April 2015

# REPLI-g® Single Cell DNA Library Handbook

## For DNA library construction from single cells for Illumina® sequencing applications

Sample to Insight

QIAGEN

# Contents

# Kit Contents

| **REPLI-g Single Cell DNA Library Kit** | **(48)** |
|---|---|
| **Catalog no.** | **150354** |
| **Number of preps** | **48** |
| REPLI-g sc DNA Polymerase (blue lid) | 2 x 48 µl |
| REPLI-g sc Reaction Buffer (yellow lid) | 2 x 700 µl |
| Buffer DLB (clear lid) | 2 x 1 tube |
| Stop Solution (red lid) | 2 x 1.8 ml |
| PBS sc 1x (clear lid) | 2 x 1.5 ml |
| DTT, 1 M (lilac lid) | 2 x 1 ml |
| $H_2O$ sc | 3 x 1.5 ml |
| T4 DNA Ligase | 204 µl |
| Klenow Fragment | 152 µl |
| A-Addition Buffer, 10x | 200 µl |
| End Repair Buffer, 10x | 200 µl |
| Ligation Buffer, 2x | 2 x 1.7ml |
| End Repair Enzyme Mix | 104 µl |
| Quick Start Protocol | 1 |

# Storage

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

# Introduction

Single cell genomic analysis enables researchers to gain novel insights across a diverse set of applications, including, developmental biology, tumor heterogeneity and disease pathogenesis and progression. Conducting single cell genomic analysis using next-generation sequencing (NGS) methods has traditionally been challenging since the amount of genomic DNA present in a single cell is very limited. PCR-based whole genome amplification methods normally have high error rates, low coverage uniformity, extensive allelic drop-outs and limited amplification yields. The REPLI-g Single Cell DNA Library Kit leverages QIAGEN's unique multiple displacement amplification (MDA) technology and efficient GeneRead™ library construction technology to overcome these challenges by allowing preparation of a sequencing library with high fidelity and minimal bias, while ensuring retention of the sample's genomic diversity.

With the REPLI-g Single Cell DNA Library Kit, reaction setup is straightforward and handling time is greatly reduced, allowing DNA amplification and library preparation to be completed in a single working day. The kit provides a time-saving, one-tube library preparation protocol that does not require sample cleanup between steps, minimizing starting material loss and cross-contamination risk. Co-optimization of MDA and library construction processes enables a highly streamlined and efficient protocol, reducing MDA time to only three hours and eliminating the library amplification step. Optimized enzyme and buffer compositions ensure superior yields of high-quality, NGS-ready libraries in just one working day.

In standard PCR amplification procedures, regions of DNA with high GC or AT content can result in little or no amplification, leading to misleading sequence data and NGS results. The REPLI-g Single Cell DNA Library Kit employs high-fidelity MDA technology to provide accurate amplification of genomes with negligible sequence bias and minimal genomic drop-outs. The REPLI-g Single Cell DNA Library Kit contains an optimized Phi29 DNA polymerase formulation, which, together with its proprietary buffer formulation, ensures

uniform amplification of genomic 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 Phi29 DNA polymerase, which has up to 1000-fold higher fidelity compared to normal PCR polymerases. Dedicated buffers and reagents undergo a unique, robust decontamination procedure to avoid amplification of contaminating DNA, ensuring high reliability.

The REPLI-g Single Cell DNA 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. Due to the high yields achieved during the WGA step, as well as the high ligation efficiency of the library construction reagents, library preparation can be performed without PCR-based library amplification, which can introduce bias and reduce library diversity. The kit allows construction of complex libraries from single cells or limited DNA samples, providing a high percentage of mapped reads, uniform genome coverage, high sequence complexity and low error rates, and outperforms PCR-based single cell library construction products from alternative suppliers.

The average product length of REPLI-g Single Cell (SC) amplified DNA is typically more than 10 kb, with a range between 2 kb and 100 kb, enabling all downstream applications such as complex genetic analysis, including long-range copy number variations, to be carried out.

Typical DNA yields from a REPLI-g Single Cell DNA Library Kit reaction are up to 40 µg per 50 µl reaction. Depending on the quality and number of the used cells, the resulting amount of DNA may be less (storage of cells for periods longer than 2–3 months will affect the yield of amplified DNA). For best amplification results, all cell samples must be correctly collected and stored.

## Principle and procedure

The REPLI-g Single Cell DNA Library Kit includes REPLI-g sc DNA Polymerase, an optimized formulation of the innovative, high-fidelity enzyme Phi29 DNA polymerase, to amplify complex genomic DNA using MDA technology, along with gentle alkaline incubation to ensure very low DNA fragmentation or generation of abasic sites. It is specifically designed to provide high yields of amplified DNA from single cells.

The REPLI-g Single Cell DNA Library Kit uses isothermal genome amplification, termed multiple displacement amplification, which involves the binding of random hexamers to denatured DNA. This is followed by strand displacement synthesis at a constant temperature with an optimized form of the enzyme Phi29 DNA polymerase, which has exceptionally strong strand displacement properties. Additional priming events occur on each displaced strand, which serves as a template, enabling generation of high yields of amplified DNA. Phi29 DNA polymerase, a phage-derived enzyme, 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, Phi29 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 DNA fragments up to 100 kb without sequence bias.

Genomic DNA must be denatured before use in enzymatic amplification procedures, which is often accomplished using harsh methods such as incubation at elevated temperatures (heat incubation) or increased pH (chemical alkaline incubation). The REPLI-g Single Cell DNA Library Kit uses gentle alkaline incubation, allowing effective cell lysis and uniform DNA denaturation of gDNA with very low DNA fragmentation or generation of abasic sites. This results in amplified DNA with very high integrity, and maximizes the length of amplified fragments so that genomic loci and sequences are uniformly represented.

All kit components used for WGA undergo a unique, controlled decontamination procedure to ensure elimination of all REPLI-g amplifiable contaminating DNA. Buffers and reagents are treated with an innovative and standardized procedure during manufacturing to ensure the absence of any detectable residual contaminating DNA. Following decontamination, the kits undergo stringent quality control to ensure complete functionality.

The REPLI-g Single Cell DNA Library Kit provides a simple and reliable method to efficiently generate DNA libraries, suitable for use on Illumina NGS instruments from just a single cell or as little as picograms of DNA in 5–5.5 hours. The kit provides a complete workflow for highly uniform amplification across the entire genome, with negligible sequence bias, followed by fast, one-tube library construction. Dedicated buffers and reagents have been developed to deliver high yields of DNA from single cells, limited material and purified DNA, with complete sequence representation and unbiased amplification.

In the first step of the WGA procedure, the cell sample is lysed and the DNA is denatured. After denaturation has been stopped by the addition of neutralization buffer, a master mix containing buffer and DNA polymerase is added. The isothermal amplification reaction proceeds for 3 hours at 30°C, and can be preprogrammed in a thermal cycler. REPLI-g SC amplified DNA can be stored long-term at –20°C with no negative effects, or used directly to generate sequencing libraries. For library construction, samples consisting of longer DNA 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 and A-addition, platform-specific adapters, which contain sequences essential for binding the library to a flow cell for sequencing and binding sequencing primer are ligated to both ends of the DNA fragments. The WGA procedure normally results in high yields of DNA so that library preparation can be performed with a high amount of input DNA, and subsequent PCR-based library enrichment can be avoided. However, if library enrichment is required, an optional, high-fidelity amplification step that provides highly accurate amplification of library DNA with low error rates and minimum bias, can also be performed. Barcode adapters, (GeneRead Adapter I Set A 12-plex, cat. no.180985 and GeneRead Adapter I Set B 12-plex, cat no.180986), which contain a unique identifying sequence, also available with the

GeneRead DNA Library I Core Kit (cat. nos. 180432 and 180434), allow samples to be pooled and sequenced in the same run. Following library construction, reaction cleanup and removal of adapter dimers can be achieved by using the GeneRead Size Selection Kit (cat. no. 180514), which uses an easy and precise, silica column-based method.

## Description of protocols

This handbook contains protocols for amplification of DNA from single cells, such as isolated tumor cells or bacteria, fresh blood or purified genomic DNA, as well as subsequent library preparation. The protocol "Amplification of Genomic DNA from Single Cells", page 16, is optimized for whole genome amplification from single cell material from sorted cells, tissue culture cells, laser-microdissected cells and cells or tissue from biopsies, circulating tumor cells, and all species of, for example, vertebrates, bacteria (gram positive and gram negative), plants (without the cell wall), etc. The protocol "Amplification of Purified Genomic DNA", page 20, is optimized for whole genome amplification from genomic DNA template.

**Table 1. Protocol selection according to starting material**

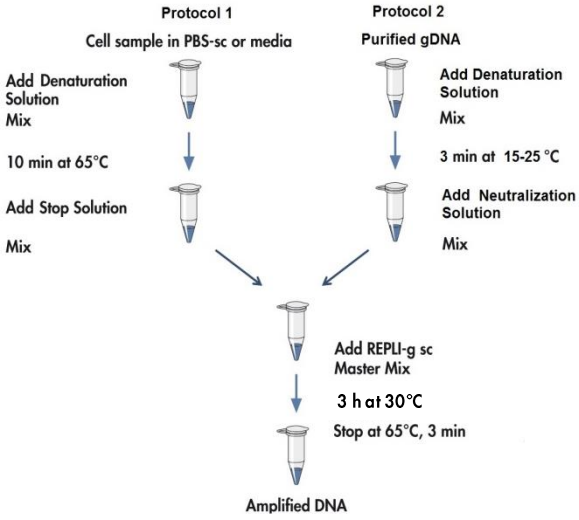
| Sample | Name | Page |
|---|---|---|
| Single cells, 1–1000 cells | Amplification of Genomic DNA from Single Cells | 16 |
| Purified genomic DNA (10 pg–10 ng) | Amplification of Purified Genomic DNA | 20 |

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 26). The prepared library can be quantified and is optimized for use on Illumina sequencing platforms.

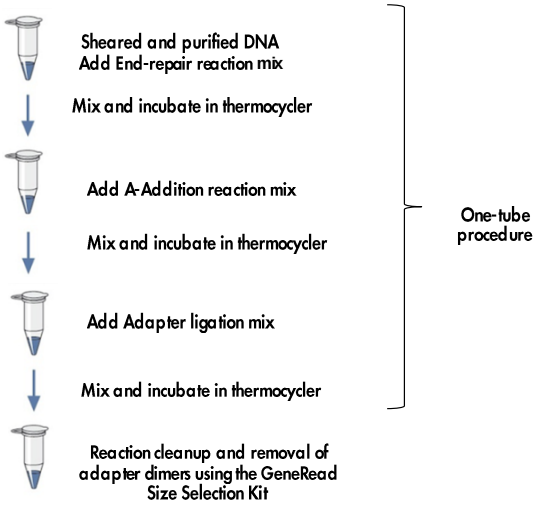
## Compatible sequencing platforms

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

## REPLI-g Single Cell DNA Library Kit procedure




DNA quantification, shearing and purification



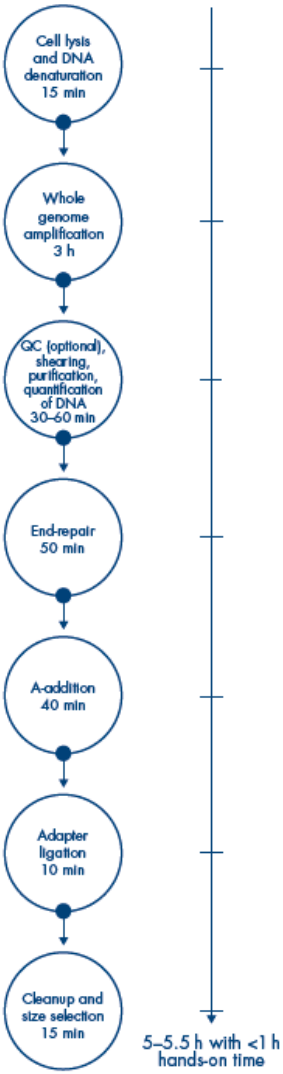



**Figure 1. A time-saving, streamlined protocol delivers ready-to-use libraries suitable for use on Illumina NGS platforms.** The REPLI-g Single Cell DNA Library Kit provides a complete workflow for highly uniform amplification across the entire genome, with negligible sequence bias, followed by fast, one-tube library construction.

# 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
- 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
- Enzymatic or physical method (e.g., Covaris® instrument) to shear DNA
- GeneRead Size Selection Kit (cat. no. 180514)
- GeneRead Adapter I Set A 12-plex (cat. no.180985)
- GeneRead Adapter I Set B 12-plex cat no.180986)
- 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 enzyme activities necessary for optimal library preparation.

### Recommendations for DNA fragmentation

DNA can be fragmented using one of the following methods:

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

To ensure the complete fragmentation of 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 Genomic DNA from Single Cells

This protocol is for the amplification of genomic DNA from 1–1000 intact cells.

## Important points before starting

- This protocol is optimized for single cell material from all species of, for example, vertebrates, bacteria (Gram positive and Gram negative), plants (without the cell wall), sorted cells, tissue culture cells, microdissected cells from frozen sections and cells. The protocol cannot be used with 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., eukaryotic or bacterial cells) are optimal for whole genome amplification reactions using the REPLI-g Single Cell DNA Library Kit.
- Avoid DNA 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 DNA.
- For the amplification of purified genomic DNA, refer to page 20.
- REPLI-g sc DNA Polymerase should be thawed on ice (see step 6). All other components can be thawed at room temperature (15–25°C).
- Buffer D2 (denaturation buffer) should not be stored for longer than 3 months.
- DNA yields of up to 40 µg will be present in negative (no-template) controls because the DNA generated during the REPLI-g Single Cell reaction by random extension of primer dimers is a high-molecular-weight product. This DNA will not affect the quality of the actual samples and will not give a positive result in downstream assays.

## Things to do before starting

- Prepare Buffer DLB by adding 500 µl $H_2O$ sc to the tube provided. Mix thoroughly and centrifuge briefly to dissolve.

  **Note**: Reconstituted Buffer DLB can be stored for 6 months at –20°C. Buffer DLB is pH-labile.

- All buffers and reagents should be vortexed before use to ensure thorough mixing.
- Set a water bath, heating block or a programmable thermal cycler to 30°C.
- If a thermal cycler is used with a heated lid, the temperature of the lid should be set to 70°C.

## Procedure

1. Prepare sufficient Buffer D2 (denaturation buffer) for the total number of whole genome amplification reactions (Table 2).

   **Note**: The total volume of Buffer D2 given in Table 2 is sufficient for 12 reactions. If performing fewer reactions, store residual Buffer D2 at –20°C. Buffer D2 should not be stored longer than 3 months.

**Table 2. Preparation of Buffer D2 for 12 reactions**

| Component | Volume for 12 reactions |
|---|---|
| DTT, 1 M | 3 µl |
| Buffer DLB (reconstituted)* | 33 µl |
| **Total volume** | **36 µl** |

* Reconstitution of Buffer DLB is described in "Things to do before starting".

2. Place 4 µl cell material (supplied with PBS) into a microcentrifuge tube. If using less than 4 µl of cell material, add PBS sc to bring the volume up to 4 µl.

   **Note**: The amount of PBS sc supplied with the REPLI-g Single Cell Kit is insufficient to prepare serial dilutions of cell material. During pipetting, avoid contact of pipet tips and cell material.

   Alternatively, 0.5 µl whole blood can be used.

3. Add 3 µl Buffer D2. 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 buffer line. During pipetting, avoid any contact of pipet tips with cell material.

4. Incubate for 10 min at 65°C.
5. Add 3 µl Stop Solution. Mix carefully by flicking the tube and centrifuge briefly. Store on ice.
6. Thaw REPLI-g sc DNA Polymerase on ice. Thaw all other components at room temperature, vortex and then centrifuge briefly.

   The REPLI-g sc Reaction Buffer may form a precipitate after thawing. The precipitate will dissolve by vortexing for 10 s.

7. Prepare a master mix according Table 3. Mix and centrifuge briefly.

   **IMPORTANT**: Add the master mix components in the order listed in Table 3. After the addition of water and REPLI-g sc Reaction Buffer, briefly vortex and centrifuge the mixture before adding REPLI-g sc DNA Polymerase.

   **Note**: Scale up accordingly if performing several reactions at once, by preparing a master mix sufficient for the total number of reactions.

   The master mix should be kept on ice and used immediately upon addition of REPLI-g sc DNA Polymerase.

**Table 3. Preparation of master mix***

| Component | Volume/reaction |
|---|---|
| $H_2O$ sc | 9 µl |
| REPLI-g sc Reaction Buffer | 29 µl |
| REPLI-g sc DNA Polymerase | 2 µl |
| **Total volume** | **40 µl** |

* To prepare a master mix for multiple reactions, scale up according to the number of reactions and add 10% more.

8. For each amplification reaction, add 40 µl master mix to 10 µl denatured DNA (from step 5).
9. Incubate at 30°C for 3 h.

**Note**: Incubating the sample for 3 hours generates sufficient DNA for PCR-free library prep using this kit.

After incubation at 30°C, heat the water bath or heating block up to 65°C, if the same water bath or heating block will be used in step 10.

**Note**: If a thermal cycler is used with a heated lid, the temperature of the lid should be set to 70°C.

10. Inactivate REPLI-g sc DNA Polymerase at 65°C for 3 min.
11. If not being used directly, store amplified DNA at 4°C for short-term storage or –20°C for long-term storage.

    DNA amplified using the REPLI-g Single Cell DNA Library Kit should be treated as genomic DNA and undergo minimal freeze-thaw cycles. We recommend storage of nucleic acids at a concentration of at least 100 ng/µl.
12. Amplified DNA can be directly used for the library construction or target-directed amplification and library construction.

    **Note**: To proceed with library preparation, quantify the amplified DNA following the instructions in Appendix B (page 37). Optical density (OD) measurements overestimate REPLI-g amplified DNA and should not be used. Typical DNA yields are approximately 40 µg per 50 µl reaction.

# Protocol: Amplification of Purified Genomic DNA

This protocol is for whole genome amplification of purified DNA using the REPLI-g Single Cell DNA Library Kit.

## Important points before starting

- This protocol is optimized for whole genome amplification of >10 ng of purified genomic DNA template. The template DNA should be suspended in TE buffer. If the DNA is of sufficient quality (e.g., high-molecular-weight DNA with no inhibitors [e.g., detergents or organic solvents]), smaller amounts (1–10 ng for eukaryotic DNA or 10–100 pg for bacterial DNA) may be used.
- Avoid DNA 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 DNA.
- For direct amplification of DNA from cell material, see page 16.
- For best results, the template DNA should be >2 kb.
- REPLI-g sc DNA Polymerase should be thawed on ice (see step 6). All other components can be thawed at room temperature (15–25°C).
- Buffer D1 (denaturation buffer) and Buffer N1 (neutralization buffer) should not be stored for longer than 3 months.
- DNA yields of up to 40 µg will be present in negative (no-template) controls because the DNA generated during the REPLI-g Single Cell reaction by random extension of primer dimers is a high-molecular-weight product. This DNA will not affect the quality of the actual samples and will not give a positive result in downstream assays.

## Things to do before starting

- Prepare Buffer DLB by adding 500 µl $H_2O$ sc to the tube provided. Mix thoroughly and centrifuge briefly to dissolve.

**Note**: Reconstituted Buffer DLB can be stored for 6 months at –20°C. Buffer DLB is pH-labile.

- All buffers and reagents should be vortexed before use, to ensure thorough mixing.
- Set a water bath, heating block or a programmable thermal cycler to 30°C.
- If a thermal cycler is used with a heated lid, the temperature of the lid should be set to 70°C.

## Procedure

1. Prepare sufficient Buffer D1 (denaturation buffer) and Buffer N1 (neutralization buffer) for the total number of whole genome amplification reactions (Tables 4–5).

   **Note**: The total volumes of Buffer D1 and Buffer N1 given in Tables 4–5 are sufficient for 12 reactions. If performing fewer reactions, store residual Buffer D1 and Buffer N1 at –20°C. Buffer D1 and Buffer N1 should not be stored longer than 3 months.

**Table 4. Preparation of Buffer D1**

| Component | Volume for 12 reactions |
|---|---|
| Reconstituted Buffer DLB* | 7 µl |
| Nuclease-free water | 25 µl |
| **Total volume** | **32 µl** |

* Reconstitution of Buffer DLB is described in "Things to do before starting".

**Table 5. Preparation of Buffer N1**

| Component | Volume for 12 reactions |
|---|---|
| Stop Solution | 9 µl |
| Nuclease-free water | 51 µl |
| **Total volume** | **60 µl** |

2. Place 2.5 µl template DNA into a microcentrifuge tube.

   The amount of template DNA should be >10 ng. A DNA control reaction can be set up using 10 ng (1 µl) control genomic DNA (e.g., REPLI-g Human Control Kit, cat. no. 150090). Adjust the volume by adding PBS sc (provided) to the starting volume of your sample.

3. Add 2.5 µl Buffer D1 to the DNA. Mix by vortexing and centrifuge briefly.
4. Incubate at room temperature for 3 min.
5. Add 5 µl Buffer N1. Mix by vortexing and centrifuge briefly. Store on ice.
6. Thaw REPLI-g sc DNA Polymerase on ice. Thaw all other components at room temperature, vortex, then centrifuge briefly.

   The REPLI-g sc Reaction Buffer may form a precipitate after thawing. The precipitate will dissolve by vortexing for 10 s.
7. Prepare a master mix according Table 6. Mix and centrifuge briefly.

   **IMPORTANT**: Add the master mix components in the order listed in Table 6. After the addition of water and REPLI-g sc Reaction Buffer, briefly vortex and centrifuge the mixture, before the addition of REPLI-g sc DNA Polymerase.

   **Note**: Scale up accordingly if performing several reactions at once by preparing a master mix sufficient for the total number of reactions.

   The master mix should be kept on ice and used immediately upon addition of REPLI-g sc DNA Polymerase.

**Table 6. Preparation of master mix***

| Component | Volume/reaction |
|---|---|
| $H_2O$ sc | 9 µl |
| REPLI-g sc Reaction Buffer | 29 µl |
| REPLI-g sc DNA Polymerase | 2 µl |
| **Total volume** | **40 µl** |

* To prepare a master mix for multiple reactions, scale up according to the number of reactions and add 10% more.

8. For each amplification reaction, add 40 µl master mix to 10 µl denatured DNA (from step 5).
9. Incubate at 30°C for 3 h.

   **Note**: Incubating the sample for 3 hours generates sufficient DNA for PCR-free library prep using this kit.

After incubation at 30°C, heat the water bath or heating block up to 65°C if the same water bath or heating block will be used in step 10.

**Note**: If a thermal cycler is used with a heated lid, the temperature of the lid should be set to 70°C.

10. Inactivate REPLI-g sc DNA Polymerase at 65°C for 3 min.
11. If not being used directly, store amplified DNA at 4°C for short-term storage or –20°C for long-term storage.

    DNA amplified using the REPLI-g Single Cell DNA Library Kit should be treated as genomic DNA and undergo minimal freeze-thaw cycles. We recommend storage of nucleic acids at a concentration of at least 100 ng/µl.
12. Amplified DNA can be directly used for the library construction or target-directed amplification and library construction.

    **Note**: To proceed with the library preparation, quantify the amplified DNA following the instructions in Appendix B (page 37). Optical density (OD) measurements overestimate REPLI-g amplified DNA and should not be used. Typical DNA yields are approximately 40 µg per 50 µl reaction.

# Protocol: DNA Fragmentation with Covaris - S220 Focused-ultrasonicator and Cleanup Using the GeneRead Size Selection Kit

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 7. 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 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

### Cleanup

1. Add 4 volumes of Buffer SB1 to 1 volume of fragmented DNA 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. Sheared purified DNA may be quantified by OD measurement using the 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 for 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 REPLI-g Single Cell DNA Library Kit.
- The following QIAGEN products are required also 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 8).

- See Table 8 for thermal cycling parameters.

**Table 8. 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 9, dispensing the reagents into a PCR tube of the well of a PCR plate.

   **Note**: The reaction mix should be prepared on ice.

**Table 9. 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 DNA.

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 10, adding the components to the PCR tube containing the end-repaired DNA from step 3.

**Table 10. 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 11, adding the components to the PCR tube containing DNA that has undergone end-repair and A-addition (step 6).

   **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 11. 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 the 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 9, 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.

    With 1 µg DNA input, sufficient amount of library should be generated for sequencing on Illumina platforms without further PCR amplification.
32. The purified library can be safely stored at –20°C until further applications or amplifications. LoBind tubes should be used to store the library.

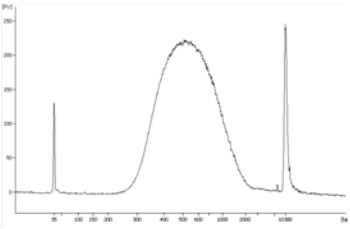


**Figure 2. Capillary electrophoresis device trace data showing the correct size distribution of library fragments and the absence of adapters or adapter-dimers.**

# 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 |
|---|---|
| **REPLI-g DNA amplification** | |
| **Reduced or no high-molecular-weight product in agarose gel in some samples but DNA yield in other samples is approximately 40 µg** | |
| a) Cells are not suitable for whole genome amplification | DNA within the cells is degraded (e.g., inappropriate storage of cells, use of fixed or apoptotic cells). |
| b) Reaction failed – possible inhibitor in the genomic DNA template | Clean up or dilute the purified genomic DNA and re-amplify. |
| c) Reaction temperature is too high | Check the incubator for correct reaction temperature (30°C) during the REPLI-g reaction. If a cycler with a heated lid is used, set temperature to 70°C. As a control, the REPLI-g reaction can be performed at a lower temperature (e.g., 25–28°C), which should give the appropriate yield. |
| d) Carryover of alcohol in isolated DNA sample | Residual alcohol in the DNA sample may reduce the yield of REPLI-g reactions. When using column-based purification procedures, ensure the duration of the drying step prior elution of DNA from the column is sufficient to evaporate residual ethanol. |

| | Comments and suggestions |
|---|---|
| **The negative (no-template) controls have DNA yields of up to 40 µg but no positive result in downstream assay (e.g., PCR)** | |
| DNA is generated during the REPLI-g reaction by random extension of primer-dimers | High-molecular-weight product can be generated by random extension of primer-dimers. This DNA will not affect the quality of actual samples or specific downstream genetic assays.<br>Decontaminate all laboratory equipment and take all necessary precautions to avoid contamination of reagents and samples with extraneous DNA.<br>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. |
| **Single cell protocol** | |
| **Reduced/no locus representation or allele dropout in downstream analysis, but DNA yield is approximately 40 µg** | |
| a) Cells are not suitable for whole genome amplification | DNA within the cells is degraded (e.g., inappropriate storage of cells, use of fixed or apoptotic cells). Cells were lost during procedure or insufficient mixing or lysis of cells occurred. Perform mixing by gently flicking the tube and avoid contact of pipet tips with the cell material. |
| b) DNA degraded after cell lysis | Perform cell lysis carefully and avoid vigorous vortexing. Do not store DNA after cell lysis. |
| c) Microdissected material does not contain the whole nucleus | When carrying out microdissection, ensure that the section thickness allows the capturing of the whole nucleus. |
| **Genome is not amplified at all, but DNA yield is up to 40 µg** | |
| Cells were not lysed | Additional cell envelope break-down is necessary for cells that have strong cell walls (e.g., plant cells and cells in dormant stages, such as spores and cysts). |
| **Genomic DNA protocol** | |
| **Reduced/no locus representation or allele dropout in downstream analysis, but DNA yield is approximately 40 µg** | |
| Genomic DNA template is degraded | Use intact genomic DNA template. Use a larger amount of genomic DNA. |
| **Library preparation protocol** | |
| **Low library yields** | |
| a) 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 |
|---|---|---|
| b) | Insufficient amount of starting DNA for direct sequencing without library amplification | Sheared DNA quantification is inaccurate. Typically, 500 ng of sheared genomic DNA generates enough Illumina-compatible libraries to use directly for sequencing without amplification. If the final library yield is not sufficient for the expected number of sequencing runs, a library amplification step can be performed following cleanup with the GeneRead Size Selection Kit (cat. no. 180514). |
| **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 | If performing library enrichment , if the fragment population shifts higher than expected after adapter ligation and PCR enrichment (e.g., more than the expected 120 bp shift), this can be a PCR artifact due to over-amplification of the DNA library. Make sure to use as few amplification cycles as possible (8–10) to avoid this effect. |
| c) | Incorrect library fragment size after adapter ligation | During library preparation, adapters of approximately 60 bp are ligated to both ends of the 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 product 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: Determination of DNA Concentration and Yield

## Quantification of DNA yield

A 50 µl REPLI-g reaction typically yields approximately 40 µg of DNA, allowing direct use of the amplified DNA in most downstream genotyping experiments and library preparation for sequencing. Depending on the quality of the input material, the resulting amount of DNA may be less (dead or apoptotic cells with fragmented or damaged DNA should not be used). However, library preparation requires a more accurate quantification of DNA, and it is important to utilize a DNA quantification method that is specific for double-stranded DNA, since REPLI-g Kit amplification products contain unused reaction primers. PicoGreen® reagent displays enhanced binding to double-stranded DNA and may be used, in conjunction with a fluorimeter, to quantify the double-stranded DNA product. For best results, the sample should be diluted with 2 volumes of water and thoroughly mixed prior to addition of PicoGreen. A protocol for the quantification of REPLI-g amplified DNA can be found in Appendix B, page 37.

## Quantification of locus representation

Locus representation for each sample can be quantified by real-time PCR. Contact QIAGEN Technical Services or visit our website at **www.qiagen.com** for a protocol.

# Appendix B: 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 DNA 1:100 when using the 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

B1. 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.

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

B3. Make 200 µl of 1.6, 0.8, 0.4, 0.2 and 0.1 µg/ml DNA standards by further diluting the 16 µg/ml genomic DNA with TE buffer.

B4. Transfer 20 µl of each DNA standard in duplicate into a 96-well plate labeled A (see figure below).

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

**96-well plate**

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

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

B5. Place 2 µl of each 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.

B6. Place 2 µl diluted REPLI-g DNA (from step B5) 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.

B7. Add 20 µl PicoGreen working solution (from step B1) 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.

B8. Centrifuge the 96-well plate briefly to collect residual liquid from the walls of the wells.

B9. 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**

B10. 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.

B11. 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.

B12. Multiply the value determined in step B11 by 1000 to show the concentration of undiluted sample DNA (since the sample DNA measured by PicoGreen fluorescence had been diluted 1 in 1000).

B13. To determine the total amount of DNA in your sample, multiply the concentration of undiluted sample DNA (µg/ml) (determined in step B12) by the reaction volume in milliliters (i.e., for a 50 µl reaction, multiply by 0.05).

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

The index sequences used in the GeneRead Adapter I Set A 12-plex (cat. no.180985) are listed in Table 12. Indices 1–12 correspond to the respective Illumina adapter indexes.

**Table 12. 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 D: Adapter Indices for the GeneRead Adapter I Set B 12-Plex

The index sequences used in the GeneRead Adapter I Set B 12-plex (cat no.180986) are listed in Table 13. Indices 13–27 correspond to the respective Illumina adapter indexes.

**Table 13. 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 E: Qualification of the REPLI-g WGA Reaction with qPCR

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

Each qPCR reaction should contain 10 ng of the gDNA amplified via WGA. Real-time PCR assays that recognize conservative gDNA regions are recommended. For example, QIAGEN's QuantiFast® Probe Assays, which detect the exon region of genes and therefore amplify gDNA, are recommended in combination with QuantiNova™ or QuantiFast mixes for such quality control assays.

For further information, please refer to the respective kit handbooks, which are available at **www.qiagen.com**.

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

PCR-based targeted enrichment can be performed using REPLI-g amplified DNA and the GeneRead DNAseq Targeted Panels V2. Proceed directly with dilution of REPLI-g amplified DNA as described in the *GeneRead DNAseq Targeted Panels V2 Handbook* (see Protocol: PCR Setup). Follow protocol "PCR Setup" in the *GeneRead DNAseq Targeted Panels V2 Handbook*, starting from step 1 (page 16) with DNA dilution

# Appendix G: Optional Amplification of Library DNA

This protocol is for optional, high-fidelity amplification of the DNA library. The GeneRead DNA I Amp Kit is used for an optional, high-fidelity library amplification step. 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 26).
- Thaw all reagents on ice.

## Procedure

G1. Prepare a reaction mix for library enrichment according to Table 14, dispensing the reagents into a PCR tube of 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 32, page 29) | Variable |
| RNase-free water | Variable |
| **Total reaction volume** | **50** |

G2. 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.

G3. Perform purification of the enriched library (see cleanup protocol on page 25) once over a column. Add 4 volumes of Buffer SB1 to 1 volume of PCR-enriched library. Usually 200 µl Buffer SB1 is added to 50 µl sample.

G4. 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).

G5. 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.

# Ordering Information

| Product | Contents | Cat. no. |
|---|---|---|
| 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 |
| 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 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 |
| **Related products** | | |
| **QIAGEN GeneRead Kits – for next-generation sequencing applications** | | |
| GeneRead Size Selection Kit (50) | For 50 reactions: Spin columns and buffers | 180514 |
| GeneRead DNAseq Targeted Panels V2 Kit | For targeted enrichment of a focused set of genes prior to NGS | Varies |

| 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**.

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.

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