April 2015

REPLI-g® Single Cell RNA Library Handbook

For RNA library construction from single cells for Illumina® sequencing applications

Sample to Insight

QIAGEN

Contents

Kit Contents

REPLI-g Single Cell RNA Library Kit	**(24)**
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Number of preps	**24**
Sample preparation	
Lysis Buffer (clear lid)	110 µl
NA Denaturation Buffer (clear lid)	80 µl
Enzymatic template preparation	
gDNA Wipeout Buffer, WTA (red lid)	55 µl
RT/Polymerase Buffer (red lid)	110 µl
Random Primer (red lid)	26 µl
Oligo dT Primer (red lid)	26 µl
Quantiscript® RT Enzyme Mix (red lid)	26 µl
Ligase Mix (blue lid)	55 µl
Ligase Buffer (blue lid)	200 µl
Amplification of cDNA	
REPLI-g sc Reaction Buffer (yellow lid)	700 µl
REPLI-g SensiPhi DNA Polymerase (yellow lid)	26 µl
H_2O sc	3 x 1500 µl
Library generation	
T4 DNA Ligase	2 x 48 µl
Klenow Fragment	2 x 36 µl
A-Addition Buffer, 10x	2 x 50 µl
End Repair Buffer, 10x	2 x 50 µl
Ligation Buffer, 2x	2 x 600 µl
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Storage

The REPLI-g 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. If stored under these conditions, the kits are stable until the date indicated on the QC label inside the kit lid.

Intended Use

The REPLI-g 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 REPLI-g 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 applications, including developmental biology, tumor heterogeneity and disease pathogenesis and progression. NGS-based gene expression analyses often require large amounts of cDNA or RNA. Whole transcriptome amplification (WTA) overcomes limited RNA availability by enabling the analysis of a very small number of cells. The REPLI-g Single Cell RNA Library Kit allows reliable investigation of the transcriptome from a single cell with minimal bias. The kit provides unique WTA chemistry in combination with a highly streamlined library construction procedure, optimized to avoid library enrichment and minimize bias associated with PCR amplification. 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, which is achieved with negligible sequence bias using innovative Multiple Displacement Amplification (MDA) technology. The REPLI-g Single Cell RNA Library Kit leverages QIAGEN's unique MDA technology and GeneRead™ library construction technology to prepare a sequencing library with high fidelity and minimal bias, while retaining the sample's unique transcriptional profile.

With the REPLI-g Single Cell RNA Library Kit, reaction setup is straightforward and handling time is greatly reduced, allowing reverse transcription, WTA and library preparation to be completed in a single working day. Co-optimization of WTA and library construction processes enables a highly streamlined and efficient workflow. The kit combines all the reaction steps for WTA in a one-tube protocol and all the reaction steps for library construction in a second one-tube protocol, greatly reducing hands-on time and minimizing starting material loss and cross-contamination risk. Optimized enzyme and buffer compositions ensure generation of high-quality, NGS-ready libraries in just one working day (Figure 1).

In standard PCR amplification procedures, regions of cDNA with high GC or AT content can result in little or no amplification, leading to unreliable NGS results. The REPLI-g Single Cell RNA Library Kit employs high-fidelity MDA technology to provide accurate amplification of all transcripts with negligible sequence bias. The kit contains REPLI-g SensiPhi DNA Polymerase, which, together with its proprietary buffer formulation, ensures uniform amplification of cDNA regions that contain highly variable GC content, thereby ensuring even coverage in subsequent sequencing reactions. Costly false-positive or -negative results are minimized with REPLI-g technology due to REPLI-g SensiPhi DNA Polymerase, which has up to 1000-fold higher fidelity compared to normal PCR polymerases. The REPLI-g Single Cell RNA Library Kit combines the advantages of REPLI-g Single Cell technology with the ligation efficiency of GeneRead technology, delivering high-quality libraries ready for NGS, without the need for any library enrichment – avoiding additional amplification bias.

The REPLI-g Single Cell RNA Library Kit contains novel REPLI-g SensiPhi DNA Polymerase, as well as an optimized set of buffers and reagents for whole transcriptome amplification (WTA) from just single cells, up to 1000 cells or equivalently small samples. Following efficient cell lysis, complete removal of genomic DNA (gDNA) and 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 REPLI-g Single Cell RNA Library Kit preserves the unique gene expression profile of each individual cell.

The ability to selectively amplify mRNA (polyA+ RNA) from total RNA preparations makes the REPLI-g 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 generation of meaningful mRNA-Seq data. Following the REPLI-g Single Cell RNA Library procedure, >80% of mapped reads belong to protein-coding RNA.

Single cell analysis can be challenging when transcript abundance varies greatly within a cell. For accurate results, it is essential that whole transcriptome amplification reliably

amplifies all transcripts, regardless of their levels within the cell. The sequencing library generated by the REPLI-g Single Cell RNA Library Kit comprises of high numbers of unique transcripts – even from single cells – providing a comprehensive picture of the transcriptome at the single cell level.

Principle and procedure

Regulation of transcription is driven by a variety of influences, such as stress, cellular environment or disease or 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 result interpretation on their average behavior – is of increasing scientific interest.

The REPLI-g 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 uniformly amplify all transcripts from single cells and very small samples, and generate a library for analysis on Illumina NGS instruments. The generated RNAseq library accurately represents the transcription pattern of a single cell with minimal amplification bias. The kit generates sufficient amounts of RNAseq library, eliminating the need to include an amplification step in the library preparation, thereby saving time.

Amplification principle

Following reverse transcription using Quantiscript RT Enzyme Mix, the cDNA is ligated and subjected to WTA. The REPLI-g 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 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 serve as a template, enabling 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 REPLI-g Single Cell 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 enables the generation of cDNA fragments of up to 100 kb without sequence bias. The REPLI-g Single Cell RNA Library Kit combines the benefits of highly uniform amplification across the entire transcriptome, with negligible sequence bias with fast library preparation — without the need for enrichment, thereby eliminating additional amplification bias.

Unique components of the REPLI-g 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. This ensures 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. For example, these processes 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, especially 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 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 buffer are specially optimized for a convenient, single-tube protocol and high-efficiency adapter ligation.

The following reactions take place in one tube during library preparation:

1. End-repair of the fragmented DNA: Ends of DNA strands will be prepared to ensure blunt ends.
2. A-addition: Blunt-ended DNA becomes A overhangs for sufficient subsequent adapter ligation.
3. Adapter ligation: Suitable adapters that allow further steps of NGS are ligated to the ends of the fragmented DNA with A overhangs.

The REPLI-g Single Cell RNA Library Kit provides a simple and reliable method to efficiently generate RNA libraries suitable for use on Illumina NGS instruments from just a single cell or as little as picograms of RNA in <7 hours, with only 1 hour hands-on time. The kit provides a complete workflow for reliable reverse transcription and highly uniform amplification across the entire transcriptome with negligible sequence bias, followed by fast, one-tube library construction (Figure 1). Dedicated buffers and reagents have been developed to deliver high-quality cDNA from single cells and purified RNA, with complete sequence representation and unbiased amplification.

In the first step of the procedure, the cell sample is lysed and the gDNA is removed. Reverse transcription is carried out for 60 minutes, followed by ligation of cDNAs (30 minutes). The isothermal amplification reaction then proceeds for 120 minutes, and can be preprogrammed in a thermal cycler. REPLI-g SC amplified cDNA can be stored long-term at –20°C with no negative effects. Samples consisting of longer cDNA fragments are first sheared into a random library of fragments. The median fragment sizes are dependent on the applications and sequencing read length. Following end-repair, platform-specific adapters, which contain sequences essential for binding library to a flow cell for sequencing, binding sequencing primer and allowing for PCR enrichment of adapter-ligated cDNA library, are ligated to both ends of the cDNA fragments. The WTA procedure normally results in high yields of cDNA so that library preparation can be performed with a high amount of input cDNA and subsequent library enrichment can be avoided. However, if

library enrichment is required, an optional, high-fidelity amplification step that provides highly accurate amplification of library cDNA with low error rates and minimum bias can also be performed (see Appendix A, page 46).

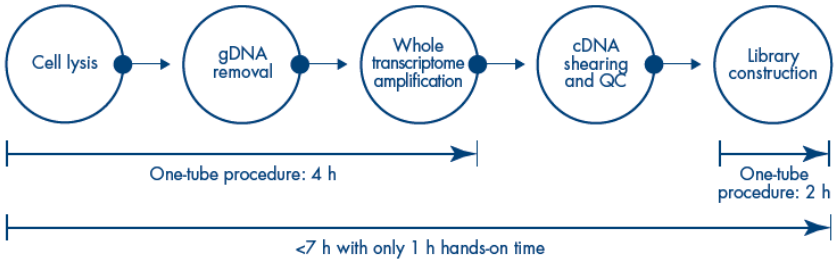


Figure 1. A time-saving, streamlined protocol delivers RNA libraries – ready for use on Illumina NGS platforms. The REPLI-g Single Cell RNA Library Kit provides a complete WTA workflow – from cell lysis, gDNA removal and cDNA synthesis to highly uniform amplification across the entire transcriptome in a one-tube protocol – with negligible sequence bias. This is followed by fast, one-tube library construction without cleanup steps between different enzymatic reactions.

Description of protocols

Depending on the protocol, the REPLI-g Single Cell RNA Library Kit is suitable for amplification of the transcriptome, 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 REPLI-g Single Cell RNA Library Kit are approximately 20 µg cDNA per 60 µl reaction, depending on the quality of the input total RNA. For best amplification results, a cell sample that has been properly collected should be used directly, since storage and collection conditions can alter transcription. 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 of sample material.

Different protocols in this handbook provide detailed instructions for using the REPLI-g Single Cell RNA Library Kit for cDNA amplification of single cells or purified RNA and construction of an NGS library.

The protocol "Amplification of Poly A+ mRNA from Single Cells" (page 19) is optimized for single cell material from all species without a cell wall – for example, cells from vertebrates, sorted cells, tissue culture cells and cells or tissue from biopsies – and avoids amplification of rRNA by omitting the Random Primer. If performing other sequence-specific detection methods, such as with qPCR or arrays, the amplified rRNA will not affect downstream application results. This is the preferred protocol if NGS is the downstream application.

The protocol "Amplification of Total RNA from Single Cells" (page 24) is used for the amplification of the complete transcriptome, including RNAs with and without poly A+ tails, all lnc RNAs and linc RNAs. Note that rRNA is also amplified when using Random and Oligo-dT Primers, and will represent a high percentage of all sequences after amplification. For further NGS analysis, please note that a high percentage of reads are derived from rRNA; therefore, sufficient reads must be obtained when performing whole transcriptome sequencing.

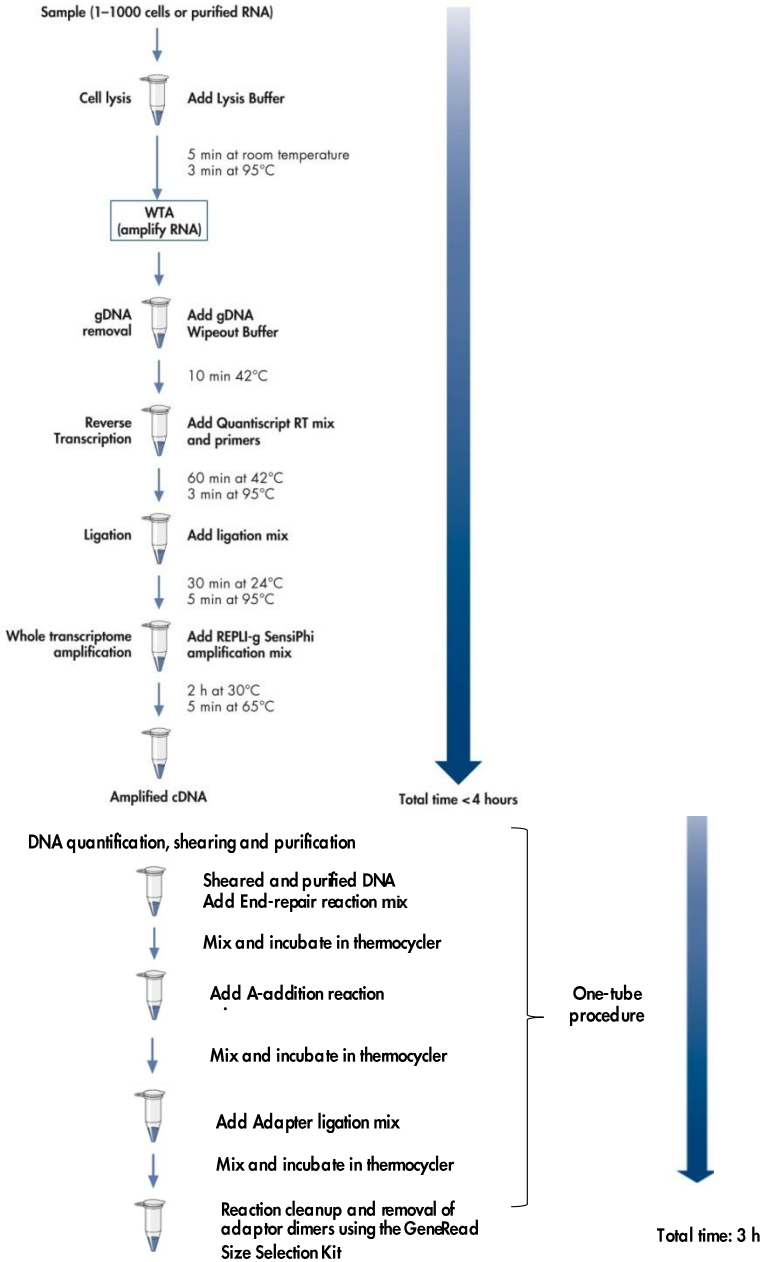
The protocol "Amplification of Purified RNA" (page 29), is optimized for whole transcriptome amplification from total or enriched RNA templates (polyA+ mRNA, rRNA-depleted mRNA) and is highly suited for NGS applications, as well as real-time PCR and microarray analysis.

The PCR-free library preparation procedure that includes end-repair, A-addition, adapter ligation, cleanup and removal of adapters and adapter dimer is described in the protocol "PCR-Free Library Preparation using REPLI-g SC Amplified DNA" (page 34). The prepared library can be quantified and is optimized for use on Illumina sequencing platforms.

Compatible sequencing platforms

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

REPLI-g Single Cell RNA library Kit procedure



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)
- Water bath or heating block
- Thermocycler
- Microcentrifuge
- Vortexer
- Pipets and pipet tips
- Ice
- Nuclease-free water or TE buffer (10 mM Tris·Cl; 1 mM EDTA, pH 8.0)
- Enzymatic or physical method (e.g., Covaris® instrument) to shear DNA
- GeneRead Adapter I Set A 12-plex (cat. no.180985)
- GeneRead Adapter I Set B 12-plex (cat no.180986)
- GeneRead Size Selection Kit (cat. no. 180514)
- Capillary electrophoresis device or comparable method to assess the quality of DNA library
- GeneRead Library Quant Kit (cat. no. 180612)

Important Notes

DNA preparation and quality control

High-quality DNA is essential for obtaining good sequencing results. The most important prerequisite for any DNA sequence analysis experiment is consistent, high-quality DNA from every experimental sample. Therefore, sample handling and DNA isolation procedures are critical to the success of the experiment. Residual traces of proteins, salts or other contaminants will degrade the DNA or decrease, if not completely block, the efficiency of the enzymatic activities necessary for optimal library preparation.

Recommendations for DNA fragmentation

DNA can be fragmented using one the following methods:

- Nebulization
- Sonication
- Using enzymes
- Using the Covaris instrument (e.g., S220™ Focused-ultrasonicator™)

To ensure complete fragmentation of the DNA that is needed for library preparation, only use the recommended parameters provided in the manufacturer's instructions. Using too much DNA in a Covaris instrument may, for example, lead to incomplete shearing of the DNA. Check the fragmented DNA for the correct size distribution using an agarose gel or capillary electrophoresis device.

For accurate DNA quantification, we recommend the QIAxpert® (cat. no. 9002340).

Use the GeneRead Size Selection Kit (cat. no. 180514) to clean up the DNA following fragmentation if the desired median fragment size is above 150 bp. Use the QIAquick® PCR

Purification Kit to clean up the DNA following fragmentation if the desired median fragment size is below 150 bp.

Recommended library quantification method

QIAGEN's GeneRead Library Quant Kit (cat. no. 180612), 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. RNA amplified using this protocol is highly suited for next-generation sequencing (RNA-Seq), as well as a wide range of applications, including, real-time PCR and microarray analysis.

For amplification of total RNA from single cell material, use the protocol "Amplification of Total RNA from Single Cells", page 24. For whole transcriptome amplification of purified total RNA or enriched mRNA, use the protocol "Amplification of Purified RNA", page 29.

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 or cells picked under the microscope).
- 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" (page 29).
- 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 REPLI-g 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., pipets, filter pipet 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 REPLI-g 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 pipet 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 if 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, 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 (see Table 1, page 21).

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	37°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 (see 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 for 12 reactions
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 (see 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 (see 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 DNA of viable cells present during WTA.

14. Amplified DNA 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: To proceed with library preparation, quantify the amplified DNA following the instructions in Appendix E (page 49). Optical density (OD) measurements overestimate REPLI-g amplified DNA 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. If working with sequence-specific methods, such as qPCR, the amplified rRNA will not affect downstream application results. If using the amplified cDNA for RNAseq, note that a high percentage of the reads will be derived from rRNA. For such applications, we recommend using the protocol "Amplification of Poly A+ mRNA from Single Cells", page 19, 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", page 29.

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 or cells picked under the microscope).
- 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" (page 30).
- 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 REPLI-g 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., pipets, filter pipet 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 REPLI-g 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 pipet 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 (see Table 5, page 26).

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	37°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 (see 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 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 generated from viable cells present during MDA.
14. Amplified DNA 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: To proceed with library preparation, quantify the amplified DNA following the instructions in Appendix E (page 49). Optical density (OD) measurements overestimate REPLI-g amplified DNA 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 (e.g., isolated using GeneRead Pure mRNA Kit) or rRNA-depleted RNA (e.g., using the GeneRead rRNA Depletion Kits). 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.
- REPLI-g amplified cDNA that is to be used in PCR or real-time PCR must be diluted 1:100. Use 2–3 µl diluted DNA for a 20 µl real-time PCR reaction volume.
- Avoid any DNA or RNA contamination of reagents by using separate laboratory equipment (e.g., pipets, filter pipet 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 REPLI-g 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 pipet 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 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 and then cool to 4°C.
4. Proceed with step 4 of the protocol "Amplification of Poly A+ mRNA from Single Cells" (page 20) or "Amplification of Total RNA from Single Cells" (page 24).

Protocol: DNA Fragmentation with Covaris - S220™ Focused-ultrasonicator™

Amplified cDNA generated with the REPLI-g Single Cell RNA Library Kit is first sheared into a random library of fragments prior to use with a wide variety of DNA library preparation kits. Specific median fragment length sizes of DNA can be prepared using a Covaris instrument, according to the manufacturer's instructions and applications.

Dilute 3–5 µg REPLI-g amplified DNA in 130 µl TE buffer. Proceed with shearing of DNA according to the manufacturer's instructions (see **http://covarisinc.com/wp-content/uploads/pn_400103.pdf**).

Example: Use the following protocol to obtain fragments with appropriate size for the described downstream application (see *Covaris quick guide DNA Shearing with S220/E220 Focused-ultrasonicator*).

Table 9. DNA fragmentation (example)

Target BP (Peak)	**300**
Peak Incident Power (W)	140
Duty Factor	10%
Cycles per Burst	200
Treatment time (s)	80
Temperature (°C)	7
Water level – S220	12
Water level – E220	6
Sample volume (µl)	130
E220 – Intensifier (pn500141)	Yes

Important points before starting

- Check the fragmented DNA for the correct size distribution using an agarose gel or capillary electrophoresis device.
- For accurate DNA quantification, we recommend the QIAxpert (cat. no. 9002340).
- Use the GeneRead Size Selection Kit (cat. no. 180514) to clean up and concentrate the sheared DNA.

Procedure

1. Add 4 volumes of Buffer SB1 to the fragmented cDNA and mix.

   Usually 520 µl Buffer SB1 is added to 130 µl sample.
2. To bind DNA, apply the mixture to the MinElute® spin column and centrifuge for 1 min.

   For maximum recovery, transfer all traces of the sample to the column.
3. Discard the flow-through and place the MinElute spin column back into the same tube.
4. To wash, add 700 µl of 80% ethanol to the MinElute spin column and centrifuge for 1 min.
5. Discard the flow-through and place the MinElute spin column back into the same tube.
6. Add 700 µl of 80% ethanol to the MinElute spin column and centrifuge for 1 min.
7. Discard the flow-through and place the MinElute spin column back into the same tube.
8. Centrifuge the MinElute spin column for an additional 1 min.

   IMPORTANT: Residual ethanol will not be completely removed unless the flow-through is discarded before this additional centrifugation step.
9. Place the MinElute spin column into a clean 1.5 ml microcentrifuge tube (provided).
10. Add 20 µl Buffer EB to the center of the membrane, let the column stand for 1 min and then centrifuge for 1 min.
11. Assess the quality of the sheared DNA using a capillary electrophoresis device or comparable method. Check for the correct size distribution of DNA fragments.

Note: The median fragment size can be used for subsequent qPCR-based quantification methods. Alternatively, sheared purified DNA may be quantified by OD measurement using a NanoDrop.

Protocol: PCR-Free Library Preparation using REPLI-g SC Amplified DNA

This protocol describes end repair, A-addition, adapter ligation and cleanup and size selection of REPLI-g SC amplified DNA, for the preparation of libraries that are ready quantification and use in next-generation sequencing on instruments from Illumina.

Important points before starting

- This protocol is for constructing sequencing libraries for Illumina NGS platforms using the REPLI-g Single Cell RNA Library Kit.
- The following QIAGEN products are also required for this protocol: GeneRead Adapters (cat. nos. 180985, 180986, 180984, 180912). For reaction cleanup and removal of adapter dimers following library construction, the GeneRead Size Selection Kit (cat. no. 180514) is required and should be ordered separately.
- Median fragment sizes depend on the applications and read length. For example, specific median fragment length sizes of DNA can be prepared using a Covaris instrument, according to the manufacturer's instructions.
- GeneRead Adapters are dissolved in duplex buffer and ready to use.
- GeneRead Adapters are fully compatible with Illumina instruments, such as MiSeq, NextSeq or HiSeq instruments. The enrichment step is not required to complete the adapter sequences.
- No heat-lid required during ligation step.
- The DNA should be in EB/Tris buffer or 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 (Table 10).
- See Table 10 for thermal cycling parameters.

Table 10. Thermal cycling parameters

Step	Time	Temperature	Additional comments
Set the heating lid to 50°C for all steps except ligation step			
End Repair	30 min	25°C	Polishing the ends of DNA fragments
	20 min	75°C	Inactivation of end-repair enzymes
	∞	4°C	Hold
A-addition	30 min	37°C	Adding A to the 3′ of the DNA fragments
	10 min	75°C	Inactivation of A-addition enzymes
Ligation	10 min	25°C	Ligation of the adapters to the DNA fragments
	∞	4°C	Hold

Procedure

End-repair of DNA fragments

1. Prepare a reaction mix for end-repair according to Table 11, dispensing the reagents into a PCR tube of the well of a PCR plate.

   Note: The reaction mix should be prepared on ice.

Table 11. Reaction mix for end-repair

Component	Volume/reaction (µl)
DNA*	1 µg
H_2O sc	Variable
End-Repair Buffer, 10x	2.5
End-Repair Enzyme Mix	2
Total reaction volume	**25**

* Purified, fragmented REPLI-g SC amplified cDNA.

2. Mix thoroughly.
3. Program a thermocycler to incubate for 30 min at 25°C, followed by 20 min at 75°C to inactivate the enzyme.

A-addition

4. Prepare a reaction mix for 3′ A-addition according to Table 12, adding the components to the PCR tube containing the end-repaired DNA from step 3.

Table 12. Reaction mix for end-repair

Component	Volume/reaction (µl)
End-repaired DNA (from step 2)	25
A-Addition Buffer, 10x	3
Klenow Fragment (3′→5′ exo-)	3
Total reaction volume	**31**

5. Mix thoroughly.
6. Program a thermocycler to incubate for 30 min at 37°C, followed by 10 min at 75°C to inactivate the enzyme.

Adapter ligation

7. Prepare a reaction mix for adapter ligation according to Table 13, adding the components to the PCR tube containing DNA that has undergone end-repair and A-addition (step 5).

   Note: When using barcode adapters, open one adapter tube at a time and change gloves between pipetting the different barcode adapters to avoid cross-contamination.

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

Table 13. Reaction setup for adapter ligation

Component	Volume/reaction (µl)
Reaction mix from step 5	31
Ligation Buffer, 2x	45
GeneRead Adapter	2.5*
T4 DNA Ligase	4
H_2O sc	Variable
Total reaction volume	**90**

*Alternatively, add the correct amount of adapter according to supplier's directions.

8. Mix thoroughly.
9. Program a thermocycler to incubate for 10 min at 25°C.

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

Reaction cleanup and removal of adapter dimers

10. Add 4 volumes of Buffer SB1 to 1 volume of the DNA library sample prepared at step 8 and mix.

    Usually 360 µl Buffer SB1 is added to 90 µl sample.

11. To bind DNA, apply the mixture to the MinElute spin column and centrifuge for 1 min.

    For maximum recovery, transfer all traces of the sample to the column.

12. Discard the flow-through and place the MinElute spin column back into the same tube.

13. To wash, add 700 µl of 80% ethanol to the MinElute spin column and centrifuge for 1 min.

14. Discard the flow-through and place the MinElute spin column back into the same tube.

15. Add 700 µl of 80% ethanol to the MinElute spin column and centrifuge for 1 min.

16. Discard the flow-through and place the MinElute spin column back into the same tube.

17. Centrifuge the MinElute spin column for an additional 1 min.

    IMPORTANT: Residual ethanol will not be completely removed unless the flow-through is discarded before this additional centrifugation step.

18. Place the MinElute spin column into a clean 1.5 ml microcentrifuge tube (provided).

19. Add 90 µl Buffer TE to the center of the membrane, let the column stand for 1 min and then centrifuge for 1 min.

    IMPORTANT: Ensure that the buffer is dispensed directly onto the centre of the membrane. Keep the spin column and the flow-through.

20. Place the same MinElute spin column into a new 2 ml collection tube (provided). Add 4 volumes of Buffer SB1 to 1 volume of the flow-through, and mix.

    Usually ~360 µl Buffer SB1 is added to ~90 µl DNA.

21. Re-apply the sample to the MinElute spin column and centrifuge for 1 min.

    For maximum recovery, transfer all traces of the sample to the column.

22. Discard the flow-through and place the MinElute spin column back into the same tube.

23. To wash, add 700 µl 80% ethanol to the MinElute spin column and centrifuge for 1 min.

24. Discard the flow-through and place the MinElute spin column back into the same tube.

25. Add 700 µl 80% ethanol to the MinElute spin column and centrifuge for 1 min.

26. Discard the flow-through and place the MinElute spin column back into the same tube.

27. Centrifuge the MinElute spin column for an additional 1 min.

IMPORTANT: Residual ethanol will not be completely removed unless the flow-through is discarded before this additional centrifugation step.

28. Place the MinElute spin column into a clean 1.5 ml microcentrifuge tube (provided).

29. For elution, add 17 µl Buffer EB to the center of the membrane, let the MinElute spin column stand for 1 min and then centrifuge for 1 min.

    IMPORTANT: Ensure that the buffer is dispensed directly onto the centre of the membrane for complete elution of bound DNA.

30. Assess the quality of the library using a capillary electrophoresis device or comparable method. Check for the correct size distribution (see 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 (step 31).

31. Quantify the library using the GeneRead Library Quant Kit (cat. no. 180612 [not provided]), or a comparable method.

    Note: For accurate library quantification of the library generated with the PCR-free library protocol, qPCR is strongly recommended. Capillary electrophoresis or Qubit® methods can overestimate library quantity as they cannot distinguish between DNA fragments with and without ligated adapters. For calculating the library concentration by qPCR, we recommend using the median size of library fragment estimated by capillary electrophoresis or the fragment size after fragmentation, plus 120 bp.

    With 1 µg DNA input, sufficient amount of library should be generated for sequencing on Illumina platforms without further PCR amplification. In the special cases where additional library amplification is needed, refer to Appendix A, page 45.

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

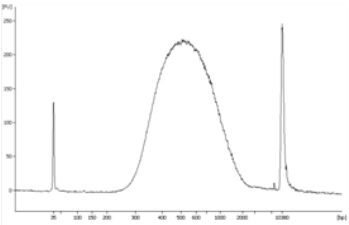


Figure 2. Capillary electrophoresis device trace data showing the correct size distribution of library fragments and the absence of adapters or adapter-dimers.

Troubleshooting Guide

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

		Comments and suggestions
Little or no amplified cDNA		
a)	Lysed cells sample not immediately used in the WTA reaction	Use the lysed cell sample immediately, without any storage prior to performing the 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	Pipetting error or missing reaction component
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	QuantiscriptRT 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 pipet tips from freshly opened boxes or bases in strongly recommended.

cDNA yields of approximately 10 µg in negative (no-template) controls, but no positive result in downstream assay (e.g., PCR)

DNA is generated during the REPLI-g reaction by contaminating RNA or DNA templates	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 positive result in downstream assay (e.g., PCR)

DNA is generated during the REPLI-g 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 pipet tips only, and keep amplification chemistry and DNA templates in separate storage locations.

Protocols using cells as a starting material
Little or no target sequence detected in real-time PCR analysis, 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 REPLI-g 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 REPLI-g 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 REPLI-g Single Cell RNA Library Kit. Only RNA transcripts longer than 500 nt can be amplified.
g)	Assays are designed to analyze large regions of cDNA	Due to random priming, amplification of full-length cDNA is not possible. We recommend analyzing smaller regions of your target cDNA.

		Comments and suggestions
Protocol using purified RNA as a starting material		
Little or no transcript detected in real-time PCR analysis, 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 non-degraded RNA or larger amounts of RNA, if possible. Only RNA transcripts longer than 500 nucleotides can be amplified.
c)	Low-abundance transcript analyzed	The REPLI-g 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.
Downstream application results not optimum		
	Little or no cDNA amplified	RT mix, ligation mix and amplification mix should always be freshly prepared before use. Storage of these mixes prior to use may affect whole transcriptome amplification.
Library preparation protocol		
Low library yields		
	Suboptimal reaction conditions due to low DNA quality	Make sure to use high-quality DNA to ensure optimal activity of the library enzymes.

		Comments and suggestions
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. 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	When 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 is due to a PCR artifact because of 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 DNA library fragments. This should be reflected on a capillary electrophoresis device by a shift in size of all library fragments of 120 bp. If using library 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 DNA.

Appendix A: Optional Amplification of Library DNA

This protocol is for optional, high-fidelity amplification of the DNA library using the GeneRead DNA I Amp Kit. The proprietary HiFi PCR Master Mix can evenly amplify DNA regions with vastly different GC content, minimizing sequencing bias caused PCR.

Things to do before starting

- Prepare library DNA (see the protocol on page 34).
- Thaw all reagents on ice.

Procedure

1. Prepare a reaction mix for library enrichment according to Table 14, dispensing the reagents into a PCR tube or the well of a PCR plate.

   Note: The reaction mix should be prepared on ice.

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 29, page 39)	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, 5 amplification cycles are sufficient for >500 ng input DNA.

3. Perform purification of the enriched library following the steps of the cleanup protocol (page 32). Add 4 volumes of Buffer SB1 to 1 volume of PCR enriched library. Usually, 200 µl Buffer SB1 is added to 50 µl sample.
4. Assess the quality of the enriched library using a capillary electrophoresis device or comparable method. Check for the correct size distribution of DNA fragments.

   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 Sets).

   Note: The median fragment size can be used for subsequent qPCR-based quantification methods (step 5).
5. Quantify the library using the GeneRead Library Quant Kit (cat. no. 180612 [not provided]), or a comparable method.

   Note: The library DNA can be stored at –20°C until ready to use for sequencing.

Appendix B: Adapter Indices for the GeneRead Adapter I Set A 12-Plex

The index sequences used in the GeneRead Adapter I Set A 12-Plex are listed in Table 16. Indices 1–12 correspond to the respective Illumina adapter indices.

Table 16. Adapter indices

Adapter name	Indices
Adapter Bc1 Illumina	ATCACG
Adapter Bc2 Illumina	CGATGT
Adapter Bc3 Illumina	TTAGGC
Adapter Bc4 Illumina	TGACCA
Adapter Bc5 Illumina	ACAGTG
Adapter Bc6 Illumina	GCCAAT
Adapter Bc7 Illumina	CAGATC
Adapter Bc8 Illumina	ACTTGA
Adapter Bc9 Illumina	GATCAG
Adapter Bc10 Illumina	TAGCTT
Adapter Bc11 Illumina	GGCTAC
Adapter Bc12 Illumina	CTTGTA

Appendix C: Adapter Indices for the GeneRead Adapter I Set B 12-Plex

The index sequences used in the GeneRead Adapter I Set B 12-Plex are listed in Table 17. Indices 13–27 correspond to the respective Illumina adapter indices.

Table 17. Adapter indices

Adapter name	Indices
Adapter Bc13 Illumina	AGTCAA
Adapter Bc14 Illumina	AGTTCC
Adapter Bc15 Illumina	ATGTCA
Adapter Bc16 Illumina	CCGTCC
Adapter Bc18 Illumina	GTCCGC
Adapter Bc19 Illumina	GTGAAA
Adapter Bc20 Illumina	GTGGCC
Adapter Bc21 Illumina	GTTTCG
Adapter Bc22 Illumina	CGTACG
Adapter Bc23 Illumina	GAGTGG
Adapter Bc25 Illumina	ACTGAT
Adapter Bc27 Illumina	ATTCCT

Appendix D: Determination of DNA Concentration and Yield

Using the REPLI-g Single Cell RNA Library Kit, a 60 µl reaction typically yields up to 20 µg of DNA, depending on the quality of RNA within the cell sample. For accurate quantification of the amplification product, it is important to use a DNA quantification method that is specific for double-stranded DNA, since the DNA sample contains unused reaction primers. Alternatively, purify the reaction according to instructions in Appendix F (page 54). Avoid using any other purification method as it will result in reduced yields. Following purification, determine the amount of DNA using a standard OD measurement.

PicoGreen® reagent displays enhanced binding to double-stranded DNA and may be used, in conjunction with a fluorimeter, to quantify the amount of amplified DNA. A protocol for the quantification of amplified DNA is provided in Appendix E.

Appendix E: PicoGreen Quantification of REPLI-g Amplified DNA

This protocol is designed for quantification of double stranded REPLI-g amplified DNA using PicoGreen reagent.

Alternatively, Qubit quantification might be also performed according to manufacturer's protocol. We recommend diluting the REPLI-g 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
- 96-well plates (suitable for use in a fluorescence microplate reader)
- Fluorescence microplate reader (e.g., TECAN® Ultra)

Procedure

Setup and reading of microplate

E1. In a 2 ml microcentrifuge tube, make a 1:150 dilution of PicoGreen stock solution in TE buffer. Each quantification reaction requires 20 µl. Cover the microcentrifuge 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 13.3 µl PicoGreen to 1986.7 µl TE buffer.

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

E2. Prepare a 16 µg/ml stock solution of genomic DNA in TE buffer.

E3. 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 genomic DNA with TE buffer.

E4. Transfer 20 µl of each DNA standard in duplicate into a 96-well plate labeled A (see figure on the next page).

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			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).

E5. Place 2 µl of each REPLI-g amplified DNA sample for quantification into a new 96-well plate and add 198 µl TE buffer to make a 1:100 dilution. Store the remaining REPLI-g amplified DNA at –20°C.

E6. Place 2 µl diluted REPLI-g DNA (from step E5) into an unused well of 96-well plate A and add 18 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.

E7. Add 20 µl PicoGreen working solution (from step E1) to each sample (amplified DNA and DNA standards) in 96-well plate A. Gently shake the plate on the bench top to mix the samples and reagent.

E8. Centrifuge the 96-well plate briefly to collect residual liquid from the walls of the wells.

E9. 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 fluorimeter's maximum.

Calculation of DNA concentration and yield

E10. Generate a standard curve by plotting the concentration of DNA standards (µg/ml) (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.

E11. Use the standard curve to determine the concentration (µg/ml) of the diluted REPLI-g amplified DNA 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 DNA 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 REPLI-g amplified DNA concentrations.

E12. Multiply the value determined in step E11 by 1000 to show the concentration of undiluted sample DNA (since the sample DNA measured by PicoGreen fluorescence had been diluted 1 in 1000).

E13. To determine the total amount of DNA in your sample, multiply the concentration of undiluted sample DNA (µg/ml) (determined in step E12) by the reaction volume in milliliters (i.e., for a 50 µl reaction, multiply by 0.05).

Appendix F: Purification of Amplified cDNA

Since the amplified cDNA is particularly long (2–100 kb), standard purification methods, such as ethanol precipitation or the use of QIAamp® Kits, cannot be applied as they will result in low recovery of <20%. Two protocols have been developed for purification of long DNA with good recovery and yield based on either LiCl/EtOH or the use of Agencourt®

AMPure® XP Beads (Beckman Coulter, cat. no. A63880). Both are available as supplementary protocols at **www.qiagen.com/us/products/catalog/assay-technologies/next-generation-sequencing/repli-g-single-cell-rna-library-kit#resources**.

Appendix G: Qualification of the WTA Reaction with qPCR

If cDNA amplified via WTA is to be used in costly and labor-intensive downstream applications such as NGS, we strongly recommend first controlling the quality of the WTA samples using qPCR.

Each qPCR reaction should contain 1–10 ng of the cDNA amplified via WTA. Real-time PCR assays that specifically recognize housekeeping genes or genes known to be expressed in the cell type used for WTA are recommended. Furthermore, qPCR assays that specifically detect cDNA, and not gDNA, should be selected. QIAGEN's QuantiTect® Primer Assays, in combination with QuantiNova™, QuantiFast® or QuantiTect SYBR® Green PCR mixes are recommended for such quality control assays. Commonly used housekeeping genes that are widely expressed in various cell types are B2M, EEFC and UBC.

Note: Due to the high molecular weight of the amplified cDNA, heat the diluted cDNA at 95°C for 5 min and then cool on ice prior to PCR to ensure reproducible real-time PCR results.

For further information, please refer to the *QuantiTect Primer Assay Handbook* or *QuantiTect SYBR Green PCR Handbook* available at **www.qiagen.com**.

Ordering Information

Product	Contents	Cat. no.
REPLI-g 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 end-repair, A-addition and ligation; for use with Illumina instruments	150073
GeneRead DNA I Amp Kit (100)	For 100 reactions: Buffers and reagents for library amplification; for use with Illumina instruments	180455
GeneRead Adapter I Set 1-plex (12)	For 12 reactions: Adapters for ligation to DNA library; for use with Illumina instruments	180912
GeneRead Adapter I Set A 12-plex (144)	For 144 reactions: 12 barcoded adapters for ligation to DNA library; for use with Illumina instruments	180985
GeneRead Adapter I Set B 12-plex (144)	For 144 reactions: 12 barcoded adapters for ligation to DNA library; for use with Illumina instruments	180986
REPLI-g Single Cell DNA Library Kit (48)	REPLI-g sc Polymerase, Buffers and Reagents for 48 x whole genome amplification reactions and subsequent end-repair, A-addition and ligation; for use with Illumina instruments	150343
Related products		
QIAGEN GeneRead Kits – for next-generation sequencing applications		
GeneRead Size Selection Kit (50)	For 50 reactions: Spin columns and buffers	180514

Product	Contents	Cat. no.
GeneRead Library Quant Kit	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	180612
GeneRead DNAseq Panel PCR Kit V2	PCR chemistry for use with the GeneRead DNAseq Panel V2 System	Varies
GeneRead DNAseq Mix-n-Match Panels V2	Pools containing wet-bench verified primer sets for targeted enrichment of a custom panel of genes	181905
GeneRead DNAseq Custom Panel V2	Pools containing primer sets for targeted enrichment of a customized panel of genes or genomic regions	181902
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**.

Product	Contents	Cat. no.
REPLI-g Single Cell Kit – for highly uniform whole genome amplification (WGA) from single cells or limited sample material		
REPLI-g Single Cell Kit (24)*	REPLI-g sc Polymerase, Buffers and Reagents for 24 x 50 µl whole genome amplification reactions (typical yield: 40 µg per reaction)	150343
REPLI-g Cell WGA & WTA Kit – for parallel whole genome and whole transcriptome amplification from cells and limited samples		
REPLI-g Cell WGA & WTA Kit (12)*	REPLI-g SensiPhi DNA Polymerase, Buffers and Reagents for 12 x 60 µl whole genome amplification reactions and 12 x 60 µl whole transcriptome amplification reactions (typical yield: 20 µg from each reaction)	150052
REPLI-g WTA Single Cell Kit – for whole transcriptome amplification of total RNA or mRNA from single cells		
REPLI-g WTA Single Cell Kit (24)*	REPLI-g SensiPhi DNA Polymerase, Buffers and Reagents for 24 x 60 µl whole transcriptome amplification reactions (typical yield: 20 µg)	150063
QIAquick PCR Purification Kit – for purification of up to 10 µg PCR products, 100 bp to 10 kb		
QIAquick PCR Purification Kit (50)*	For purification of 50 PCR reactions: 50 QIAquick Spin Columns, Buffers, Collection Tubes (2 ml)	28104

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

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