September 2023

Supplementary Protocol

QIAamp® PowerFecal® Pro and DNeasy® PowerSoil® Pro WGS SeqSets

The QIAamp PowerFecal Pro WGS SeqSets and DNeasy PowerSoil Pro WGS SeqSets are comprehensive all-in-one solutions for microbiome research. These kits offer a streamlined process from nucleic acid extraction to whole genome metagenomics using the QIAseq FX DNA Library Prep Kit and easy-to-use bioinformatic analysis via the Microbial Analysis Portal.

These kits are specifically designed to maximize the efficiency and reproducibility of microbiome research workflows, starting from extraction of high-quality DNA from fecal (QIAamp PowerFecal Pro DNA) or soil (DNeasy PowerSoil Pro) samples, through comprehensive whole genome metagenomics and analysis. The bundled Microbial Analysis Portal provides powerful capabilities for taxonomic identification of microbes and antimicrobial resistance (AMR) analysis, making these kits a complete Sample to Insight® solution for microbiome research.

Kit Components

Component number	Component name	51825 QIAamp PowerFecal Pro WGS SeqSet A (96)	51835 QIAamp PowerFecal Pro WGS SeqSet B (96)	51845 QIAamp PowerFecal Pro WGS SeqSet C (96)	51855 QIAamp PowerFecal Pro WGS SeqSet D (96)
51804	QIAamp PowerFecal Pro Kit (50)	2 pc	2 pc	2 pc	2 pc
1120146	QIAseq® FX DNA Library Core Kit (96)	1 pc	1 pc	1 pc	1 pc
180312	QIAseq UDI Y-Adapter Kit A (96)	1 pc	–	–	–
180314	QIAseq UDI Y-Adapter Kit B (96)	–	1 pc	–	–
180316	QIAseq UDI Y-Adapter Kit C (96)	–	–	1 pc	–
180318	QIAseq UDI Y-Adapter Kit D (96)	–	–	–	1 pc
1124693	QIAseq Beads (25mL)	1 pc	1 pc	1 pc	1 pc
1129599	QIAseq Normalizer Reagent Kit (96)	1 pc	1 pc	1 pc	1 pc
1129601	QIAseq Normalizer Primer Kit (96)	1 pc	1 pc	1 pc	1 pc
333785	GeneGlobe® Analysis Credits Kit (96)	1 pc	1 pc	1 pc	1 pc

Component number	Component name	47165 DNeasy PowerSoil Pro WGS SeqSet A (96)	47175 DNeasy PowerSoil Pro WGS SeqSet B (96)	47185 DNeasy PowerSoil Pro WGS SeqSet C (96)	47195 DNeasy PowerSoil Pro WGS SeqSet D (96)
47014	DNeasy PowerSoil Pro Kit (50)	2 pc	2 pc	2 pc	2 pc
1120146	QIAseq FX DNA Library Core Kit (96)	1 pc	1 pc	1 pc	1 pc
180312	QIAseq UDI Y-Adapter Kit A (96)	1 pc	–	–	–
180314	QIAseq UDI Y-Adapter Kit B (96)	–	1 pc	–	–
180316	QIAseq UDI Y-Adapter Kit C (96)	–	–	1 pc	–
180318	QIAseq UDI Y-Adapter Kit D (96)	–	–	–	1 pc
1124693	QIAseq Beads (25mL)	1 pc	1 pc	1 pc	1 pc
1129599	QIAseq Normalizer Reagent Kit (96)	1 pc	1 pc	1 pc	1 pc
1129601	QIAseq Normalizer Primer Kit (96)	1 pc	1 pc	1 pc	1 pc
333785	GeneGlobe Analysis Credits Kit (96)	1 pc	1 pc	1 pc	1 pc

Component number	Component name	51863 QIAamp PowerFecal Pro WGS SeqSet (24)	47203 DNeasy PowerSoil Pro WGS SeqSet (24)
51804	QIAamp PowerFecal Pro Kit (50)	1 pc	–
47014	DNeasy PowerSoil Pro Kit (50)	–	1 pc
1120145	QIAseq FX DNA Library Core Kit (24)	1 pc	1 pc
180310	QIAseq UDI Y-Adapter Kit (24)	1 pc	1 pc
333923	QIAseq Beads (10mL)	1 pc	1 pc
1126900	QIAseq Normalizer Reagent Kit (24)	1 pc	1 pc
1129602	QIAseq Normalizer Primer Kit (24)	1 pc	1 pc
333782	GeneGlobe Analysis Credits Kit (24)	1 pc	1 pc

Protocol: Sample Extraction Using DNeasy PowerSoil Pro or QIAamp PowerFecal Pro

Important notes before starting

- Ensure that the PowerBead Pro Tubes rotate freely in the centrifuge without rubbing.
- If Solution CD3 has precipitated, heat at 60°C until precipitate dissolves.
- Perform all centrifugation steps at room temperature (15–25°C).
- Blue text (marked with a ▲) applies to DNeasy PowerSoil; red text (marked with a ●) applies to QIAamp PowerFecal Pro.

Procedure

1. Spin the PowerBead Pro Tube briefly to ensure that the beads have settled at the bottom. Add up to ▲ 250 mg of soil or ● 250 mg of stool and 800 µL of Solution CD1. Vortex briefly to mix.

   Note: After the sample has been loaded into the PowerBead Pro Tube, the next step is a homogenization and lysis procedure. The PowerBead Pro Tube contains a buffer that will help disperse the soil or stool particles, begin to dissolve ▲ humic acids or ● inhibitors, and protect nucleic acids from degradation. Gentle vortexing mixes the components in the PowerBead Pro Tube and begins to disperse the sample in the buffer.

2. Homogenize samples thoroughly using one of the following methods:

   2a. Secure the PowerBead Pro Tube horizontally on a Vortex Adapter for 1.5–2 mL tubes (cat. no. 13000-V1-24). Vortex at maximum speed for 10 min.

   Note: If using Vortex Adapter for more than 12 preps simultaneously, increase the vortexing time by 5–10 min.

   Note: Using the Vortex Adapter will maximize homogenization, which can lead to higher DNA yields. Avoid using tape, which can become loose and result in reduced homogenization efficiency, inconsistent results, and reduced yields.

   2b. Use a PowerLyzer 24 Homogenizer. PowerBead Pro Tubes must be properly balanced in the tube holder of the PowerLyzer 24 Homogenizer. We recommend homogenizing the soil at 2000 rpm for 30 s, pausing for 30 s, then homogenizing again at 2000 rpm for 30 s.

   Note: Homogenizing samples at higher speeds (up to 4000 rpm) may increase yields but result in more fragmented DNA.

   2c. Use a TissueLyser II. Place the PowerBead Pro Tube into the TissueLyser Adapter Set 2 x 24 (cat. no. 69982) or 2 mL Tube Holder (cat. no. 11993) and Plate Adapter Set (cat. no. 11990). Fasten the adapter into the instrument and shake for 5 min at 25 Hz. Reorient the adapter so that the side that was closest to the machine body is now furthest from it. Shake again for 5 min at 25 Hz.

   Note: Vortexing/shaking is critical for complete homogenization and cell lysis. Cells are lysed by a combination of chemical agents from step 1 and mechanical shaking introduced at this step. Randomly shaking the beads in the presence of disruption agents will cause the beads to collide with microbial cells and lead to the cells breaking open.

3. Centrifuge the PowerBead Pro Tube at 15,000 x *g* for 1 min.

4. Transfer the supernatant to a clean 2 mL Microcentrifuge Tube (provided).

   Note: Expect 500–600 µL. The supernatant may still contain some soil or stool particles.

5. Add 200 µL of Solution CD2 and vortex for 5 s.

   Note: Solution CD2 contains Inhibitor Removal Technology® (IRT), which is a reagent that can precipitate non-DNA organic and inorganic material including ▲ humic substances, ● polysaccharides, cell debris, and proteins. It is important to remove contaminating organic and inorganic matter that may reduce DNA purity and inhibit downstream DNA applications.

6. Centrifuge at 15,000 x *g* for 1 min. Avoiding the pellet, transfer up to 700 µL of supernatant to a clean 2 mL Microcentrifuge Tube (provided).

   Note: Expect 500–600 µL.

   Note: The pellet at this point contains non-DNA organic and inorganic material including ▲ humic acids, ● polysaccharides, cell debris, and proteins. For best DNA yields and quality, avoid transferring any of the pellet.

7. Add 600 µL of Solution CD3 and vortex for 5 s.

   Note: Solution CD3 is a high-concentration salt solution. Because DNA binds tightly to silica at high salt concentrations, Solution CD3 will adjust the DNA solution salt concentration to allow binding of DNA, but not non-DNA organic and inorganic material that may still be present at low levels, to the MB Spin Column filter membrane.

8. Load 650 µL of the lysate onto an MB Spin Column and centrifuge at 15,000 x *g* for 1 min.

   Note: DNA is selectively bound to the silica membrane in the MB Spin Column in the presence of high salt solution. Contaminants pass through the filter membrane, leaving only DNA bound to the membrane.

9. Discard the flow-through and repeat step 8 to ensure that all of the lysate has passed through the MB Spin Column.
10. Carefully place the MB Spin Column into a clean 2 mL Collection Tube (provided). Avoid splashing any flow-through onto the MB Spin Column.
11. Add 500 µL of Solution EA to the MB Spin Column. Centrifuge at 15,000 x *g* for 1 min.

    Note: Solution EA is a wash buffer that removes protein and other non-aqueous contaminants from the MB Spin Column filter membrane.

12. Discard the flow-through and place the MB Spin Column back into the same 2 mL Collection Tube.
13. Add 500 µL of Solution C5 to the MB Spin Column. Centrifuge at 15,000 x *g* for 1 min.

    Note: Solution C5 is an ethanol-based wash solution used to further clean the DNA that is bound to the silica filter membrane in the MB Spin Column. This wash solution removes residual salt, ▲ humic acid, ● inhibitors, and other contaminants, while allowing the DNA to stay bound to the silica membrane.

14. Discard the flow-through and place the MB Spin Column into a new 2 mL Collection Tube (provided).
15. Centrifuge at up to 16,000 x *g* for 2 min. Carefully place the MB Spin Column into a new 1.5 mL Elution Tube (provided).

    Note: This spin removes residual Solution C5. It is critical to remove all traces of Solution C5 because the ethanol in it can interfere with downstream DNA applications, such as PCR, restriction digests, and gel electrophoresis.

16. Add 50–100 µL of Solution C6 to the center of the white filter membrane.

    Note: Placing Solution C6 in the center of the small white membrane will make sure the entire membrane is wet. This will result in a more efficient and complete release of the DNA from the MB Spin Column filter membrane. As Solution C6 passes through the silica membrane, DNA that was bound in the presence of high salt is selectively released by Solution C6 (10 mM Tris), which lacks salt.

17. Centrifuge at 15,000 x *g* for 1 min. Discard the MB Spin Column. The DNA is now ready for downstream applications.

    Note: We recommend storing the DNA frozen (–30°C to –15°C or –90°C to –65°C) as Solution C6 does not contain EDTA. To concentrate DNA, refer to the respective troubleshooting guides of ▲ *DNeasy PowerSoil Pro Kit Handbook* and ● *QIAamp PowerFecal Pro DNA Kit Handbook*.

Protocol: QIAseq FX DNA Library Preparation and Normalization using QIAseq Library Normalizer Kits

QIAseq Normalizer Kits provide a fast and easy way to normalize and pool NGS libraries for Illumina®, while skipping library qualification and quantification steps. QIAseq Normalizer will accurately normalize over a broad range of library concentrations, but it cannot fully compensate for libraries of poor quality or libraries at lower concentrations of less than 15 nmol/L. Therefore, we recommend to adhere to the below guidelines and to use a minimum gDNA input of 5 ng for library preparation. If working with less than 5 ng of input, it is recommended to skip the QIAseq Library Normalizer procedure and perform library normalization manually. Please refer to procedure in *QIAseq FX DNA Library Kit Handbook* (**qiagen.com/HB-2015**) if working with <5 ng of gDNA.

> **Note**: Higher input amounts will generally enable more complex libraries. While 5 ng input comprise a high number of copies for small genomes such as many microbes, larger genomes such as human or many plant genomes will benefit from higher input amounts.

- Make sure the used input material for library preparation is of good quality and/or is accurately quantified.
- The laboratory staff should be experienced with performing QIAseq FX library preparation.
- The conditions for the library preparation should be well established in your laboratory (e.g., input amount, adapter concentrations/dilution, PCR settings, cleanup procedures and other workflow parameters) so your library yields are consistent with concentrations well above 15–20 nmol/L in a minimal volume of 15 µL.
- Do not use QIAseq Normalizer Kits when using libraries for downstream hybridization capture.

This modified QIAseq FX DNA Library protocol is optimized for subsequent library normalization using QIAseq Library Normalizer Kits. Normalizing libraries after preparation allows pooling and sequencing without the need for library quantification by qPCR or alternative methods.

Further reading on library normalization:

- QIAseq Normalizer Kits (**qiagen.com/qiaseq-normalizer**)
- *QIAseq Normalizer Kit Handbook* (**qiagen.com/HB-2015**)

Fragmentation, End-Repair, A–addition, and Adapter Ligation

This protocol describes the FX reaction for single-tube fragmentation, end-repair, and A-addition.

Important points before starting

- Before setting up the reaction, it is critical to accurately determine the amount of the input DNA.
- Do not use less than 5 ng of input DNA if using QIAseq Normalizer Kits
- Ensure input DNA is in water, 10 mM Tris, QIAGEN's Buffer EB, or low TE (0.1x TE, 0.1 mM EDTA). If input DNA is in 1x TE, please set up the FX reaction according to the protocol in Appendix B.

Table 1. Guideline for choosing the initial fragmentation time

Fragment peak size	250 bp	350 bp	450 bp	550 bp
	Fragmentation time (min) at 32°C			
10 ng input DNA†	24	16	14	10
100 ng input DNA	16	10	8	6
1000 ng input DNA	14	8	6	4

Note: The same FX fragmentation time will produce a consistent fragment size within an approximately 5-fold range of input DNA amounts. The exact reaction time may need to be optimized for DNA samples of variable quality.

† For input DNA <10 ng, FX Enhancer is required for optimal performance (Table 12). To produce a fragment distribution centered around 300 bp from 5 ng input, incubate the FX reaction including FX Enhancer for 10 min.

Things to do before starting

- Refer to Table 9 to determine the time required to fragment input DNA to the desired size. If input DNA is less than 10 ng, add FX Enhancer according to the protocol and use half the reaction time listed for 10 ng input DNA.
- Make fresh 80% ethanol.
- Thaw reagents 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 thermocyclers. For increased speed and convenience, all incubation steps of the protocol can be preprogrammed and saved in advance.
- Equilibrate QIAseq Beads to room temperature (15–25°C) for 20–30 min before use.
- Vortex and spin down the thawed adapter plate before use.

Procedure

1. Program a thermocycler according to Table 10 using the predetermined FX fragmentation time for step 2. Be sure to use the instrument's heated lid, and if possible, set the temperature of the heated lid to 70°C.

Table 2. Input DNA (5 ng – 1000 ng) free of EDTA, Buffer EB, or in 0.1x TE

Step	Incubation temperature	Incubation time
1	4°C	1 min
2	32°C	1–30 min*
3	65°C	30 min
4	4°C	Hold

* To determine the reaction time for step 2, refer to Table 9.

2. Start the program. When the thermocycler block reaches 4°C, pause the program.
3. Prepare the FX reaction mix in a PCR plate or tube on ice according to Table 11 for >10 ng input DNA or Table 12 (next page) for <10 ng input DNA. Mix well by gently pipetting (do not vortex to mix).

Table 3. FX reaction mix setup (per sample) for >10 ng input DNA

Component	Volume (µL)
FX Buffer, 10x	5
Purified DNA	Variable
Nuclease-free water	Variable
Total without FX Enzyme Mix	**40**

Table 4. FX reaction mix setup (per sample) for <10 ng input DNA

Component	Volume (µL)
FX Buffer, 10x	5
Purified DNA (≥5 ng)	Variable
FX Enhancer	2.5
Nuclease-free water	Variable
Total without FX Enzyme Mix	**40**

4. Add 10 µL FX Enzyme Mix to each reaction and mix well by pipetting up and down 20 times. It is critical to keep the reactions on ice for the entire time during reaction setup.
5. Briefly spin down the PCR plate/tubes and immediately transfer to the prechilled thermocycler (4°C). Resume the cycling program.
6. When the thermocycler program is complete and the sample block has returned to 4°C, remove samples and place them on ice.
7. Immediately proceed with adapter ligation as described in the next protocol.
8. Pierce the foil seal for each adapter well to be used, and transfer 5 µL from one DNA adapter well to each 50 µL sample from the previous protocol. Track the barcodes from each adapter well used for each sample.

   Note: If your DNA input is <10 ng, dilute the adapters according to Table 13.

Table 5. Adapter dilution factors

Sample DNA amount	Adapter dilution
5–9 ng	1:10
≥10 ng	Undiluted

9. Freeze the adapter plate containing unused adapters. The QIAseq adapters are stable for a minimum of 10 freeze-thaw cycles.

   Important: Only 1 single adapter should be used per ligation reaction. If adapters from another supplier are used, follow the manufacturer's instructions. Do not reuse adapter wells once the foil seal has been pierced.
10. Prepare the ligation Master Mix (per DNA sample, Table 14) in a separate PCR plate or tube on ice, and mix well by pipetting.

Table 6. Ligation master mix setup (per sample)

Component	Volume (µL)
Ligation buffer, 5x	20
DNA ligase	10
Nuclease-free water	15
Total	**45**

11. Add 45 µL of the ligation Master Mix to each sample, for a total of 100 µL, and mix well by pipetting. Incubate the ligation reaction at 20°C for 15 min.

    Important: Do not use a thermocycler with a heated lid.
12. Proceed immediately to adapter ligation cleanup using 0.8x (80 µL) QIAseq Beads.

13. Add 80 µL of resuspended QIAseq Beads to each ligated sample and mix well by pipetting.
14. Incubate the mixture for 5 min at room temperature. Pellet the beads on a magnetic stand (e.g., DynaMag™, Thermo Fisher Scientific) for 2 min, then carefully discard the supernatant.
15. Wash the beads by adding 200 µL of 80% ethanol. Pellet the beads on the magnetic stand and discard the supernatant. Repeat the wash once, for a total of 2 ethanol washes. Remove as much excess ethanol as possible.
16. Incubate the beads on the magnetic stand for 5–10 min or until the beads are dry.
17. Elute by resuspending in 52.5 µL of Buffer EB or 10 mM Tris·Cl, pH 8.0. Pellet the beads on the magnetic stand. Carefully transfer 50 µL of supernatant into a new plate or tube.
18. Perform a second purification using 1.1x (55 µL) of QIAseq Beads following steps 14–16 for DNA binding and washing. Elute DNA by adding 26 µL Buffer EB or 10 mM Tris·Cl, pH 8.0. Pellet the beads and carefully collect 23.5 µL of purified DNA sample in a DNA LoBind® tube (Thermo Fisher Scientific) for library amplification. If not proceeding immediately, the sample can be stored at –30°C to –15°C.

Amplification of Library DNA

This protocol requires library amplification primers provided in the QIAseq Normalizer Kits. Replace the amplification primer mix provided with the QIAseq FX DNA Library Kit by the Normalizer Primer Mix.

Always perform PCR-based library amplification before normalizing libraries using the QIAseq Normalizer Kits. Do not use PCR-free libraries together with QIAseq Normalizer Kits. This protocol is for high-fidelity amplification of the DNA library using the HiFi PCR Master Mix provided in the QIAseq FX DNA Library Kit together with the Normalizer Primer Mix from the QIAseq Library Normalizer Kit.

Things to do before starting

- Thaw QIAseq HiFi PCR Master Mix from the QIAseq FX DNA Library Kit and the Normalizer Primer Mix from the QIAseq Library Normalizer Kit on ice. Once reagents are thawed, mix them thoroughly by quick vortexing to avoid any localized concentrations.
- Always start with the cycling conditions specified in this protocol. The cycling has been optimized for use with QIAseq HiFi PCR Master Mix for even and high-fidelity amplification of sequencing libraries.

Procedure

1. Program a thermocycler with a heated lid according to Table 15.

Table 7. Library amplification cycling conditions

Time	Temperature	Number of cycles
2 min	98°C	1
20 s	98°C	
30 s	60°C	3 (1 µg input DNA) 4 (500 ng input DNA) 5 (200 ng input DNA) 6 (100 ng input DNA) 7 (50 ng input DNA) 8 (20 ng input DNA) 9 (10 ng input DNA) 11 (5 ng input DNA)
30 s	72°C	
1 min	72°C	1
∞	4°C	Hold

2. Prepare a reaction mix on ice according to Table 16. Mix the components in a PCR tube or 96-well PCR plate.

   Important: Make sure to use the Normalizer Primer Mix to amplify the libraries. Using the library amplification primers included in the QIAseq FX DNA Library Kits will lead to normalization failure.

Table 8. Reaction mix for library enrichment

Component	Volume (µL)
HiFi PCR Master Mix, 2x	25
Normalizer Primer Mix*	1.5
Library DNA	23.5
Total reaction volume	**50**

3. Transfer the PCR tube or plate to the thermocycler and start the program.
4. Once PCR is complete, add 60 µL of resuspended QIAseq Beads to each reaction (50 µL) and pipette up and down thoroughly to mix.
5. Incubate the mixture for 5 min at room temperature. Pellet the beads on a magnetic stand (e.g., DynaMag) and carefully discard the supernatant.
6. Wash the beads by adding 200 µL of 80% ethanol. Pellet the beads on the magnetic stand and discard the supernatant. Repeat the wash once, for a total of 2 ethanol washes. Remove as much excess ethanol as possible.
7. Incubate on the magnetic stand for 5–10 min or until the beads are dry.
8. Remove the beads from the magnetic stand. Elute by resuspending in 32.5 µL of Buffer EB or 10 mM Tris·Cl, pH 8.0. Pellet the beads on the magnetic stand. Carefully transfer 30 µL of the supernatant into a new tube.

   Note: If required, the elution volume can be reduced to 20 µL to increase the library concentration. The QIAseq Normalizer procedure will require 15 µL of modified library. Library concentrations should consistently be greater than 15 nmol/L.
9. Proceed to the library normalization protocol (next page). Alternatively, the purified libraries can be safely stored at –20°C.

   Note: Optionally, the libraries can be analyzed using a capillary electrophoresis device such as QIAGEN QIAxcel® or Agilent® BioAnalyzer (Agilent Technologies, Inc.). Quantification may be performed using qPCR (e.g., using the QIAseq Library Quant Assay

Kit). Analysis by electrophoresis is recommended when using the QIAseq FX DNA Library Kit for the first time or while optimizing conditions, but is generally not required prior to library normalization.

Library normalization and pooling

This protocol describes the normalization of previously modified Illumina libraries. Only use libraries that have been amplified in presence of the Normalizer Primer Mix. Using non-modified libraries will lead to normalization failure. QIAseq Normalization will yield double-stranded libraries at a concentration of approximately 4 nmol/L. Normalized libraries can be pooled for sequencing at equal volumes without further quantification.

To perform QIAseq Normalization in 96-well format instead of tubes, please refer to the plate protocol included in the *QIAseq Normalizer Kit Handbook* (**www.qiagen.com/HB-3361**).

Things to do before starting

- Thoroughly mix the Normalizer Reagent by vortexing for 60 s. Make sure the pellet of beads is complete dissolved and the solution is well homogenized.
- Homogenize the bottle(s) of Normalizer Wash Buffer by vortexing and preheat the whole reagent bottle(s) to 55°C in a water bath. Alternatively, use a heating block for 1.5 mL or 2.0 mL tubes or Falcon® tubes (Corning, Inc.). Fill the homogenized wash buffer into compatible receptacles to preheat. Prepare 450 µL wash buffer (400 µL are required) per normalization reaction.
- Preheat a heating block for 1.5 mL tubes to 55°C.
- It is not required to equilibrate the Normalizer Reagent and Normalizer Elution Buffer to room temperature before use.

Procedure

1. Homogenize the bottle(s) of Normalizer Wash Buffer by vortexing and preheat the whole reagent bottle(s) to 55°C in a water bath. Alternatively, use a heating block for 1.5 mL or 2.0 mL tubes or Falcon tubes. Fill the homogenized wash buffer into compatible receptacles to preheat. Prepare 450 µL wash buffer (400 µL are required) per normalization reaction. Leave the wash buffer at 55°C until use.
2. Thoroughly mix the Normalizer Reagent by vortexing for 60 s. Make sure the pellet of beads is completely dissolved and the solution is well homogenized.
3. Directly before use, pulse spin the Normalizer Reagent at low force to collect all liquid at the bottom. Then use a 200 µL pipette to homogenize the reagent. Be sure to disperse a sediment of beads that may have formed during pulse spin.
4. For every library to be normalized, pipette 5 µL of homogenized Normalizer Reagent to a 1.5 mL tube. Leave the tubes at room temperature.
5. Add 15 µL of the modified library to be normalized to a 1.5 mL tube containing Normalizer Reagent. Mix well by pipetting or vortexing.
6. Incubate for 10 min at room temperature.
7. Add 200 µL pre-warmed Normalizer Wash Buffer (55°C) to each tube.
8. Pellet the beads on a magnetic stand for 2 min and wait until the solution is clear.

   Important: Make sure all beads have pelletized. If you are uncertain, leave the tubes on the magnet for 5 min.

9. Carefully discard the supernatant without disturbing the pellet.
10. With the tube on the magnet add 200 µL pre-warmed Normalizer Wash Buffer (55°C) to each pellet.
11. Switch the tube position on the magnet to wash the beads. Then wait until all beads have pelletized. Alternatively, turn the tube by 180° to force the beads to opposite side of the tube.
12. Carefully discard the supernatant without disturbing the pellet. Remove as much remaining liquid as possible, then proceed to the next step immediately.

    Note: Drying the beads is not required.
13. Add 26 µL Normalizer Elution Buffer to each pellet and mix well by vortexing. Make sure the pellet is completely dissolved.
14. Tap the tube on the benchtop to collect the liquid at the bottom. If there is liquid remaining in the lid, pulse spin the tube at low force. Do not spin to form a compact bead pellet.
15. Incubate for 5 min at 55°C in a heating block.
16. Pulse spin to collect all liquid at the bottom, then pellet the beads on a magnetic stand for 2 min and wait until the solution is clear.
17. Carefully transfer 25 µL of the supernatant to a new tube.

    Important: Do not discard the supernatant. The supernatant contains the ready-to-sequence library.
18. Pool the normalized libraries for sequencing following the below guidelines. Alternatively, the normalized libraries can be stored at –20°C for up to 3 months.

Pooling guidelines

- If normalized libraries were frozen, thaw them completely, mix by pulse vortexing, and spin down to avoid localized concentrations.
- For a homogeneous distribution of sequence reads, combine equal volumes of normalized libraries into a pool. For example, combine 5 µL of each normalized library. The pool will have an overall concentration of approximately 4 nmol/L.
- For joint sequencing, only combine libraries that have different indexes to allow complete demultiplexing of sequence data.

Library pool quantification (optional)

- Quantifying the library pool is optional, but may be considered if using QIAseq Normalizer for the first time.
- QIAseq normalized libraries are double-stranded and can be quantified using gel electrophoresis systems such as the QIAxcel or the Agilent 2100 Bioanalyzer. To prevent interference caused by the Normalizer Elution Buffer, dilute 5 µL of the library pool with 5 µL of nuclease-free water prior to electrophoresis.
- Quantification using colorimetric assays (e.g., Qubit™, Thermo Fisher Scientific) or a photospectrometer (e.g., Nanodrop™, Thermo Fisher Scientific) can be performed without diluting the normalized library pool. Colorimetric or photospectrometric assays require knowledge about the average library fragment size to calculate the approximate molarity. Table 17 lists the expected concentration for typical library fragment sizes.

Table 9. Theoretical library concentrations of normalized libraries for typical library fragment sizes

Ave. library fragment size (bp)	Expected concentration (ng/µL)
250	0.66
300	0.79
350	0.92
400	1.06
450	1.19
500	1.32

Sequencing

- Libraries normalized using the QIAseq Normalizer Kits are double-stranded and require denaturation prior to sequencing.
- For complete instructions on how to denature sequencing libraries, prepare custom sequencing primers, and to set up a sequencing run, please refer to the system-specific Illumina documents.
- QIAseq normalized libraries have a concentration of approximately 4 nmol/L. Follow the system-specific Illumina documents for denaturing and loading – starting from a concentration of 4 nmol/L.

Analysis

- Redeem analysis credits provided in GeneGlobe Analysis Credits Kit at **www.qiagen.com/redeem**
- Login and upload **.fastq** files to **microbialanalysisportal.qiagen.com**

Document Revision History

Date	Changes
09/2023	Initial release

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.