Polymerase, Buffers and Reagents for 24 x 60 µl whole transcriptome amplification reactions and subsequent enzymatic fragmentation end-repair, A-addition and ligation – for use with Illumina instruments	180733
QIAseq FX Single Cell RNA Library Kit (96)	REPLI-g SensiPhi DNA Polymerase, Buffers and Reagents for 96 x 60 µl whole transcriptome amplification reactions and subsequent enzymatic fragmentation end-repair, A-addition and ligation – for use with Illumina instruments	180735
GeneRead DNA I Amp Kit (100)	For 100 reactions: Buffers and reagents for library amplification – for use with Illumina instruments	180455

Related products

QIAGEN QIAseq Kits – for next-generation sequencing applications

Product	Contents	Cat. no.
GeneRead Size Selection Kit (50)	For 50 reactions: Spin columns and buffers	180514
QIAseq Library Quant Assay Kit (product number 333314)	Laboratory-verified forward and reverse primers for 500 x 25 µl reactions (500 µl); DNA Standard (100 µl); Dilution Buffer (30 ml); (1.35 ml x 5) GeneRead qPCR SYBR® Green Mastermix	333314
QIAseq FX Single Cell	For 24 reactions: Buffers and reagents for cell	180713

DNA Library Kit (24)	lysis, whole genome amplification, and library preparation including DNA fragmentation, end-repair and adapter ligation. Includes a plate containing 24 barcoded adapters for use with Illumina instruments	
QIAseq FX Single Cell DNA Library Kit (96)	For 96 reactions: Buffers and reagents for cell lysis, whole genome amplification and library preparation including DNA fragmentation, end-repair and adapter ligation. Includes a plate containing 96 barcoded adapters for use with Illumina instruments	180715
QuantiNova Probe PCR Kit – for highly sensitive, specific and ultrafast, probe-based real-time PCR		
QuantiNova Probe PCR Kit (100)*	For 100 x 25 µl reactions: 1 ml 2x QuantiNova Probe PCR Master Mix, 500 µl QuantiNova Yellow Template Dilution Buffer, 250 µl QN ROX Reference Dye, 1.9 ml RNase-Free Water	208252
QuantiFast Probe PCR Kits – For fast, real-time PCR and two-step qRT-PCR using sequence-specific probes		
QuantiFast Probe PCR Kit (400)*	For 400 x 25 µl reactions: 3 x 1.7 ml 2x QuantiFast Probe PCR Master Mix (contains ROX dye), 2 x 2 ml RNase-Free Water	204254
QuantiFast Probe Assays – for qPCR and qRT-PCR gene expression analysis using predesigned assays together with QuantiFast Kits		
QuantiFast Probe Assays*	For qPCR and qRT-PCR gene expression analysis using predesigned assays together with QuantiFast Kits	Varies

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

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