June 2016

QIAseq FX Single Cell DNA Library Kit

For DNA library construction from single cells for Illumina® platforms

Sample to Insight

QIAGEN

Contents

Kit Contents

QIAseq FX Single Cell DNA Library Kit	**(24)**	**(96)**
Catalog no.	**180713**	**180715**
Number of preps	**24**	**96**
REPLI-g sc DNA Polymerase (blue lid)	48 µl	4 x 48 µl
REPLI-g sc Reaction Buffer (yellow lid)	700 µl	4 x 700 µl
Buffer DLB (clear lid)	1 tube	2 tubes
Stop Solution (red lid)	1.8 ml	1.8 ml
PBS sc 1x (clear lid)	1.5 ml	1.5 ml
DTT, 1 M (lilac lid)	1 ml	1 ml
H_2O sc	3 x 1.5 ml	8 x 1.5 ml
FX Enzyme Mix	1 tube	1 tube
FX Buffer, 10x	1 tube	1 tube
FX Enhancer	1 tube	1 tube
DNA Ligase	1 tube	1 tube
5x DNA Ligase Buffer	1 tube	2 tubes
Adapter Plate Illumina*	1x 24 plex	1x 96 plex
Quick Start Protocol	1	1

* For adapter sequences, refer to Appendix C, page32.

Storage

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

Intended Use

The QIAseq FX Single Cell 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 QIAseq FX 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 research areas – 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 QIAseq FX Single Cell DNA Library Kit applies an optimized protocol using QIAGEN's unique multiple displacement amplification (MDA) technology to amplify gDNA out of single cells. The amplified DNA is subsequently fragmented using the QIAGEN QIAseq FX technology which incorporates enzymatic DNA fragmentation into a streamlined, optimized protocol that does not require sample cleanup between fragmentation and adapter ligation, saving time and reducing material loss. Optimized enzyme and buffer compositions ensure high library yield. Streamlined library construction protocols also enable straightforward automation of library preps on different liquid-handling platforms.

With the QIAseq FX 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 only a few hours. The kit provides a time-saving, one-tube library preparation protocol that does not require extra DNA fragmentation and 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 2 hours and eliminating the library amplification step. Optimized enzyme and buffer compositions ensure superior yields of high-quality, NGS-ready libraries without the need of PCR enrichment.

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

QIAseq FX Single Cell DNA Library Kit employs high-fidelity MDA technology to provide accurate amplification of genomes with negligible sequence bias and minimal genomic dropouts. The QIAseq FX Single Cell DNA Library Kit contains an optimized Phi29 DNA polymerase formulation, which – together with its proprietary buffer formulation and an optimized protocol – 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 QIAseq FX Single Cell DNA Library Kit combines the advantages of REPLI-g Single Cell technology with the fast and fully enzymatic procedure of QIAseq FX. High-quality libraries ready for NGS are delivered without the need for any library enrichment, thereby 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 materials with a high percentage of mapped reads, uniform genome coverage, high sequence complexity and low error rates. It also outperforms PCR-based single cell library construction products from alternative suppliers.

Dual-barcoded, plate-format adapters are included with the QIAseq FX Single Cell DNA Library Kit (24) and QIAseq FX Single Cell DNA Library Kit (96). Each adapter well contains a single-use adapter consisting of a unique combination of two eight-nucleotide identification barcodes. By combining one of eight D5 barcodes and one of twelve D7 barcodes in each ready-to-use adapter, this kit supports up to 96-plexing prior to sequencing (see Appendix C for barcode IDs). By supplying adapters in a single-use plate format, the possibility of adapter cross-contamination, and the resulting misassignment of reads between different libraries, is eliminated.

The average product length of REPLI-g Single Cell (SC) amplified DNA using high-quality single cells or intact gDNA 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 QIAseq FX Single Cell DNA Library Kit reaction depending on incubation time are approximately 20-50 µg per 50 µl reaction. Only a part of this amplified gDNA is required for the library preparation, the remainder can be frozen and stored for future experiments and follow-up studies. The quality and number of the used cells will also affect the resulting amount of DNA (storage of cells for periods longer than 2–3 months will lead to lower yields of amplified DNA). For best amplification results, cells must be correctly collected and stored.

Principle and procedure

The QIAseq FX Single Cell DNA Library Kit provides a fast, fully enzymatic procedure for cell lysis, DNA amplification, amplified DNA fragmentation, and NGS library construction with no need for PCR-based library enrichment. The QIAseq FX Single Cell DNA Library Kit uses MDA technology to amplify complex genomic DNA. It includes a modified REPLI-g Amplification protocol using high-fidelity REPLI-g sc DNA Polymerase and an optimized Buffer formulation, along with gentle alkaline incubation, to ensure high DNA integrity and optimal amplification. It is specifically designed to provide high yields of uniformly amplified DNA from single cells and to maximize genome coverage, ensuring regions potentially containing sequence variants or features of interest are not missed.

The QIAseq FX Single Cell DNA Library Kit uses isothermal genome amplification, termed multiple displacement amplification, which involves the binding of random hexamers to denatured DNA. This procedure is followed by strand displacement synthesis at a constant temperature with an optimized form of the enzyme Phi29 polymerase – a polymerase that has exceptionally strong strand displacement properties. Additional priming events occur on each displaced strand that serve as a template, enabling generation of high yields of

amplified DNA. Phi29 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 polymerase easily solves secondary structures such as hairpin loops – thereby preventing slipping, stoppage and dissociation of the polymerase during amplification. This feature enables the generation of DNA fragments up to 100 kb without sequence bias.

Genomic DNA must be denatured before use in enzymatic amplification procedures. Denaturation of genomic DNA is often accomplished using harsh methods such as incubation at elevated temperatures (heat incubation) or increased pH (chemical alkaline incubation). The QIAseq FX 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 step 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 MDA 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 QIAseq FX Single Cell DNA Library Kit provides a simple and reliable method to efficiently generate DNA libraries in less than 3.5 hours. These libraries are suitable for sequencing on any Illumina NGS instrument. The kit provides a complete workflow for highly uniform amplification across the entire genome – with negligible sequence bias – followed by a fast, one-tube library construction including enzymatic fragmentation.

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 2 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 it can be used directly to generate sequencing libraries. For library construction, the samples consisting of long WGA-DNA strands are first enzymatically sheared into smaller fragments. The median fragment size is dependent on the desired application and sequencing read length, and can be adjusted by varying the QIAseq FX DNA fragmentation reaction conditions. The fragmented DNA is directly end-repaired and an 'A' is added to the 3' ends in the same tube following enzymatic shearing, making the DNA fragments ready for ligation. Following this step, platform-specific adapters are ligated to both ends of the DNA fragments. These adapters contain sequences essential for bridge amplification and sequencing on any Illumina sequencer.

The WGA procedure normally results in high yields of DNA so that library preparation can be performed with a high amount of input DNA, and so subsequent PCR-based library enrichment can be avoided. However, if library enrichment is required, an optional, high-fidelity amplification step using the GeneRead DNA I Amp Kit can also be performed that provides highly accurate amplification of library DNA with low error rates and minimum bias.

Dual-barcoded, plate-format adapters are included with the QIAseq FX Single Cell DNA Library Kits. Each adapter well contains a single-use adapter consisting of a unique combination of two eight-nucleotide identification barcodes. For guidelines on multiplexing low numbers of samples, please refer to guidelines provided by your sequencing platform provider. By combining one of eight D5 barcodes and one of twelve D7 barcodes in each ready-to-use adapter, this kit supports up to 96-plexing prior to sequencing (see Appendix C for barcode IDs). Following library construction, the reaction cleanup and removal of adapter dimers can be achieved either by: 1) using the GeneRead™ Size Selection Kit (cat. no. 180514) – based on a simple, easy and precise silica column-based method, or 2) by using Agencourt® AMPure® XP Beads (Beckman Coulter, cat. no. A63880).

Description of protocols

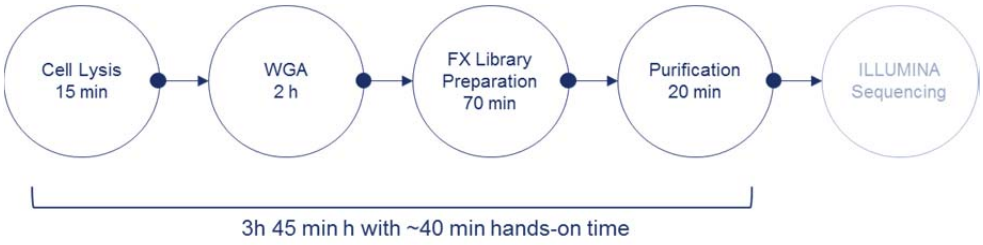
This handbook contains protocols for the amplification of DNA from single cells – such as eukaryotic cells or bacteria – for the enzymatic fragmentation of amplified DNA, and for subsequent library preparation. The genome amplification protocol is described in "Whole Genome Amplification" (page 15).

The PCR-free library preparation procedure that includes end-repair, A-addition, adapter ligation and cleanup and removal of adapters and adapter dimers is described in the protocol "Enzymatic fragmentation and library preparation" (page 19). The prepared library can be quantified with the QIAseq Library Quant system and is optimized for use on Illumina sequencing platforms.

Compatible sequencing platforms

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

Workflow chart



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.

- Agencourt AMPure XP Beads (cat. no. A63880, A63881) for bead-based size selection OR the GeneRead Size Selection Kit (QIAGEN, cat. no. 180514) for column-based size selection
- Microcentrifuge tubes
- 100% ethanol (ACS grade)
- Nuclease-free water
- Buffer EB (QIAGEN, cat. no. 19086) or 10 mM Tris-HCl pH 8.0
- PCR tubes or plates
- LoBind tubes (e.g., from Axygene or Eppendorf)
- Pipettes and pipette tips
- Magnetic racks for magnetic beads separation (e.g., Thermo Fisher Scientific/Life Technologies, DynaMag™-2 Magnet, cat. no. 12321D)
- Heating block
- Thermocycler
- Microcentrifuge
- Vortexer
- Ice
- QIAxcel, Agilent® 2100 Bioanalyzer or similar to evaluate the DNA fragmentation profile (optional) or other comparable Capillary electrophoresis device or method to assess the quality of DNA library
- QIAseq Library Quant Assay Kit (product number 333314)

- GeneRead DNA I Amp Kit (100) (optional)
- Quant-iT™ PicoGreen® dsDNA Assay Kit (optional)

Important Notes

Cells, DNA preparation and quality control

High-quality DNA is essential for obtaining good amplification and sequencing results. The most important prerequisite for any DNA sequence analysis experiment is consistent, high-quality DNA from every experimental sample. Therefore, cell handling and DNA isolation procedures are critical to the success of the experiment. Low integrity DNA decreases the efficiency of amplification and the quality of the generated libraries.

General precautions

- Use good laboratory practices to minimize cross-contamination of nucleic acid products.
- Always use PCR tubes, microcentrifuge tubes and pipette tips that are certified sterile, DNase- and RNase-free.
- Before starting, wipe down work area and pipettes with an RNase and DNA cleaning product such as RNase Away® (Molecular BioProducts, Inc., San Diego, CA) or LookOut® DNA Erase (Sigma-Aldrich).
- For consistent genome amplification, library construction and amplification ensure that the thermal cycler used in this protocol is in good working order and has been calibrated according to the manufacturer's specifications.
- Read the entire protocol before beginning. Take note of stopping points where samples can be frozen at –20°C and plan your workflow accordingly.
- Enzyme-based DNA fragmentation is sensitive to many factors, such as reaction temperature, time and setup conditions, as well as the quality of the input DNA.

Recommended library quantification method

QIAGEN's QIAseq Library Quant Assay Kit (product number 333314) – which contains laboratory-verified forward and reverse primers together with a DNA standard – is highly recommended for accurate quantification of the prepared library. Alternatively Qubit may be used but it is not recommended for non-enriched libraries.

Protocol: Whole Genome Amplification

This protocol is for the amplification of genomic DNA from 1–1000 intact cells, or >6 pg purified gDNA.

Important points before starting

- This protocol is optimized for single cell material from all species, including: vertebrates, bacteria (gram positive and gram negative, flow-sorted cells, tissue culture cells, micromanipulated cells and laser-microdissected cells from frozen sections. The protocol cannot be used with cells fixed 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 QIAseq FX Single Cell DNA Library Kit.
- If starting with purified DNA and not intact cells, ensure that the DNA is of high quality (high molecular weight DNA and free of inhibitors, such as solvents and detergents) and suspended in TE. If working with eukaryotic DNA, we recommend using 1–10 ng; for bacterial gDNA, we recommend using 6–100 pg.
- Avoid DNA contamination of reagents by using separate laboratory equipment (e.g., pipettes, filter pipette tips, reaction vials, etc.). Set up the REPLI-g Single Cell reaction in a location free of DNA.
- 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 20 µg may be present in negative (no template) controls, as DNA is generated during the REPLI-g Single Cell reaction by random extension of primer dimers – generating 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 programmable thermal cycler, or a heating block, 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. Thaw H2O sc, DTT and RepliG reaction buffer 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.
2. 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," page 16.

3. Prepare sufficient 1:10 dilution DTT in H2O sc for the total number of reactions (Table 3).

Table 3. Preparation of DTT 1:10 dilution for 12 reactions

Component	Volume for 12 reactions*
DTT, 1 M	3 µl
H2O sc	27 µl
Total volume	**30 µl**

* Add 10%.

4. Place 4 µl cell material (supplied with PBS) or gDNA into each well of a 96-well plate or microcetrifuge tube. If using less than 4 µl of starting material, add PBS sc to bring the volume up to 4 µl.

   Note: During pipetting, avoid contact of pipette tips and cell material.

5. 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 pipette tips with cell material.

6. Incubate for cell preparations 10 min at 65°C and gDNA preparations 3 min at room temperature, then cool down to 4°C.

   Note: If a thermal cycler is used, the temperature of the heating lid should be set at 70°C to avoid evaporation. Alternatively, incubation can be performed in a heating block.

7. Add 3 µl Stop Solution. Mix carefully by flicking the tube and centrifuge briefly. Store on ice.

8. Thaw REPLI-g sc DNA Polymerase on ice. Tip gently on the tube to mix and centrifuge briefly.

9. Prepare a master mix according Table 4. Mix and centrifuge briefly.

   IMPORTANT: Add the master mix components in the order listed in Table 4. After the addition of water, REPLI-g sc Reaction Buffer and DTT, briefly vortex and centrifuge the mixture before adding REPLI-g sc DNA Polymerase. After adding REPLI-g sc DNA Polymerase, flick carefully and centrifuge briefly. The master mix should be kept on ice and used immediately upon 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.

Table 4. Preparation of master mix*

Component	Volume/reaction
H_2O sc	6.5 µl
REPLI-g sc Reaction Buffer	29 µl
DTT (1:10)	2.5 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%.

10. For each amplification reaction, add 40 µl master mix to 10 µl denatured DNA (from step 7). Mix by flicking the tube and centrifuge briefly.
11. Incubate at 30°C for 2 h.

    Note: Incubating the sample for 2 hours generates sufficient DNA for PCR-free library prep using this kit. Incubation of 1 hour is also possible but leads to reduced yields and may not be appropriate for all types of cells.

    After incubation at 30°C, heat the heating block up to 65°C if the same heating block will be used in step 11.

    Note: If a thermal cycler is used with a heated lid, the temperature of the lid should be set to 70°C.
12. Inactivate REPLI-g sc DNA Polymerase at 65°C for 3 min.
13. If not being used directly, store amplified DNA either at 4°C for short-term storage, or at –20°C for long-term storage.

    DNA amplified using the QIAseq FX 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.

14. Amplified DNA can be directly used for library construction. Additionally, extra amplified DNA can be used for PCR analysis or for targeted resequencing.

    Note: To proceed with library preparation, you may quantify the amplified DNA following the instructions in Appendix B (page 29). 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: Enzymatic fragmentation and Library Preparation Using QIAseq FX Single Cell Amplified DNA

This protocol describes the FX reaction for single-tube fragmentation, end-repair, A-addition and size selection of QIAseq FX Single Cell 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 the QIAseq FX Single Cell DNA Library Kit.
- The following QIAGEN products are required also for this protocol: for reaction cleanup and removal of adapter dimers following library construction, either Agencourt AMPure XP Beads (cat. no. A63880, A63881) or the GeneRead Size Selection Kit (cat. no. 180514) are required and should be ordered separately.
- The amplified DNA should be diluted in H_2O before starting.

Things to do before starting

- Program thermal cyclers. For increased speed and convenience, all incubation steps of the protocol can be preprogrammed and saved on a thermal cycler in advance (Table 5).

  Refer to Table 5 to determine the time and protocol required to fragment input DNA to the desired size.

- Prepare fresh 80% ethanol.

Prepare Buffer 10 mM Tris-HCl, pH 8.0

Procedure: Enzymatic Fragmentation and Library Preparation

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

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

   Program a thermocycler according to Table 5 and start the program. If possible, set the temperature of the heated lid to ~70°C.

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

Table 5. Amplified gDNA fragmentation reaction conditions

Step	Temperature	Incubation time (Fragment size 300 bp)	Incubation time (Fragment size 500 bp)
1	4°C	1 min	1 min
2	32°C	15 min*	10 min
3	65°C	30 min	30 min
4	4°C	Hold	Hold

* The insert size of the completed libraries is determined by the duration of step 2. Using 200–1000 ng input DNA and the FX enhancer, 15 min fragmentation time produces a fragment distribution of around 300 bp, 500 bp if the enhancer is not used. Fragmentation time can be increased or decreased to generate shorter or longer inserts, respectively. Use a thermocycler with a heated lid.

3. Dilute amplified gDNA 1:10 in H2O sc. This step should give 200–1000 ng total amplified DNA in 10 µl H2O sc (20–100 ng/µl). If you have quantified the DNA obtained from the WGA, do not exceed 5 µl undiluted DNA input in the FX reaction. Pipette 10 µl of the diluted DNA in PCR tubes or stripes, and place them on ice or on a cooling block.
4. Prepare the FX Reaction Mix on ice according to Table 6 if the desired fragment size of library is 300 bp, or according to Table 7 for library fragment size of 500 bp – and mix by pipetting. Add the components of the FX Reaction Mix in the same order as stated in the table. Before adding the FX Enzyme Mix, pipette up and down the Buffer Mix. You can scale up the FX Reaction Mix according to the number of samples required.

Table 6. FX Reaction Setup for inserting fragment size of 300 bp

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

* Mix by pipetting and keep on ice.

Table 7. FX Reaction Setup for inserting fragment size of 500bp

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

* Mix by pipetting and keep on ice.

5. Add 40 µl FX Reaction Mix to each diluted amplified gDNA sample on ice and gently vortex to mix.

6. Briefly spin down the PCR plate/tubes, immediately transfer to the pre-chilled thermocycler (4°C) and resume the program. Once the fragmentation program is complete, transfer samples to ice.
7. Immediately proceed with adapter ligation as described in the next protocol.

Adapter ligation

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

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

11. Prepare the Ligation master mix (per DNA sample) in a separate tube on ice according to Table 8. Mix well by gently vortexing at low rpm.

Table 8. Ligation master mix (per sample)

Component	Volume/reaction*
DNA Ligase Buffer, 5x	20 µl
H2O sc	15 µl
DNA Ligase	10 µl
Total reaction volume	**45 µl**

* Mix by pipetting and keep on ice.

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

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

13. Proceed immediately to adapter ligation cleanup (steps 14–23) using 0.8x (80 µl) Agencourt AMPure XP beads.

14. Add 80 µl resuspended Agencourt AMPure XP beads slurry to each ligated sample and mix well by pipetting or gently vortexing.
15. Incubate the mixture for 5 min at room temperature.
16. Pellet the beads on a magnetic stand for 2 min and carefully discard the supernatant.
17. Wash the beads by adding 200 µl fresh 80% ethanol to each pellet. Pellet the beads on the magnetic stand for 2 min, then carefully discard the supernatant.
18. Repeat the wash step 17 once for a total of 2 ethanol washes.
19. Incubate on the magnetic stand for 5–10 min or until the beads are dry. Avoid over-drying, which may result in lower DNA recovery. Remove from the magnetic stand.
20. Elute by resuspending in 52.5 µl 10 mM Tris-HCl, pH 8.0. Pellet beads on the magnetic stand. Carefully transfer 50 µl supernatant to a new PCR plate.
21. Perform a second purification. Add 50 µl of resuspended 1x Agencourt AMPure XP beads to each sample and mix.
22. Follow steps 15–19.
23. Elute by resuspending in 26 µl 10 mM Tris-HCl, pH 8.0. Pellet the beads on the magnetic stand. Carefully transfer 23.5 µl of supernatant into a new PCR plate. Store purified libraries at –20°C until ready for sequencing.
24. Assess the quality of the library using a capillary electrophoresis device or comparable method. Check for the correct size distribution (Figure 1) of library fragments and for the absence of adapters or adapter dimers.

    Note: The median size of the DNA fragment should be shifted by the size of the adapters that were ligated to the library fragments (~120 bp).

    Note: The median fragment size can be used for subsequent qPCR-based quantification methods.

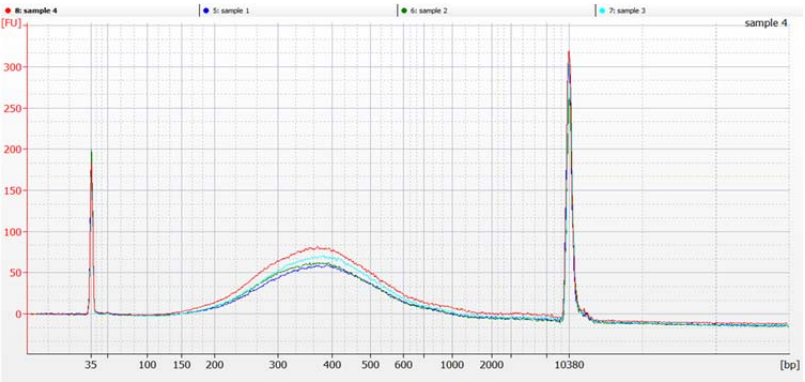


Figure 1. Capillary electrophoresis device trace of generated libraries. Capillary electrophoresis device trace data showing the correct size distribution of 4 replicate completed libraries and the absence of adapters or adapter dimers.

25. Quantify the library using the QIAseq Library Quant Assay Kit (product number 333314, not provided by this kit), or other comparable method.

    Note: Library quantitation with qPCR is strongly recommended in order to ensure accurate library dilution and flow-cell loading. Inaccurate library quantitation may result in under- or over-clustering the flowcell. Capillary electrophoresis or Qubit® methods can overestimate library quantity since they cannot distinguish DNA fragments with and without adapters. However if Qubit® quantification is required, we recommend amplification of the library first (see Appendix D, page 37) prior Qubit quantification.

    With 200 ng – 1 µg WGA DNA input, sufficient library should be generated for clustering on Illumina platforms without further PCR amplification (Figure 2).

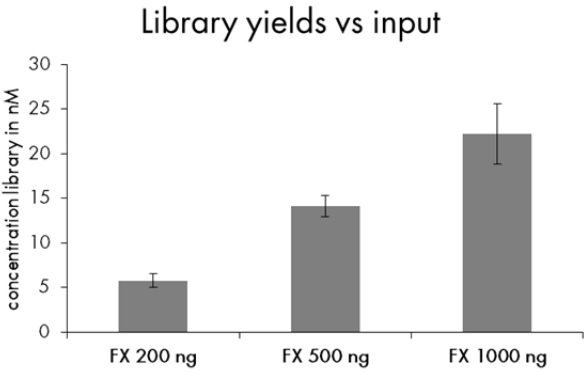


Figure 2: Library yields vs input of WGA DNA. Plotted are means of triplicate reactions with SD.

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

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 yields and 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). If plant or fungal cells were used, lysis may not be complete due to the presence of cell walls.
b) gDNA not suitable for whole genome amplification	Purified gDNA is degraded (e.g., inappropriate storage, long storage).
c) Reaction failed – possible inhibitor in the genomic DNA template	Clean up or dilute the purified genomic DNA and reamplify.
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 that the duration of the drying step prior to elution of DNA from the column is sufficient to evaporate residual ethanol.
e) Reaction time is too short	2h is sufficient to amplify gDNA from bacterial and eukaryotic cells. While sufficient yield is possible with shorter incubation in some experimental systems, this may not be appropriate for all cell types and should be confirmed experimentally.
Reduced yields	

		Comments and suggestions
a)	Reaction temperature is too high	Check the incubator for correct reaction temperature (30°C) during the whole genome amplification reaction. If cycler with heated lid is used, set temperature to 70°C. As a control, the whole genome amplification reaction can be performed at a lower temperature (e.g., 25–28°C), which should give the appropriate yield.
The negative (no template) controls have DNA yields of up to 20 µg but no positive result in downstream assay (e.g., PCR)		
a)	DNA is generated during the whole genome amplification reaction by random extension of primer-dimers or amplification of contaminating DNA	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. Decontaminate all laboratory equipment and take all necessary precautions to avoid contamination of reagents and samples with extraneous DNA. If possible, work in a laminar flow hood. Use sterile equipment and barrier pipette tips only, and keep amplification chemistry and DNA templates in separate storage locations.
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 of lysis of cells occurred. Perform mixing by gently flicking the tube and avoid contact of pipette tips with the cell material.
b)	DNA degraded after cell lysis	Perform cell lysis carefully according to the protocol and avoid vigorous vortexing. Do not store DNA after cell lysis.
c)	Genomic DNA template is degraded	Use intact genomic DNA template and avoid repeated pipetting of DNA. Use a larger amount of genomic DNA.
d)	Microdissected material does not contain the whole nucleus	When carrying out microdissection, ensure that the section thickness allows the capturing of the whole nucleus and that the nucleus is not damaged
Genome is not amplified at all, but DNA yield is up to 40 µg		
a)	Cells were not lysed. DNA is generated during the whole genome amplification reaction by random extension of primer-dimers	Additional cell envelope breakdown is necessary for cells that have strong cell walls (e.g., plant cells and cells in dormant stages, such as spores and cysts).
b)	Sample is contaminated	Contaminating DNA may out compete the amplification of single cells genome.
A higher than expected proportion of reads map to the mitochondrial genome		

		Comments and suggestions
a)	Cell lysis was incomplete, exposing mtDNA but leaving the nucleus partially intact	During cell lysis, ensure the lysis buffer and cell are mixed gently but thoroughly.

Using cells isolated with laser-capture microdissection, coverage of a particular chromosome is absent, or a complete loss of heterozygosity is observed

a)	Chromosomes were damaged during laser capture	During microdissection, take extra care not to damage the nucleus.

Library preparation protocol

Low library yields

a)	WGA yields were lower than expected	Quantify the yield of WGA using PicoGreen® Reagent. Typically, 100 ng of WGA DNA generates enough Illumina-compatible library 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 the adapter ligation step.
b)	Suboptimal reaction conditions due to low DNA quality	Follow the protocol recommendation; do not exceed 10 µl DNA input in the FX reaction.

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. Agencourt AMPure Beads or the GeneRead Size Selection Kit (cat. no. 180514) efficiently removes adapter dimers as well as free adapter molecules.
b)	Presence of larger library fragments after library enrichment	If performing library enrichment and the fragment population shifts higher than expected after adapter ligation and PCR enrichment (e.g., more than the expected 120 bp shift) –a PCR artifact may be the cause, due to over-amplification of the DNA library. Make sure to use as few amplification cycles as possible (8–10) to avoid this effect or avoid PCR enrichment completely.

		Comments and suggestions
c)	Incorrect library fragment size after adapter ligation	During library preparation, adapters of approximately 60 bp are ligated to both ends of the DNA library fragments. This ligation should be reflected on a capillary electrophoresis device by a shift in size of all library fragments of 120 bp. If using library adapters from other suppliers, please refer to the size information given in the respective documentation. The fragment distribution of non- amplified libraries may differ from the distribution of amplified libraries. 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.
d)	Mean fragment size is smaller than expected	The temperature of the fragmentation reaction is higher than 32°C, check for the correct temperature and calibrate the thermocycler if necessary. Alternatively, to increase fragment size reduce incubation time to 10-12min.

Appendix A: Determination of Concentration and Quality of Amplified DNA

Quantification of DNA yield

A 50 µl QIAseq WGA 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). For a more accurate quantification of the amplified DNA, it is important to utilize a DNA quantification method that is specific for double-stranded DNA, since amplification products contain unused reaction primers. Quant-iT™ PicoGreen® dsDNA reagent displays enhanced binding to double-stranded DNA and may be used in conjunction with a fluorometer, to quantify the double-stranded DNA product. A protocol for the quantification of QIAseq FX Single Cell amplified DNA can be found in Appendix B, page 29.

Quantification of locus representation

Locus representation for each sample can be quantified by real-time PCR.

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 5-10 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 exon region of the genes and therefore

amplify gDNA, in combination with QuantiNova™ or QuantiFast mixes are recommended for such quality control assays.

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

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

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

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

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

Equipment and reagents to be supplied by user

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

Procedure

B1. Make a 1:200 dilution of PicoGreen stock solution in TE buffer. Each quantification reaction requires 50 µl. Depending on the final volume, use a 2 ml microcentrifuge tube

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

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

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

B2. Prepare a 16 µg/ml stock solution of 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 50 µl of each DNA standard in duplicate into a 96-well plate labeled A (figure below).

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

96-well plate

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

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

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

B6. Place 5 µl diluted QIAseq FX Single Cell amplified DNA (from step B5) into an unused well of 96-well plate A and add 45 µl TE buffer to make a 1:1000 dilution.

The 1:100 dilutions from step B5 can be stored at –20°C and used for future downstream sample analysis.

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

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

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

B10. Measure the sample fluorescence using a fluorescence microplate reader and standard fluorescence filters (excitation approximately 480 nm; emission approximately 520 nm).

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

Calculation of DNA concentration and yield

B11. Generate a standard curve by plotting the concentration (µg/ml) of DNA standards (x-axis) against the fluorescence reading generated by the microplate reader (y-axis). Plot an average of the fluorescence recorded for each DNA standard of the same concentration.

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

B13. 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 1:1000).

B14. 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 Barcodes for the QIAseq FX Libraries

The barcode sequences used in the QIAseq FX Single Cell DNA Library Kit 96-plex adapter and 24-plex adapter plate are listed in Table 9. Indices 501–508 and 701–712 correspond to the respective Illumina adapter barcodes. The layouts of the 96-plex and 24-plex single use adapter plate are displayed in Figure 3A and Figure 3B.

Table 9. Adapter barcodes used in the QIAseq FX DNA Library Kit 96-plex Adapter Plate

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

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

Figure 3A. QIAseq FX 96-plex Adapter layout.

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

Figure 3B. QIAseq FX 24-plex Adapter Plate layout.

Appendix D: Amplification of Library DNA

PCR-based library amplification is not normally required for the QIAseq FX Single Cell DNA Library kit, since the WGA reaction typically generates more than sufficient material for PCR-free library construction. However, if insufficient library is available from precious samples, libraries may be amplified using the GeneRead DNA I Amp Kit (cat no. 180455). The proprietary HiFi PCR Master Mix can evenly amplify DNA regions with vastly different GC content, minimizing sequencing bias caused by PCR.

Things to do before starting

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

Procedure

1. Prepare a reaction mix according to Table 10.

Table 10. Reaction mix for library enrichment

Component	Volume/reaction (µl)
HiFi PCR Master Mix, 2x	25
Primer Mix (10 µM each)	1.5
Library DNA (from step 26, page 24)	Variable
RNase-free water	Variable
Total reaction volume	**50**

2. Program a thermocycler according to Table 11.

Table 11. Thermal cycling parameters

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

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

3. Perform size selection as described in the GeneRead Size Selection Handbook (Protocol: GeneRead Size selection of sheared DNA in common elution buffers, page 11).
4. Assess the quality of the library using a capillary electrophoresis device or other comparable method. Check for the correct size distribution (Figure 4; blue line) of library fragments, and for the absence of adapters or adapter dimers. Note: The median fragment size can be used for subsequent qPCR-based quantification methods.

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

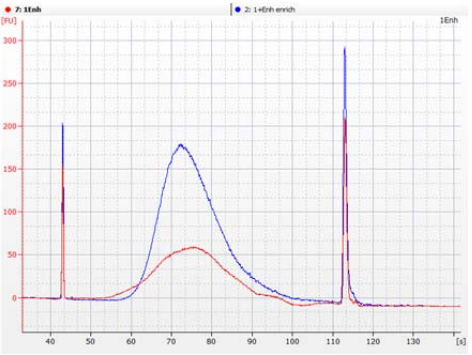


Figure 4: Capillary electrophoresis device trace of generated libraries. Capillary electrophoresis device trace data showing the correct size distribution of completed libraries and the absence of adapters or adapter dimers. Red line: library without/before enrichment. Blue line: same library after PCR enrichment.

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

Appendix E: Multiplex PCR-Based Targeted Enrichment Using QIAseq FX Single Cell Amplified DNA and Library Construction for Sequencing on Illumina Platforms

For follow-up studies using targeted resequencing, PCR-based targeted enrichment can be performed using the stored QIAseq FX Single Cell amplified DNA and the QIAseq 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. Libraries can be prepared from the resulting PCR amplicons using the QIAseq 1-Step Amplicon Library Kit (cat. no 180415).

Appendix F: Manual 96-well protocol QIAseq FX Single Cell library generation

This procedure has been adapted from the single tube protocol. It is suitable for generation of PCR-free libraries from single cells or from low amounts of purified gDNA in 96-well format using the QIAseq FX Single Cell DNA library kit.

Equipment and reagents to be supplied by user

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

Protocol: Whole Genome Amplification

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.

- Thaw all buffer and reagents except the RepliG sc Polymerase. All buffers and reagents should be vortexed before use to ensure thorough mixing.

- Set a programmable thermal cycler, or a heating block 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. Thaw H2O sc, DTT and RepliG reaction buffer 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.
2. Prepare sufficient Buffer D2 (denaturation buffer) for the total number of whole genome amplification reactions (Table 12).

   Note: The total volume of Buffer D2 given in Table 12 is sufficient for 96 reactions. If performing fewer reactions, store residual Buffer D2 at –20°C. Buffer D2 should not be stored longer than 3 months.

Table 12. Preparation of Buffer D2 for 96 reactions

Component	Volume for 96 reactions
DTT, 1 M	24 µl
Buffer DLB (reconstituted)*	264 µl
Total volume	**288 µl**

* Reconstitution of Buffer DLB is described in "Things to do before starting," page 16. Add 10%.

3. Prepare sufficient 1:10 dilution DTT in H2O sc for the total number of 96 reactions (Table 13).

Table 13. Preparation of DTT 1:10 dilution for 96 reactions

Component	Volume for 96 reactions*
DTT, 1 M	24 µl
H2O sc	216 µl
Total volume	**240 µl**

* Add 10%.

4. Place 4 µl cell material (supplied with PBS) or gDNA into each well of the 96-well plate. If using less than 4 µl of starting material, add PBS sc to bring the volume up to 4 µl.

   Note: During pipetting, avoid contact of pipette tips and cell material.

5. Add 3 µl Buffer D2 into each well and then seal the plate using a tape sheet. Centrifuge the plate briefly at 1000 rpm to ensure that the cell material and Buffer D2 are collected at the bottom of the wells.

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

   Ensure that after centrifugation the cell material and Buffer D2 are collected at the bottom of the wells.

7. Incubate cell preparations10 min at 65°C and gDNA preparations 3 min at room temperature, then cool down at 4°C.

   Note: If a thermal cycler is used, the temperature of the heating lid should be set at 70°C to avoid evaporation. Alternatively, incubation can be performed in a water bath.

8. Briefly centrifuge at 1000 rpm to ensure that cell material and Buffer D2 are collected at the bottom of the well.

9. Remove the tape sheet and add 3 µl Stop Solution. Seal the plate, centrifuge briefly, and store on ice.

   Ensure that after centrifugation the lysed cell material and Stop Solution are collected at the bottom of the wells.

10. Thaw REPLI-g sc DNA Polymerase on ice. Tip gently on the tube to mix and centrifuge briefly.

11. Prepare a master mix according Table 14. Mix and centrifuge briefly.

    IMPORTANT: Add the master mix components in the order listed in Table 14. After the addition of water, REPLI-g sc Reaction Buffer and DTT, briefly vortex and centrifuge the mixture before adding REPLI-g sc DNA Polymerase. After adding REPLI-g sc DNA

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

Table 14. Preparation of master mix*

Component	Volume/96 reactions*
H_2O sc	624 µl
REPLI-g sc Reaction Buffer	2784 µl
DTT (1:10)	240 µl
REPLI-g sc DNA Polymerase	192 µl
Total volume	**3840 µl**

* Add 10%.

12. For each amplification reaction, add 40 µl master mix to 10 µl denatured DNA (from step 7). Seal the plate with a tape sheet and centrifuge briefly at 1000 rpm.
13. Mix by a short vortexing step and centrifuge again briefly.

    Ensure that after centrifugation the lysed cell material and REPLI-g sc master mix are collected at the bottom of the wells.
14. Incubate at 30°C for 2 h.

    Note: Incubating the sample for 2 hours generates sufficient DNA for PCR-free library prep using this kit. Incubation of 1 hour leads to reduced yields and has to be verified for the individual cell types.

    After incubation at 30°C, heat the heating block up to 65°C if the same heating block will be used in step 11.

    Note: If a thermal cycler is used with a heated lid, the temperature of the lid should be set to 70°C.
15. Inactivate REPLI-g sc DNA Polymerase at 65°C for 3 min.
16. 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 QIAseq FX 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.

17. Amplified DNA can be directly used for the library construction or target-directed amplification and library construction.

    Note: To proceed with library preparation, you may quantify the amplified DNA following the instructions in Appendix B (page 29). 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: Enzymatic Fragmentation and Library Preparation Using QIAseq FX SC Amplified DNA

This protocol describes the FX reaction for single-tube fragmentation, end-repair, A-addition and size selection of QIASeq FX Single Cell amplified DNA for: 1) the preparation of 96 libraries that are ready for quantification, and 2) use in next-generation sequencing on instruments from Illumina.

Important points before starting

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

Things to do before starting

- Program thermal cyclers. For increased speed and convenience, all incubation steps of the protocol can be preprogrammed and saved on a thermal cycler in advance (Table 15).

Refer to Table 15 to determine the time and protocol required to fragment input DNA to the desired size.

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

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

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

    Program a thermocycler according to Table 15 and start the program. If possible, set the temperature of the heated lid to ~70°C.

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

Table 15. Amplified gDNA fragmentation reaction conditions

Step	Temperature	Incubation time (Fragment size 300 bp)	Incubation time (Fragment size 500 bp)
1	4°C	1 min	1 min
2	32°C	15 min*	10 min
3	65°C	30 min	30 min
4	4°C	Hold	Hold

* The insert size of the completed libraries is determined by the duration of step 2. Using 200–1000 ng input DNA, 15 min fragmentation time produces a fragment distribution of around 300. Use a thermocycler with a heated lid.

20. Dilute amplified gDNA 1:10 in H2O sc. This should give 200–1000 ng total amplified DNA in 10 µl H2O sc (20–100 ng/µl). If you have quantified the DNA obtained from the WGA, do not exceed 5 µl undiluted DNA input in the FX reaction. Pipette 10 µl of the diluted DNA into each well of the 96-well plate, and place them on ice or a cooling block.

21. Prepare the FX Reaction Mix on ice according to Table 16 if the desired library fragment size is 300 bp – or according to Table 17 for a library fragment size of 500 bp – and mix by pipetting. Add the components of the FX Reaction Mix in the same order as stated in the table. Before adding the FX Enzyme Mix, pipette the Buffer Mix up and down. You can upscale the FX Reaction Mix according to the number of samples processed.

Table 16. FX Reaction Setup for inserting fragment sizes of 300 bp

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

*Add 10%,mix by pipetting and keep on ice.

Table 17. FX Reaction Setup for inserting fragment sizes of 500 bp

Component	Volume/96 reactions*
FX Buffer, 10x	480 µl
H2O sc	2400 µl
FX Enzyme Mix	960 µl
Total reaction volume	**3840 µl**

* Mix by pipetting and keep on ice.

22. Add 40 µl FX Reaction Mix to each diluted amplified gDNA sample on cooling block using a repeater pipette by pipetting on the upper inner walls of the wells.
23. Seal the plate with a tape sheet and centrifuge briefly at 1000 rpm.
24. Mix by short vortexing.
25. Briefly spin down the PCR plate again, immediately transfer to the pre-chilled thermocycler (4°C) and resume the program. Once the fragmentation program is complete, transfer plate to ice.

26. Immediately proceed with adapter ligation as described in the next protocol.

Adapter ligation

27. Equilibrate Agencourt AMPure XP beads to room temperature for 20–30 min before use.
28. Vortex and spin down the adapter plate. Remove the protective adapter plate lid, carefully pierce the foil seal and transfer 5 µl from one DNA adapter well to each 50 µl sample from the previous protocol using a multichannel pipette. Ensure the right orientation of both adapter and sample plates and track the barcodes used for each sample.

    IMPORTANT: Only one single adapter should be used per ligation reaction. If adapters from another supplier are used, follow the manufacturer's instructions.
29. Prepare the Ligation master mix on ice according to Table 18. Mix well by vortexing at low rpm.

Table 18. Ligation master mix

Component	Volume/96 reactions*
DNA Ligase Buffer, 5x	1920 µl
H2O sc	1440 µl
DNA Ligase	960 µl
Total reaction volume	**4320 µl**

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

    IMPORTANT: Do not use a thermocycler with a heated lid.
34. Proceed immediately to adapter ligation cleanup (steps 35–47) using 0.8x (80 µl) Agencourt AMPure XP beads.

35. Add 80 µl resuspended Agencourt AMPure XP beads slurry to each ligated sample using a repeater pipette and by pipetting beads on the upper side of the well; seal the plate and mix well by vortexing.
36. Incubate the mixture for 5 min at room temperature.
37. Pellet the beads on a magnetic stand for 2 min and carefully discard the supernatant.
38. Wash the beads by adding 200 µl fresh 80% ethanol to each pellet on the magnetic rack, then carefully discard the supernatant.
39. Repeat the wash step 38 once for a total of 2 ethanol washes.
40. Incubate on the magnetic rack for 5–10 min or until the beads are dry. Avoid over-drying, which may result in lower DNA recovery. Remove from the magnetic stand.
41. Elute by resuspending in 52.5 µl 10 mM Tris-HCl, pH 8.0. Pellet beads on the magnetic stand. Carefully transfer 50 µl supernatant to a new PCR plate.
42. Perform a second purification. Add 50 µl of resuspended 1x Agencourt AMPure XP beads to each sample; seal the plate and mix.
43. Follow steps 36–40.
44. Elute by resuspending in 26 µl 10 mM Tris-HCl, pH 8.0. Pellet the beads on the magnetic rack. Carefully transfer 23.5 µl of supernatant into a new PCR plate. Store purified libraries at –20°C until ready for sequencing.
45. Assess the quality of the libraries using a capillary electrophoresis device or comparable method. Check for the correct size distribution (Figure 1) of library fragments, and for the absence of adapters or adapter dimers.

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

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

Note: qPCR method is strongly recommended for the library generated with PCR-free library protocol to give accurate library quantification. Capillary electrophoresis or Qubit® methods can overestimate library quantity since they cannot distinguish DNA fragments with and without adapters ligated. However if Qubit® quantification is required, we recommend amplification of the library first (see Appendix F, page 34) prior Qubit quantification.

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

Appendix G: Purification of Amplified DNA after Whole Genome Amplification

If purification of the amplified DNA is required for additional downstream applications not described in this handbook, the following purification protocol may be used.

Procedure

1. Dilute amplified DNA from step 13 (page 18) 1:2 with H2O sc.
2. Add 50 µl resuspended Agencourt AMPure XP beads slurry to 50 µl diluted WGA sample, and mix well by pipetting.
3. Incubate the mixture for 5 min at room temperature.
4. Pellet the beads on a magnetic stand for 2-5 min and carefully discard the supernatant.
5. Wash the beads by adding 200 µl fresh 80% ethanol to each pellet. Pellet the beads on the magnetic stand for 2-5 min, and then carefully discard the supernatant.
6. Repeat the wash step 5 once for a total of 2 ethanol washes.
7. Incubate on the magnetic stand for 5–10 min or until the beads are dry. Avoid over-drying, which may result in lower DNA recovery. Remove from the magnetic stand.
8. Elute by resuspending in 20 µl 10 mM Tris-HCl, pH 8.0. Pellet beads on the magnetic stand. Carefully transfer 17 µl supernatant to a new PCR plate.
9. Store purified amplified DNA at –20°C until further processing.

Ordering Information

Product	Contents	Cat. no.
QIAseq FX Single Cell DNA Library Kit (96)	For 96 reactions: Buffers and reagents for cell lysis, whole genome amplification and library preparation including DNA fragmentation, end-repair and adapter ligation. Includes a plate containing 96 barcoded adapters for use with Illumina instruments.	180715
QIAseq FX Single Cell DNA Library Kit (24)	For 24 reactions: Buffers and reagents for cell lysis, whole genome amplification, and library preparation including DNA fragmentation, end-repair and adapter ligation. Includes a plate containing 24 barcoded adapters for use with Illumina instruments.	180713
Related products		
QIAGEN QIAseq and GeneRead Kits – for next-generation sequencing applications		
GeneRead Size Selection Kit (50)	For 50 reactions: Spin columns and buffers	180514
GeneRead DNA I Amp Kit (100)	For 100 reactions: Buffers and reagents for library amplification, for use with Illumina instruments	180455
QIAseq FX Single Cell RNA Library Kit (24)	For 24 reactions: Buffers and reagents for mRNA-seq library preparation from single cells	180733
QIAseq FX Single Cell RNA Library Kit (96)	For 96 reactions: Buffers and reagents for mRNA-seq library preparation from single cells	180735

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www.qiagen.com

Australia ■ techservice-au@qiagen.com

Austria ■ techservice-at@qiagen.com

Belgium ■ techservice-bnl@qiagen.com

Brazil ■ suportetecnico.brasil@qiagen.com

Canada ■ techservice-ca@qiagen.com

China ■ techservice-cn@qiagen.com

Denmark ■ techservice-nordic@qiagen.com

Finland ■ techservice-nordic@qiagen.com

France ■ techservice-fr@qiagen.com

Germany ■ techservice-de@qiagen.com

Hong Kong ■ techservice-hk@qiagen.com

India ■ techservice-india@qiagen.com

Ireland ■ techservice-uk@qiagen.com

Italy ■ techservice-it@qiagen.com

Japan ■ techservice-jp@qiagen.com

Korea (South) ■ techservice-kr@qiagen.com

Luxembourg ■ techservice-bnl@qiagen.com

Mexico ■ techservice-mx@qiagen.com

The Netherlands ■ techservice-bnl@qiagen.com

Norway ■ techservice-nordic@qiagen.com

Singapore ■ techservice-sg@qiagen.com

Sweden ■ techservice-nordic@qiagen.com

Switzerland ■ techservice-ch@qiagen.com

UK ■ techservice-uk@qiagen.com

USA ■ techservice-us@qiagen.com