September 2014

REPLI-g® WTA Single Cell Handbook

For cDNA generation and whole transcriptome amplification from single cells

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Contents

Kit Contents

REPLI-g WTA Single Cell Kit	**(24)**
Catalog no.	**150063**
Number of 60 µl single cell WTA reactions	**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	1000 µl
Quick-Start Protocol	1

* Tube lids are colored for easy reference. Tubes with clear lids are used for sample lysis, tubes with red lids are used for gDNA removal and generation of cDNA suitable for use in a WTA reaction, tubes with blue lids are used in the ligation reaction, and tubes with yellow lids are used for whole transcriptome amplification of cDNA.

REPLI-g WTA Single Cell Kit	**(96)**
Catalog no.	**150065**
Number of 60 µl single cell WTA reactions	**96 each**
Sample preparation	
Lysis Buffer (clear lid)	4 x 110 µl
NA Denaturation Buffer (clear lid)	4 x 80 µl
Enzymatic template preparation	
gDNA Wipeout Buffer, WTA (red lid)	4 x 55 µl
RT/Polymerase Buffer (red lid)	4 x 110 µl
Random Primer (red lid)	4 x 26 µl
Oligo dT Primer (red lid)	4 x 26 µl
Quantiscript RT Enzyme Mix (red lid)	4 x 26 µl
Ligase Mix (blue lid)	4 x 55 µl
Ligase Buffer (blue lid)	4 x 200 µl
Amplification of cDNA	
REPLI-g sc Reaction Buffer (yellow lid)	4 x 700 µl
REPLI-g SensiPhi DNA Polymerase (yellow lid)	4 x 26 µl
H_2O sc	4 x 1000 µl
Quick-Start Protocol	1

* Tube lids are colored for easy reference. Tubes with clear lids are used for sample lysis, tubes with red lids are used for gDNA removal and generation of cDNA suitable for use in a WTA reaction, tubes with blue lids are used in the ligation reaction, and tubes with yellow lids are used for whole transcriptome amplification of cDNA.

Storage

The REPLI-g WTA Single Cell Kit is shipped on dry ice. The kit, including all reagents and buffers, should be stored immediately upon receipt at –15°C to –30°C in a constant-temperature freezer. When stored under these conditions and handled correctly, the products can be kept for at least 6 months after shipping without showing any reduction in performance. For longer storage, the kit should be stored at –65°C to –90°C.

Intended Use

The REPLI-g WTA Single Cell 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 WTA Single Cell Kit is tested against predetermined specifications to ensure consistent product quality.

Introduction

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 WTA Single Cell Kit offers researchers unique WTA chemistry that enables investigation of the transcriptome from a single cell.

The REPLI-g WTA Single Cell Kit contains a novel Phi 29 polymerase, REPLI-g SensiPhi Polymerase, and an optimized set of buffers and reagents for whole transcriptome amplification of total RNA or Poly A+ mRNA from a single cell to up to 1000 cells. Following efficient cell lysis, effective removal of genomic DNA (gDNA), and sensitive reverse transcription of the entire RNA, the kit uses Multiple Displacement Amplification (MDA) for accurate amplification of cDNA. REPLI-g SensiPhi Polymerase was engineered to achieve high sensitivity, fidelity, and processivity and is especially suited for effective MDA from very small amounts of starting material.

The straightforward reaction setup, streamlined processing steps totaling only 20 minutes hands-on time, and overall reaction time of just 4 hours for complete amplification of RNA, make the REPLI-g WTA Single Cell Kit procedure an easy and reliable method to prepare samples for the investigation of transcriptional patterns using a variety of downstream analysis methods, including NGS.

The REPLI-g WTA Single Cell Kit can be used with a range of samples, including those often analyzed in clinical research, such as tumor cells, stem cells, sorted cells, or other small samples. This handbook contains protocols for optimized use of the REPLI-g WTA Single Cell Kit to efficiently generate and amplify cDNA from single cells, purified total RNA, or purified poly A+ mRNA, which are used in a wide variety of research areas (Table 1, page 8).

Table 1. Range of sample material and research areas

Sample material (cells/tissue)	Research area
Cancer sample*	Tumor progression
	Tumor stem cells
	Tumor evolution
	Tumor heterogeneity
	Cancer driver genes and driver pathways
	Circulating tumor cells (CTCs) and disseminated tumor cells (DTCs)
Human/animal†	Mosaicism studies
	Stem cell research
	Genetic predisposition studies
	Allele-specific gene expression
	Expression of quantitative trait loci (eQTLs)
	Gene expression regulation

* Biopsies, sorted or cultured cells, LCMs, fine needle aspirate and others.

† The REPLI-g WTA Single Cell Kit does not lyse cell walls. Therefore, it cannot be used for bacterial cells. Plant or other cells that have cell walls are also not suitable.

Biological Background

Regulation of transcription in each cell is driven by a variety of external and internal factors, such as stress and cellular environment, as well as somatic, genomic and epigenomic variation (e.g., point mutations, copy number variations, structural variations, methylation). 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 is of increasing scientific interest, rather than analyzing a larger number of cells and basing result interpretation on their average behavior.

The REPLI-g WTA Single Cell Kit was specifically designed to reliably investigate effects on transcription regulation at the single-cell transcriptome level.

Target nucleic acids and downstream applications

The REPLI-g WTA Single Cell Kit uniformly amplifies all transcripts from very small samples, accurately representing the transcription pattern of a single cell with very limited, or no, amplification bias. The kit generates up to 20 µg cDNA which accommodates a great variety and number of analyses to be performed. Amplified cDNA is highly suited for next-generation sequencing* (mRNA-Seq), gene expression arrays, or quantitative PCR analysis. The resulting high cDNA yield is particularly important for NGS because it eliminates the need to include an amplification step in the library preparation, thereby avoiding the introduction of PCR-derived bias and saving time compared to typical processing of small samples.

Depending on the protocol (see pages 17, 23, and 29), the REPLI-g WTA Single Cell Kit is suitable for amplification of the transcriptome for analysis of:

- mRNA with poly A+ tails
- Total RNA
- All regions of RNA transcripts
- 3′ ends of mRNAs
- Long non-coding (lnc) and long intergenic non-coding (linc) RNAs

* If performing next-generation sequencing, be aware that a high percentage of reads are derived from rRNA; therefore, sufficient reads must be obtained when performing whole transcriptome sequencing following amplification of total RNA. Amplification of rRNA is efficiently reduced by omitting random primers (refer to protocol "Amplification of Poly A+ mRNA from Single Cells", page 16) or amplifying mRNA-enriched (poly A+) 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 a REPLI-g WTA Single Cell Kit reaction 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.

When following the protocol "Amplification of Total RNA from Single Cells" on page 23, the WTA reaction amplifies cDNA from all regions of RNA transcripts, including the 5′ ends. Depending on the reverse transcription primers used, all transcripts or only polyadenylated transcripts are amplified:

- If performing NGS, use the protocol "Amplification of Poly A+ mRNA from Single Cells" with Oligo-dT priming only (see Table 4, page 20) to avoid amplification of rRNA. This is the preferred protocol if NGS is the downstream application.
- Using both Random and Oligo-dT Primers (Protocol "Amplification of Total RNA from Single Cells", page 23) co-amplifies all ribosomal RNA (rRNA) to the same level as in the cell. The rRNA is detected at high percentage in the subsequent NGS run, which dramatically affects expression results, particularly of low-abundance genes. This is the preferred protocol for PCR, qPCR, or Pyrosequencing®, because poly A+ mRNAs and non-poly A RNAs (mRNAs and Incs) are represented.

The REPLI-g WTA Single Cell Kit can be used successfully to amplify transcripts with low copy number (e.g., less than ~10 copies/sample or from a single cell). Low-copy–number transcripts are often defined as transcripts with a C_T value greater than 30 in real-time PCR, when using 10–100 pg RNA (depending on primers and detection type). Note that at such low copy numbers, stochastic variation in the amplification may impact quantification results. In these cases, transcript copy number results determined by NGS or real-time PCR should be considered qualitative.

Principle and procedure

The REPLI-g WTA Single Cell Kit provides highly uniform amplification across the entire transcriptome, with negligible sequence bias (2). The method is based on MDA technology, which carries out isothermal cDNA amplification utilizing a uniquely processive DNA polymerase capable of replicating up to 70 kb without dissociating from the cDNA template. 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* Polymerase during replication. Because MDA technology makes use of large DNA molecules for amplification, the ligation reaction of cDNA is necessary to obtain high-molecular-weight DNA for WTA.

Genetic analyses often require large amounts of cDNA. WTA overcomes the limits of low RNA quantity, allowing a small number of cells, or even single cells, to be analyzed. The easy reaction setup, logical and streamlined processing steps, low handling time of just 20 minutes, and overall reaction time of just 4 hours for the complete amplification of total RNA, make the REPLI-g WTA Single Cell Kit procedure an easy and reliable method. The resulting amplified cDNA is stable during long-term storage for up to several years with no structural changes or degradation effects, enabling later analysis of sample material.

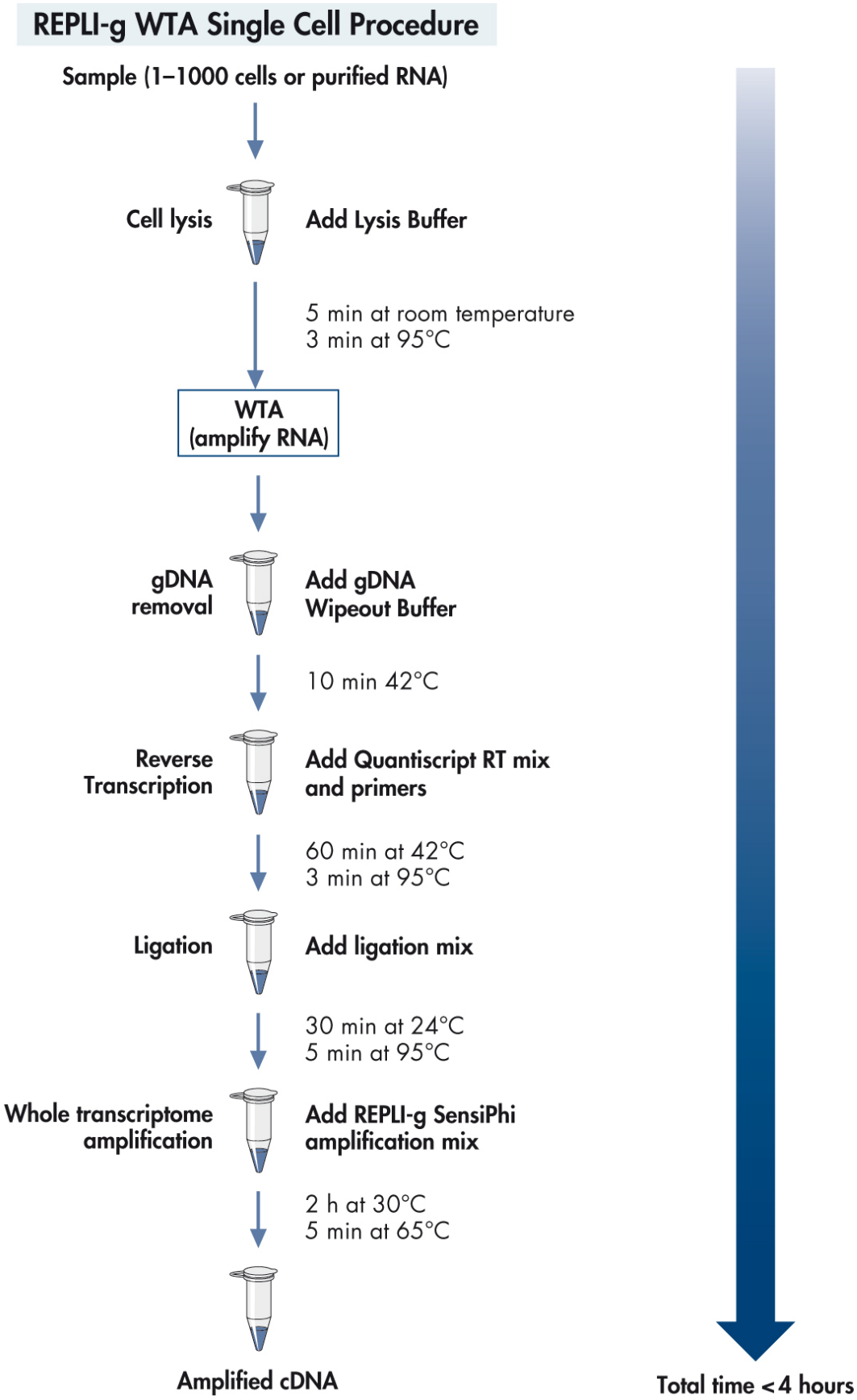
The REPLI-g WTA Single Cell Kit contains reagents for the following sequential reactions (refer to flowchart on page 13):

- Lysis of cells: a single cell sample (containing 1–1000 cells) is lysed efficiently within 5 minutes, with no effect on RNA integrity. The lysed sample is used for WTA of total RNA or, optionally, mRNA-enriched RNA.
- Generation of cDNA: following cell lysis, gDNA is removed prior to the WTA process, since accurate measurement of transcript levels depends on the elimination of false-positive results caused by gDNA contamination. Depending on the primer chosen during the subsequent reverse transcription reaction, all transcripts (if performing total RNA enrichment using random and Oligo dT primers) or only polyadenylated transcripts (if performing poly A+ mRNA enrichment using Oligo dT primers) will be amplified. Consequently, the reaction will contain a mixture of random and Oligo dT primers, or Oligo dT primers only, which reduces rRNA amplification and ensures the 3’ ends of cDNA are reverse transcribed (transcript sizes are approximately 700–1000 bp).

- Ligation: the synthesized cDNA is ligated using a high-efficiency ligation mix. Due to the nature of the ligation reaction, cDNA fragments are not assembled in the order in which they originally existed in the cell. However, kit chemistry is designed to make these events very rare and detection of nucleic acid sequences (e.g., polymorphisms) is not affected in downstream applications, such as NGS, array analysis, or qPCR. Error rates (e.g., chimeras, etc.) observed during kit optimization were in normal range.
- Whole transcriptome amplification: The ligated cDNA is amplified utilizing MDA technology with the novel REPLI-g SensiPhi DNA Polymerase in an isothermal reaction lasting 2 hours.

Unique components of the REPLI-g WTA Single Cell Kit

- All kit components undergo a unique, controlled decontamination procedure to block amplification of contaminating DNA or RNA by the REPLI-g method. Buffers and reagents are exposed to an innovative and standardized DNA decontamination process. Following this process, the kits undergo stringent quality control to ensure complete functionality.
- The innovative lysis buffer, which can be used with as little as 1 cell, effectively stabilizes cellular RNA. The stabilization ensures that the resulting RNA accurately reflects the *in vivo* gene expression profile, and that RNA stays intact to maximize even coverage and retention of all transcripts from the transcriptome for subsequent analyses.
- 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 addition, REPLI-g SensiPhi DNA Polymerase has strong proofreading activity that results in over 1000-fold fewer errors than *Taq*-based WTA methods. It also has strong strand-displacement activity, enabling replication of cDNA through stable hairpin structures that are resistant to *Taq*-based whole genome amplification procedures.


REPLI-g WTA Single Cell Procedure
Sample (1–1000 cells or purified RNA)
Cell lysis
Add Lysis Buffer
5 min at room temperature
3 min at 95°C
WTA
(amplify RNA)
gDNA
removal
Add gDNA
Wipeout Buffer
10 min 42°C
Reverse
Transcription
Add Quantiscript RT mix
and primers
60 min at 42°C
3 min at 95°C
Ligation
Add ligation mix
30 min at 24°C
5 min at 95°C
Whole transcriptome
amplification
Add REPLI-g SensiPhi
amplification mix
2 h at 30°C
5 min at 65°C
Amplified cDNA
Total time < 4 hours

Description of protocols

Different protocols in this handbook provide detailed instructions for using the REPLI-g WTA Single Cell Kit for cDNA amplification of single cells or purified RNA. Use Table 2 (page 15) to select the appropriate protocol based on starting material and downstream analysis.

The protocol "**Amplification of Poly A+ mRNA from Single Cells**", page 17, 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. The protocol amplifies mRNAs (and other RNAs) with poly A+ tails only and is highly suited for a wide range of applications, including RNA-Seq, real-time PCR, and microarray analysis.

The protocol "**Amplification of Total RNA from Single Cells**", page 23, 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. The protocol amplifies 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. If working with sequence-specific detection methods, such as with qPCR or arrays, the amplified rRNA will not affect downstream application results. If performing RNAseq, be aware 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 Poly A+ mRNA from Single Cells" avoids amplification of rRNA by omitting Random Primer.

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 a wide range of applications, including NGS, real-time PCR, and microarray analysis.

Table 2. Protocol selection according to starting material

Starting material	Protocol	Downstream analysis
Single cells (1–1000 cells)	Amplification of PolyA+ mRNA from Single Cells, page 17	NGS (RNA-Seq)*, qPCR, microarray
Single cells (1–1000 cells)	Amplification of Total RNA from Single Cells, page 23	qPCR
Purified RNA (50 pg–100 ng)	Amplification of Purified RNA, page 29	NGS (RNA-Seq)*, qPCR, microarray

* Note that the amplified product will be cDNA; therefore, subsequent library preparation must be performed using a DNA library prep kit. Refer to Appendix A for more information on using amplified cDNA for NGS.

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
- Thermal cycler
- Microcentrifuge
- Vortexer
- Pipets and pipet tips
- Wet ice
- Nuclease-free water or TE buffer (10 mM Tris·Cl; 1 mM EDTA, pH 8.0)

Protocol: Amplification of Poly A+ mRNA from Single Cells

This protocol is for amplification of polyadenylated mRNA using single cell material. RNA amplified using this protocol is highly suited for a wide range of applications, including next-generation sequencing (RNA-Seq), 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 23. 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 WTA Single Cell Kit. Avoid using more than 1000 cells in the reaction, since samples containing too many cells may not be lysed effectively.
- REPLI-g amplified cDNA 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.
- Purification of amplified cDNA is only necessary for labeling of cDNA, for example, to be used in microarrays. Use one of the 2 purification protocols specially developed for REPLI-g amplified DNA (see Appendix D, page 44). All other downstream applications, such as NGS or PCR, are not affected by remaining nucleotides.
- 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 WTA 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 WGA.
- Because the REPLI-g WTA 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.

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 3, page 19).

Table 3. Thermal cycling parameters

Step	Time	Temperature	Additional comments
Set the heating lid to 50ºC 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 flicking the tube, and centrifuge briefly.**

   Note: Ensure that the cell material does not stick to the tube wall 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 the Quantiscript RT mix (see Table 4). 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 4. Preparing 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**

* Scale up accordingly if performing several reactions at one time.

† 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 5). 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 5.

   Note: The ligation mix must be prepared fresh.

Table 5. Preparing ligation mix*

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

* Scale up accordingly if performing several reactions at one time.

† 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 6). 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 6. Preparing 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**

* Scale up accordingly if performing several reactions at one time.

† 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 ºC to –30ºC until required for downstream applications. We recommend storage of the amplified DNA at a minimum concentration of 100 ng/µl.**

    Optical density (OD) measurements overestimate REPLI-g amplified DNA. Refer to Appendix C (page 41) for PicoGreen® measurements. Alternatively, purify the reaction with one of the 2 purification protocols specially developed for REPLI-g amplified DNA (see Appendix D, page 44). Afterwards determine the amount of DNA by standard OD measurement.

    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 WGA.

14. **Amplified cDNA behaves like purified genomic DNA and has an approximate length of 2000–70,000 bp. It is highly suited for use in a variety of downstream applications, including next-generation sequencing, microarrays (cDNA arrays, no RNA arrays), and quantitative PCR. Follow the application-specific advice given in Table 7.**

Note: The amplified product will be double-stranded cDNA. Therefore, subsequent library preparation must be performed with a DNA library prep kit. Refer to Appendix A, page 37, for more information about using amplified cDNA for NGS.

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.

Table 7. Advice for downstream applications

Downstream applications	Use of amplified DNA/cDNA	QIAGEN products
Real-time PCR, PCR	Dilute amplified DNA/cDNA 1:100 and use 2–3 µl for real-time PCR	QuantiTect®, QuantiFast®, QuantiNova™ Kits QuantiTect Primer Assays
NGS	Use 3–10 µl for shearing, purify sheared DNA/cDNA and start DNA library preparation	GeneRead™ Library Prep Kits

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, be aware 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 17, 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 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 WTA Single Cell Kit. Avoid using more than 1000 samples in the reaction, since samples containing too many cells may not be lysed effectively.
- REPLI-g amplified cDNA 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.
- Purification of amplified cDNA is only necessary for labeling of cDNA, for example, to be used in microarrays. Use one of the 2 purification protocols specially developed for REPLI-g amplified DNA (see Appendix D, page 44). All other downstream applications, such as NGS or PCR, are not affected by remaining nucleotides.
- 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 WTA 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 WTA 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 8, page 25).

Table 8. Thermal cycling parameters

Step	Time	Temperature	Additional comments
Set the heating lid to 50ºC 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.**
2. **Add 4 µl Lysis Buffer. Mix carefully by 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 9). 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 9. Preparing 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**

* Scale up accordingly if performing several reactions at one time.

† Mix by vortexing and centrifuge briefly.

7. **Incubate at 42ºC for 60 min. Stop the reaction by incubating at 95ºC for 3 min, then cool on ice.**
8. **Prepare the ligation mix (Table 10). 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 10.

   Note: The ligation mix must be prepared fresh.

Table 10. Preparing ligation mix*

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

* Scale up accordingly if performing several reactions at one time.

† Mix by vortexing and centrifuge briefly.

9. **Incubate at 24ºC 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 11). 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 11. Preparing 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**

* Scale up accordingly if performing several reactions at one time.

† Mix by vortexing and centrifuge briefly.

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

    Optical density (OD) measurements overestimate REPLI-g amplified DNA. Refer to Appendix C (page 41) for PicoGreen measurements. Alternatively, purify the reaction with one of the 2 purification protocols specially developed for REPLI-g amplified DNA (see Appendix D, page 44). Afterwards determine the amount of DNA by standard OD measurement.

    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 WGA.

14. **Amplified cDNA behaves like purified genomic DNA and has an approximate length of 2000–70,000 bp. It is highly suited for use in a variety of downstream applications, including next-generation sequencing, microarrays (cDNA arrays, no RNA arrays), and quantitative PCR. Follow the application-specific advice given in Table 12.**

Note: The amplified product will be double-stranded cDNA. Therefore, subsequent library preparation must be performed with a DNA library prep kit. Refer to Appendix A, page 37, for more information about using amplified cDNA for NGS.

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.

Table 12. Advice for downstream applications

Downstream applications	Use of amplified DNA/cDNA	QIAGEN products
Real-time PCR, PCR	Dilute amplified DNA/cDNA 1:100 and use 2–3 µl for real-time PCR	QuantiTect, QuantiFast, QuantiNova, Type-it Kits
NGS	cDNA amplified with this protocol is not recommended for NGS. The preferred protocol is "Amplification of Poly A+ mRNA from Single Cells" on page 17.	GeneRead Library Prep Kits

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 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 or rRNA depletion Nano Kit).It is not suited for degraded RNA, as that derived from FFPE tissues.
- The specific protocol used for WTA of purified RNA depends on the starting material and the downstream application. See Table 13 (page 31) for details.
- Use >50 pg of purified RNA for the WTA protocol.
- REPLI-g amplified cDNA 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.
- Purification of amplified cDNA is only necessary for labeling of cDNA, for example, to be used in microarrays. Use one of the 2 purification protocols specially developed for REPLI-g amplified DNA (see Appendix D, page 44). All other downstream applications, such as NGS or PCR, are not affected by remaining nucleotides.
- 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 WTA 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 WGA.
- Because the REPLI-g WTA 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 applications, such as NGS, array analysis, or qPCR. Error rates (e.g., chimeras, etc.) observed during kit optimization were in normal range.

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 (see Table 13, page 31).

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 17) or "Amplification of Total RNA from Single Cells" (page 23). Refer to Table 13 (page 31) to select the correct protocol based on the type of RNA to be amplified.**

Table 13. Selection of correct WTA protocol using purified RNA

Starting material	Protocol	Region amplified and downstream application
Total RNA (>50 pg)	Amplification of Total RNA from Single Cells, page 23. Start with step 4.	All mRNA and rRNA regions from a sample PCR and Pyrosequencing
Total RNA (> 50 pg)	Amplification of Poly A+ mRNA from Single Cells, page 17. Start with step 4.	mRNA (poly A+) NGS
Poly A+ RNA (> 50 pg)	Amplification of Total RNA from Single Cells, page 23 Start with step 4.	All regions of mRNA within the poly A+ region Any application
mRNA-enriched RNA (rRNA-depleted) (> 50 pg)	Amplification of Total RNA from Single Cells, page 23. Start with step 4.	All regions of an enriched mRNA sample Any application

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 protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit 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) Excess cells in the sample causes inefficient lysis	Use 1–1000 cells
d) Incorrect reaction temperature	Be sure to carry out the RT, 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	Check your pipets. Be sure to mix all reagents well after thawing and to store them on ice.
f) Incorrect incubation time	For the RT, ligation, and amplification reactions, be sure to use the incubation times specified in the protocol.
g) RT mix, ligation mix, and amplification mix not freshly prepared	Always prepare Quantiscript RT mix, ligation mix, and REPLI-g SensiPhi amplification mix fresh before use. Storage of these mixes prior to use may affect whole transcriptome amplification.

	Comments and suggestions
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.
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 REPLI-g reaction by random extension of primer–dimers	High-molecular-weight product can be generated by random extension of primers, forming primer-multimers. This DNA will not affect the quality of actual samples or specific downstream 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 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 or RNA. If possible, work in a laminar-flow hood. Use sterile equipment and barrier pipet tips only, and keep amplification chemistry and 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 volumes less than a single cell due to Poisson distribution.

	Comments and suggestions
b) Cells are not intact	Use viable cells for REPLI-g WTA Single Cell 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 which 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 having 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 Poisson distribution, the REPLI-g WTA Single Cell 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 WTA Single Cell 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.

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 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 REPLI-g WTA Single Cell Kit amplifies low-abundance transcripts to a variable extent due to Poisson distribution.

	Comments and suggestions
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 3′ Regions of mRNA (Poly A+) from Single Cells"	In the protocol "Amplification of the 3′ Regions of mRNA (Poly A+) 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

a) Sensitive downstream applications may require DNA cleanup after REPLI-g reaction	Contact QIAGEN Technical Services for DNA cleanup recommendations suitable for your application.
b) Little or no cDNA amplified	Always prepare RT mix, ligation mix, and amplification mix fresh before use. Storage of these mixes prior to use may affect whole transcriptome amplification.

References

1. Dean, F.B. et al (2002) Comprehensive human genome amplification using multiple displacement amplification. Proc. Natl. Acad. Sci. USA **99**, 5261.
2. Hosono, S. et al (2003) Unbiased whole-genome amplification directly from clinical samples. Genome Res. **13**, 954.

Appendix A: Use of Amplified cDNA or gDNA for Next-Generation Sequencing

Next-generation sequencing (NGS) is a driving force for numerous applications, including cancer research, stem cell research, metagenomics, population genetics, and medical research.

Library preparation can be done either with intact DNA, using tagmentation methods like Nextera DNA library prep, or the amplified DNA can be fragmented by mechanical shearing prior to library construction (e.g., Covaris® instrument). A dedicated protocol has been developed for Nextera library prep using cDNA amplified with the REPLI-g WTA Single Cell kit. Please check the supplementary protocols available at www.qiagen.com.

The efficient QIAGEN GeneRead Library Prep Kits are based on a streamlined, optimized, one-tube protocol that does not require sample cleanup between each step, which saves time and prevents handling errors and loss of valuable samples. The following describes use of these kits.

The GeneRead library prep procedure includes an optional, high-fidelity amplification step to ensure high yields of DNA library that are reproducibly generated with minimal sequence bias and low error rates. This step can be omitted because the amount of DNA derived from the REPLI-g amplification is sufficient for several library preps without further amplification.

Since the NGS process is relatively long and complex, it is advisable to control the quality of WTA samples by qPCR prior to preparing the NGS library. We recommend using QuantiTect Primer Assays in combination with QuantiTect SYBR® Green, QuantiFast SYBR Green, or QuantiNova SYBR Green mix for quality control because the assays are designed to detect only cDNA. Select QuantiTect Primer Assays that detect either housekeeping or genes known to be expressed in the specific cells used in the experiment. As template, use 1–10 ng WTA cDNA in each qPCR reaction.

Fragmentation of amplified cDNA generated by the REPLI-g WTA Single Cell Kit

Amplified cDNA generated with the REPLI-g WTA Single Cell Kit is first sheared into a random library of fragments prior to use with a wide variety of DNA library preparation kits. The median fragment sizes optimal for various NGS instruments are listed in Table 14, page 38. Specific median fragment length sizes of DNA can be prepared using a Covaris instrument, according to the manufacturer's instructions.

Table 14: Optimal fragment size for various instruments*

Instrument	Optimal fragment size (bp)
Illumina MiSeq®, version 1	300 bp
Illumina MiSeq, version 2	500 bp
Ion Proton™ instrument	200 bp
Ion Torrent PGM™ instrument	400 bp

* For other instruments, please check the supplier's manual.

Use of fragmented cDNA with GeneRead Library Prep Kits

A1. Use 2–5 µg of amplified cDNA generated with the REPLI-g WTA Single Cell Kit.

Note: Follow instructions in the Covaris manual to determine the correct instrument settings to achieve the optimal fragment size.

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, QIAxcel® Advanced, or Agilent® Bioanalyzer®.

A2. Purify fragmented cDNA using the QIAquick PCR Purification Kit (cat. no. 28104)

Note: Follow "**QIAquick PCR Purification Kit Protocol, using a microcentrifuge**" in the *QIAquick Spin Handbook*.

A3. Prepare GeneRead Library following the protocols in the kit handbooks.

Note: Choose the correct kit for your application and NGS instrument (Table 15, page 39).

Table 15: GeneRead Library Preparation

Instrument	GeneRead Library	Cat. no.
Illumina MiSeq, Illumina HiSeq® 2000 or 2500 Illumina Genome Analyzer	GeneRead DNA Library I Core Kit	180432
	GeneRead Adapter I Set 1-plex	180912
	GeneRead Adapter I Set 12-plex	180984
	GeneRead DNA I Amp Kit	180455
Ion Proton instrument Ion Torrent PGM instrument	GeneRead DNA Library L Core Kit	180462
	GeneRead Adapter L Set 1-plex	180922
	GeneRead Adapter L Set 12-plex	180994
	GeneRead DNA L Amp Kit	180485

Appendix B: Determining DNA Concentration and Yield

Using the REPLI-g WTA Single Cell 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 D (page 44). 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 fluorometer, to quantify the amount of amplified DNA. A protocol for the quantification of amplified DNA is provided in Appendix C.

Appendix C: PicoGreen Quantification of Amplified DNA

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

Note: Degraded or old PicoGreen reagent may result in inaccurate DNA quantification. DNA yields in excess of 80 µg should be ignored and, if necessary, quantification should be repeated using fresh PicoGreen reagent.

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

Equipment and reagents to be supplied by user

- Quant-iT™ PicoGreen dsDNA reagent (Invitrogen, cat. no. P7581)
- TE buffer (10 mM Tris·Cl; 1 mM EDTA, pH 8.0)
- Human genomic DNA (e.g., REPLI-g Control DNA, QIAGEN cat. no. 150090)
- 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

C1. 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.

C2. Prepare a 16 µg/ml stock solution of genomic DNA in TE buffer.

DNA standards will be prepared from this stock solution.

C3. 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.

C4. Transfer 20 µl of each DNA standard in duplicate into a 96-well plate labeled A (see Figure 1).

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

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

Figure 1. 96-well plate. Gray squares: genomic DNA standard (µg/ml).

C5. Place 2 µl of each amplified DNA sample for quantification into a new
96-well plate and add 98 µl TE buffer to make a 1:50 dilution. Store the remaining amplified DNA at –20°C.

C6. Place 2 µl diluted DNA (from step C5) into an unused well of 96-well plate A and add 18 µl TE to make a 1:500 dilution.

The 1:50 dilutions can be stored at –20°C and used for future downstream sample analysis.

C7. Add 20 µl PicoGreen working solution (from step 1) 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.

C8. Centrifuge the 96-well plate briefly to collect residual liquid from the walls of the wells.

C9. Measure the sample fluorescence using a fluorescence microplate reader and standard fluorescence filters (excitation approximately 485 nm; emission approximately 538 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

C10. 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.

C11. Use the standard curve to determine the concentration (µg/ml) of the diluted 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 amplified DNA concentrations.

C12. Multiply the value determined in step 11 by 500 to show the concentration of undiluted sample DNA (as the sample DNA measured by PicoGreen fluorescence had been diluted 1 in 500).

C13. To determine the total amount of DNA in your sample, multiply the concentration of undiluted sample DNA (µg/ml) from step C12 by the reaction volume in milliliters (i.e., for a 50 µl reaction, multiply by 0.05).

Appendix D: Purification of Amplified cDNA for use in labeling reactions or OD measurement

Since the amplified cDNA is particularly long (2–100 kb), standard purification methods, like ethanol precipitation or 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 AmPure beads. Both are available as supplementary protocols on the REPLI-g WTA Single Cell Kit product page at www.qiagen.com.

Appendix E: 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.

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

Ordering Information

Product	Contents	Cat. no.
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
REPLI-g WTA Single Cell Kit (96)	REPLI-g SensiPhi DNA Polymerase, Buffers, and Reagents for 96 x 60 µl whole transcriptome amplification reactions (typical yield: 20 µg)	150065
Related products		
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 Cell WGA & WTA Kit (48)	REPLI-g SensiPhi DNA Polymerase, Buffers, and Reagents for 48 x 60 µl whole genome amplification reactions and 48 x 60 µl whole transcriptome amplification reactions (typical yield: 20 µg from each reaction)	150054
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 Single Cell Kit (96)	REPLI-g sc Polymerase, Buffers, and Reagents for 96 x 50 µl whole genome amplification reactions (typical yield: 40 µg per reaction)	150345
GeneRead DNA Library I Core Kit (12)	For 12 reactions: Buffers and reagents for end-repair, A-Addition, and ligation for use with Illumina instruments	180432
GeneRead DNA I Amp Kit (100)	For 100 reactions: Ready-to-use library amplification master mix and primer ix, for use with illumine instruments	180455

Product	Contents	Cat. no.
GeneRead DNA Library L Core Kit (12)	For 12 reactions: Buffers and reagents for end-repair, ligation, and nick-repair for use with Life Technology instruments	180462
GeneRead DNA L Amp Kit (100)	For 100 reactions: Ready-to-use library amplification master mix and primer mix, for use with Life Technology instruments	180485

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

Notes

Limited License Agreement for the REPLI-g WTA Single Cell Kit

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

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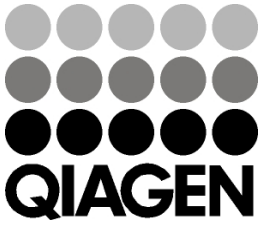

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Sample & Assay Technologies