QIAGEN

July 2023

# QIAseq® Normalizer Kit Handbook

For the normalization of next-generation sequencing (NGS) libraries for sequencing on Illumina® instruments without prior library quantification.

Sample to Insight

# Contents

# Kit Contents

| QIAseq Library Normalizer Kit | (96) | (24) |
|---|---|---|
| Catalog no. | 180605 | 180603 |
| Number of reactions | 96 | 24 |
| **QIAseq Normalizer Reagent Kit** | | |
| Normalizer Reagent | 1 x 580 µL | 1 x 135 µL |
| Normalizer Wash Buffer | 4 x 14 mL | 1 x 14 mL |
| Normalizer Elution Buffer | 2 x 1.9 mL | 1 x 1.9 mL |
| RNase-free Water | 1 x 1.9 mL | 1 x 1.9 mL |
| **QIAseq Normalizer Primer Kit** | | |
| Normalizer Primer Mix | 1 x 165 µL | 1 x 40 µL |

| QIAseq Universal Normalizer Kit | (96) | (24) |
|---|---|---|
| Catalog no. | 180615 | 180613 |
| Number of reactions | 96 | 24 |
| **QIAseq Normalizer Reagent Kit** | | |
| Normalizer Reagent | 1 x 580 µL | 1 x 135 µL |
| Normalizer Wash Buffer | 4 x 14 mL | 1 x 14 mL |
| Normalizer Elution Buffer | 2 x 1.9 mL | 1 x 1.9 mL |
| RNase-free Water | 1 x 1.9 mL | 1 x 1.9 mL |
| **QIAseq Normalizer Primer Kit** | | |
| Normalizer Primer Mix | 1 x 165 µL | 1 x 40 µL |
| **QIAseq Normalizer PCR Kit** | | |
| HiFi PCR Master Mix | 2 x 1.5 mL | 2 x 300 µL |
| RNase Free Water | 2 x 1.9 mL | 1 x 1.9 mL |
| **QIAseq Beads** | | |
| QIAseq Beads | 1 x 10 mL | 1 x 5 mL |

# Shipping and Storage

The QIAseq Library Normalizer Kit is shipped in two boxes at different temperatures. The QIAseq Universal Normalizer Kit is shipped in four boxes at different temperatures.

Store the QIAseq Normalizer Reagent Kit and QIAseq Beads at 2–8°C upon arrival (do not freeze). Store the QIAseq Normalizer Primer Kit and QIAseq Normalizer PCR Kit at –15 to –30°C upon arrival.

When stored correctly, QIAseq Library Normalizer Kit is good until the expiration date printed on the outer package

# Intended Use

QIAseq Normalizer Kits are intended for molecular biology applications. These products are 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 Normalizer Kit is tested against predetermined specifications to ensure consistent product quality.

# Introduction

NGS is a driving force for numerous new and exciting applications, including cancer research, stem cell research, metagenomics, population genetics, and medical research. Library preparation methods are available for diverse applications featuring advanced workflows and streamlined protocols allowing to generate high quality NGS libraries with ease in a short period of time. Likewise, the sequencing technology is constantly improving with strongly increased throughput and decreased cost per yield. For that reason, today most NGS applications are unthinkable without massive multiplexed sequencing, which allows a high number of samples to be sequenced at once on a single sequencing instrument or flow cell.

## Why normalization is important

Cost-efficient multiplexed sequencing requires accurate pooling of libraries to achieve comparable sequencing depths for all libraries. Failure to combine libraries at equal ratios will lead to under- or overrepresentation of libraries in terms of sequencing yield. Underrepresented libraries will yield too little sequence reads, overrepresented libraries will waste sequencing capacity. Today's gold standard to enable library pooling at acceptable accuracies is to quantify each library prior to pooling.

## Why QIAseq Normalizer

Typical methods for library quantification include PCR (qPCR/dPCR), capillary gel electrophoresis and colorimetric DNA assays. While qPCR has proven to quantify libraries with high accuracy, it is laborious, time-intensive and costly to perform (Figure 1). Pre-diluting libraries for qPCR and manually diluting libraries based on the assayed concentration provides much room for error.

The QIAseq Normalizer Kits offer a fast and easy workflow to adjust many different types of libraries to a fixed concentration. Once normalized, libraries can be pooled at equal volumes to achieve a homogenous read representation without the need for library quantification prior to sequencing. The QIAseq Normalizer Kits enable streamlined NGS workflows at minimized cost and shorter processing time compared to conventional methods using classical library quantification and pooling techniques.

## Principle and procedure

QIAseq Normalizer Kits make use of modified library amplification primers and a chemistry based on magnetic beads to efficiently bind and release a defined amount of library molecules in a tightly controlled fashion. With a target concentration of approximately 4 nmol/L, normalized libraries are tightly adjusted for balanced library pooling and optimal clustering on

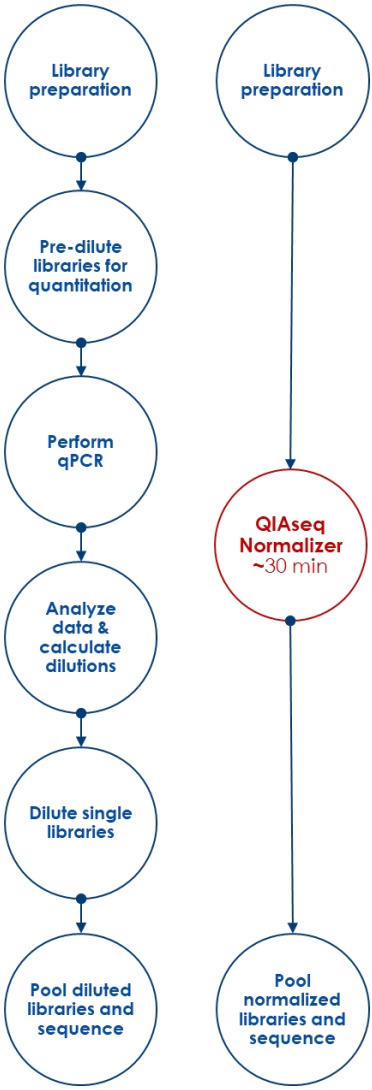


**Figure 1. Comparison of QIAseq Normalizer and qPCR-based library normalization.**

Illumina flow cells. Double-stranded DNA library molecules are eluted from the beads at non-denaturing conditions and can be safely stored at –20°C. By maintaining the libraries double-stranded during normalization, the normalized pools may optionally be assayed on standard capillary electrophoresis instruments.

The QIAseq Normalizer workflows are compatible with virtually all libraries that can be sequenced on Illumina sequencing instruments. This includes QIAGEN as well as third party library preparation workflows for Illumina.

## QIAseq Normalizer workflow

The QIAseq Normalizer workflow follows a simple bind – wash – elute routine, which is identical for all compatible types of libraries (Figure 2). Normalized libraries are double-stranded DNA and have a common concentration of approx. 4 nmol/L, which allows them to be directly used for pooling and sequencing.

Prior to normalization, libraries must be modified using the Normalizer Primer Mix. During a 2-cycle PCR in presence of Normalizer Primer Mix, modifications can be added to virtually any kind of Illumina library with intact P5/P7 ends (Workflow A). This applies to any finished library that can be sequenced on Illumina instruments.

Library preparation workflows that involve the ligation of full-length indexed-adapters enable in-process library modification and do not require an additional PCR step (Workflow B). Full-length adapters such as QIAseq CDI/UDI Y-Adapters contain full-length P5/P7 sequences. Therefore, libraries can be modified by using the Normalizer Primer Mix during the library amplification step. QIAseq Normalizer is not compatible with PCR-free library preparation.

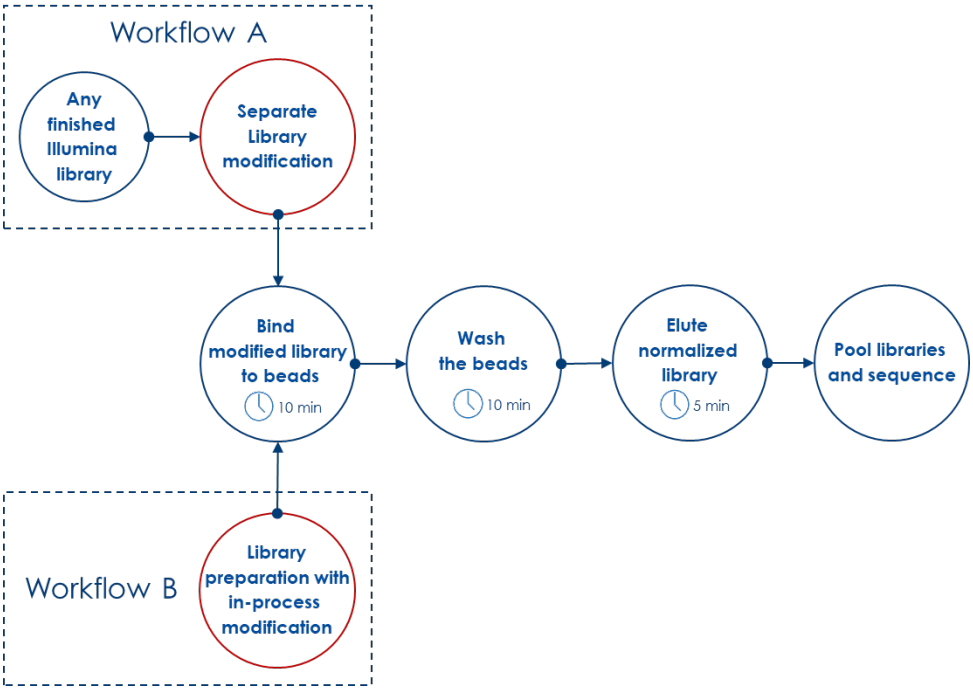


**Figure 2. QIAseq Normalizer Workflow.** Only libraries that have been amplified/modified using the QIAseq Normalizer Primer Mix can be used for normalization. Modifications can be added to virtually any finished Illumina library in a separate library modification step (Workflow A). For library preparation methods using ligation of full-length indexed-adapters, library modifications can be applied during the process of library preparation without any additional step (Workflow B). Once modified, all library types are normalized using an identical bind-wash-elute workflow.

## Pick the correct Normalizer Kit & Workflow for your library

Library modification using the Normalizer Primer Mix is always required prior to normalization. Depending on the library preparation method used, the correct way of modifying the library must be selected. There are two ways to modify your libraries – Workflow A or Workflow B (Figure 3). To choose a workflow and the needed QIAseq Normalizer Kit, answer the following questions about your library. If you are not certain which workflow to choose, please contact the QIAGEN customer support for assistance.

**Choose Workflow A and QIAseq Universal Normalizer Kits if one or more of the following statements apply (an example using QIAseq Targeted DNA Library (Pro) Kits):**

- Library preparation ends with a PCR using indexed-primers (e.g. from a 96-well plate).
- The P5/P7 library ends that enable binding to the flow-cell are added in the final PCR at the end of library preparation.
- During tagmentation or ligation a short/stubby adapter is added to your library. This adapter does not contain index motifs and is universally used for all samples.
- Your library type typically yields low concentrations of less than 15 nmol/L and you like to amplify the library further in order to perform normalization.

**Choose Workflow B and QIAseq Library Normalizer Kits if one or more of the following statements apply (an example using QIAseq FX DNA Library Kits):**

- Library preparation ends with a PCR using an amplification primer mix that is universally used for all samples.
- The P5/P7 library ends that enable binding to the flow-cell are added as part of the adapter during ligation.
- During the ligation step full-length indexed-adapters are used (e.g. from a 96-well plate).

**Note**: Please refer to the QIAseq FX Handbook for a dedicated library preparation protocol including library normalization using QIAseq Normalizer.

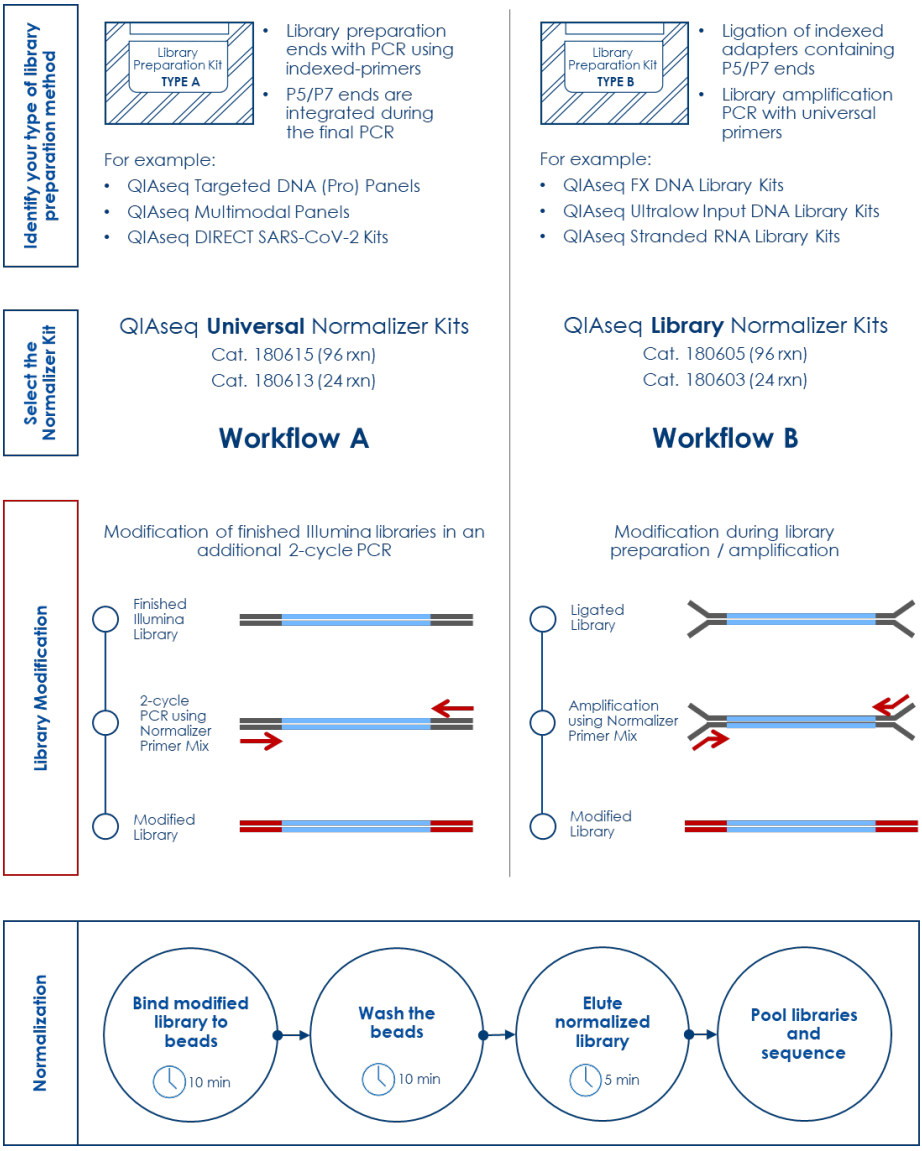


**Figure 3. Workflow overview comparing QIAseq Universal Normalizer Kits (Workflow A) and QIAseq Library Normalizer Kits (Workflow B).**

## High throughput and automated Normalization workflows

A plate protocol for manual library Normalization for the 96-well plate format is included in this handbook. Library normalization workflows using the QIAseq Library Normalizer Kits or QIAseq Universal Normalizer Kits are easily automatable on various NGS liquid-handling platforms.

# Important Notes

QIAseq Normalizer Kits provide a fast and easy way to normalize and pool NGS libraries for Illumina, while skipping library qualification and quantification steps. Even though QIAseq Normalizer will accurately normalize a broad range of library types to the target concentration, 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 to decide when to use – and when not to use – QIAseq Normalizer.

## Requirements for use of QIAseq Normalizer Kits

- The used input material for library preparation is of good quality and/or is accurately quantified;
- The laboratory staff is experienced with the library preparation method in use;
- The conditions for the library preparation method are 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;
- The planned number of libraries in the sequencing pool is 6 or greater.

## When QIAseq Normalizer Kits should not be used

If at least one of the following applies, we recommend to use conventional methods to qualify and quantify your libraries.

- The laboratory staff has little experience with the employed library preparation method or is performing it for the first time;
- Library yields are unpredictable with frequently low concentrations of 15 nmol/L or less in a minimal volume of 15 µL;

- The employed library preparation methods tend to yield libraries with strong adapter dimer contamination;
- Less than 6 libraries are planned for multiplexed sequencing;
- The libraries to be sequenced are PCR-free;
- The libraries are planned for use in target-enrichment workflows based on hybridization capture.

## Description of protocols

This handbook contains two generalized protocols for library modification and one universal protocol for the normalization of modified libraries. The normalization protocol is available as a tube protocol for use with 1.5 mL Eppendorf tubes and as an optimized plate protocol for use with standard 96-well PCR plates or PCR-strips.

**Library modification Workflow A** requires QIAseq Universal Normalizer Kits. The protocol describes a universal modification procedure that can be applied to most finished Illumina libraries that meet the recommended criteria in the "Important Notes" section. QIAseq Universal Normalizer Kits contain all reagents needed for the modification procedure.

**Library modification Workflow B** is meant for use with QIAseq Library Normalizer Kits, but can also be performed using the QIAseq Universal Normalizer Kits. The protocol describes general guidelines on replacing any universal library amplification primer (mix) of your library kit with the Normalizer Primer Mix. Workflow B requires using PCR and cleanup reagents contained in the employed library kit.

# 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.

- LoBind® tubes for storage of generated libraries (Eppendorf, cat. no. 0030108094, 0030108116 or 0030108132) or equivalent
- 96-well PCR plates or PCR tubes/strips
- Foil seal for 96-well plates, heat-resistant
- 100% ethanol (ACS grade)
- Nuclease-free water
- Pipette tips and pipettes
- Vortexer
- Microcentrifuge and/or plate centrifuge for 96-well plates
- Thermocycler
- Magnetic stand (e.g., DynaMag™,2 Magnet or DynaMag™, 96 Side Skirted Magnet, Thermo Fisher, cat. no. 12027)
- QIAGEN QIAxcel®, Agilent® Bioanalyzer®, or similar method to assess the quality of DNA library (optional)

# Important points before starting

- Ensure to have correctly chosen the modification workflow for your library type, and have purchased the appropriate QIAseq Normalizer kit. See page 10 for further guidance;
- Prepare NGS libraries for Illumina following the guidelines of the respective kit handbook (e.g. QIAseq FX DNA Library Kits, QIAseq Ultralow Input Kits, QIAseq Targeted DNA Panels, and many others - including third party library preparation methods);
- For normalization, only use libraries that were amplified/modified using the QIAseq Normalizer Primer Mix. Normalization of libraries amplified with routine amplification primers will fail;
- Do not use PCR-free libraries for normalization;
- Make sure the libraries meet the recommended criteria for use with QIAseq Normalizer on pages 14–15.

# Protocol: (Tube Format) Workflow A - Library modification for use with QIAseq Universal Normalizer Kits

This protocol describes the modification of any Illumina library that has intact P5/P7 ends. Use this protocol for libraries that meet the criteria for workflow A (page 10). After modification libraries can be normalized using the QIAseq Normalizer workflow (refer to pages 29 and 35)

## Things to do before starting

- Be sure your libraries and preparation workflow meet the recommendations for use with QIAseq Normalizer (pages 14–15)
- Complete the library preparation process for Illumina to generate sequencing-ready libraries. Follow the manufacturer's guidelines.
- If libraries were frozen, thaw them on ice. Once libraries are thawed, mix them thoroughly by quick vortexing to avoid any localized concentrations. Briefly spin down libraries.
- Thaw QIAseq HiFi PCR Master Mix and the Normalizer Primer Mix on ice. Once reagents are thawed, mix them thoroughly by quick vortexing to avoid any localized concentrations. Briefly spin down vortexed reagents before use.
- Prepare fresh 80% ethanol
- Equilibrate QIAseq beads to room temperature (15–25°C). Directly before use, vortex the beads thoroughly to homogenize.

## Procedure

1. Set up a thermal cycler with a heated lid according to Table 1.

   **Note**: If your libraries are likely to have very low concentrations (< 10 nmol/L) or only small volumes of library are available (< 15 µL), increase the PCR cycles to 3-4 cycles.

**Table 1. Thermal cycling parameters**

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

*Perform 3-4 cycles, if your input library concentrations are generally low (< 10 nmol/L) or only small volumes of input library (< 15 µL) are available.

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

**Table 2. Reaction mix for Normalizer modification PCR**

| Component | Volume/reaction (µL) |
|---|---|
| HiFi PCR Master Mix, 2x | 25 |
| Normalizer Primer Mix | 1.5 |
| Finalized Illumina library* | ≤ 23.5 |
| RNase-free water | variable |
| **Total reaction volume** | **50** |

*If available, always use 23.5 µL of finalized Illumina library. Otherwise use all available volume and fill with RNase-free water.

3. Transfer the PCR tube/strip or plate to the thermal cycler and start the program.

4. Once PCR is complete, transfer the whole reaction to a new 1.5 mL Eppendorf tube and add 50 µL of homogenized QIAseq Beads to each reaction.
5. Mix by thoroughly pipetting up and down or vortex. Then Incubate the mixture for 5 min at room temperature.
6. If required, pulse spin the tubes to collect all liquid at the bottom.
7. Pellet the beads on a magnetic stand for 5 minutes or until the solution is clear.
8. Carefully discard the supernatant without disturbing the pellet.
9. Wash the beads by adding 200 µL fresh 80% ethanol to each pellet. Pellet the beads on the magnetic stand and carefully discard the supernatant.
10. Repeat step 9 for a total of two ethanol washes.
11. Remove as much excess ethanol as possible.
12. Incubate on the magnetic stand for 5–10 min or until the beads are dry. Overdrying of beads may result in lower DNA recovery. Remove from the magnetic stand.
13. Elute modified libraries by resuspending in 32.5 µL of RNase-free water or 10 mM Tris·Cl, pH 8.0. Pellet the beads on the magnetic stand and 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.
14. The collected supernatant contains the modified library and can be used for normalization. Proceed to "Protocol: (Tube format) QIAseq Library Normalization for use with all QIAseq Normalizer Kits". Alternatively, modified libraries can be stored for later use at –20°C.

# Protocol: (Plate format) Workflow A - Library modification for use with QIAseq Universal Normalizer Kits

This protocol describes the modification of any Illumina library that has intact P5/P7 ends. Use this protocol for libraries that meet the criteria for workflow A (page 10). After modification libraries can be normalized using the QIAseq Normalizer workflow (refer to pages 29 and 35).

## Things to do before starting

- Be sure your libraries and preparation workflow meet the recommendations for use with QIAseq Normalizer (pages 14–15)
- Complete the library preparation process for Illumina to generate sequencing-ready libraries. Follow the manufacturer's guidelines.
- If libraries were frozen, thaw them on ice. Once libraries are thawed, mix them thoroughly by quick vortexing to avoid any localized concentrations. Briefly spin down libraries.
- Thaw QIAseq HiFi PCR Master Mix and the Normalizer Primer Mix on ice. Once reagents are thawed, mix them thoroughly by quick vortexing to avoid any localized concentrations. Briefly spin down vortexed reagents before use.
- Prepare fresh 80% ethanol
- Equilibrate QIAseq beads to room temperature. Directly before use, vortex the beads thoroughly to homogenize.

## Procedure

1. Set up a thermal cycler with a heated lid according to Table 3.

   Note: If your libraries are likely to have very low concentrations (< 10 nmol/L) or only small volumes of library are available (< 15 µL), increase the PCR cycles to 3-4 cycles.

**Table 3. Thermal cycling parameters**

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

*Perform 3-4 cycles, if your input library concentrations are generally low (< 10 nmol/L) or only small volumes of input library (< 15 µL) are available.

2. Prepare the library modification reaction mix on ice according to Table 4. Mix the components in a 96-well PCR plate.

**Table 4. Reaction mix for Normalizer modification PCR**

| Component | Volume/reaction (µL) |
|---|---|
| HiFi PCR Master Mix, 2x | 25 |
| Normalizer Primer Mix | 1.5 |
| Finalized Illumina library* | ≤ 23.5 |
| RNase-free water | variable |
| **Total reaction volume** | **50** |

*If available, always use 23.5 µL of finalized Illumina library. Otherwise use all available volume and fill with RNase-free water.

3. Seal the PCR plate using a foil seal, transfer to the thermal cycler and start the program.

4. During the PCR, prepare a matching number of wells of a fresh 96-well plate with each 50 µL of homogenized QIAseq Beads.
5. Once PCR is complete, pulse spin the PCR plate to collect all liquid and transfer the whole reaction to the plate containing QIAseq beads. Mix by thoroughly pipetting up and down.

   **Note**: Alternatively, use a sealing foil to close the plate and mix by vortexing. Gently tap the plate on the benchtop to collect most liquid at the bottom.
6. Incubate the mixture for 5 min at room temperature.
7. Pulse spin the plate to collect all liquid at the bottom.
8. Pellet the beads on a plate-format magnetic stand for 5 minutes or until the solution is clear.
9. Carefully discard the supernatant without disturbing the pellet.
10. With the plate on the magnet, wash the beads by adding 150 µL fresh 80% ethanol to each pellet.
11. Move the plate on the magnet to switch positions and force the pellet to the opposite side of the tube.

    **Note**: Alternatively, incubate for 2 minutes in presence of ethanol.
12. Carefully discard the supernatant without disturbing the beads.
13. Repeat steps 10-12 for a total of 2 ethanol washes.
14. Remove as much excess ethanol as possible.
15. Incubate on the magnetic stand for 5–10 min or until the beads are dry. Overdrying of beads may result in lower DNA recovery.
16. Remove from the magnetic stand and elute modified libraries by resuspending in 32.5 µL of RNase-free water or 10 mM Tris·Cl, pH 8.0.

    **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.

17. Use a sealing foil to close the plate and mix by vortexing until the beads have formed a homogeneous slurry. Gently tap the plate on the benchtop to collect most liquid at the bottom.
18. Incubate for 2 minutes at room temperature, then pulse spin the plate to collect all liquid at the bottom.
19. Pellet the beads on the magnetic stand and carefully transfer 30 µL of the supernatant into a new plate.
20. The collected supernatant contains the modified library and can be used for normalization. Proceed to protocol: QIAseq Library Normalization. Alternatively, modified libraries can be stored for later use at –20°C.

# Protocol: Workflow B - Library modification for use with QIAseq Library Normalizer Kits

This protocol describes the combined modification of compatible libraries during the amplification step of library preparation. Use this protocol for libraries that meet the criteria for workflow B (page 10). After combined amplification/modification, libraries can be normalized using the QIAseq Normalizer workflow (refer to pages 29 and 35).

**Important**: This protocol requires PCR and cleanup reagents of your library preparation kit. Replace the amplification primers or primer mix provided with your library preparation kit by the Normalizer Primer Mix.

## Things to do before starting

- Be sure your libraries and preparation workflow meet the recommendations for use with QIAseq Normalizer (pages 14-15)
- Complete the library preparation process until ligated libraries are ready for the library amplification step.
- Use input amounts that consistently yield libraries with concentrations of at least 15-20 nmol/L or more (page 14). If you opt to use the QIAseq FX DNA Library Kits, libraries with concentrations >20 nmol/L from only 1-5 ng genomic DNA can be generated.
- If ligated libraries were frozen, thaw them on ice. Once libraries are thawed, mix them thoroughly by quick vortexing to avoid any localized concentrations. Briefly spin down libraries.
- Thaw the Normalizer Primer Mix on ice. Then mix thoroughly by quick vortexing to avoid any localized concentrations. Briefly spin down before use.
- Thaw and prepare all other reagents needed for library amplification. These are either included in your library preparation kit or must be purchased separately depending on

the kit used. E.g. QIAseq FX DNA Library Kits include HiFi PCR Master Mix for library amplification.

## Procedure

1. Perform your library preparation workflow according to the manufacturer's instructions until ready for library amplification.
2. Thaw the Normalizer Primer Mix and mix thoroughly by quick vortexing to avoid localized concentrations.
3. Set up a thermal cycler with a heated lid for library amplification according to your library preparation protocol. Also use the number of cycles that is recommended for the respective amount of input material used.

   **Important**: Choose library input amount and cycle number to consistently yield libraries of 15-20 nmol/L or more.
4. Prepare the reaction mix for library amplification in a PCR tube/strip or 96-well plate using the PCR Master Mix or compatible PCR solution from your library preparation kit.
5. Replace your default amplification primers or primer mix by the Normalizer Primer Mix.

   **Note**: If you use non-QIAGEN library preparation solutions, use 1.5 µL of Normalizer Primer Mix for a typical 50 µL reaction and scale up or down proportionally for larger or smaller PCR volumes.

   **Important**: Make sure to use the Normalizer Primer Mix for library amplification. Using standard amplification primer mixes will result in normalization failure.
6. Mix the components in a PCR tube/strip or 96-well plate, load the thermal cycler and start the program.
7. Once PCR is complete, proceed to library cleanup as outlined in you library preparation protocol.
8. At the end of the cleanup procedure, elute modified libraries by resuspending in 32.5 µL of RNase-free water or 10 mM Tris·Cl, pH 8.0.

**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.

9. The collected eluate contains the modified library and can be used for normalization. Proceed to protocol: QIAseq Library Normalization. Alternatively, modified libraries can be stored for later use at –20°C.

# Protocol: (Tube format) QIAseq Library Normalization for use with all QIAseq Normalizer Kits

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

## Things to do before starting

- Modify libraries to be normalized following Workflow A or Workflow B. Do not use non-modified libraries. See page 10 for further guidance.
- Be sure your libraries and preparation workflow meet the recommendations for use with QIAseq Normalizer (pages 14–15)
- For normalization use libraries that consistently reach concentrations of at least 15-20 nmol/L or more.
- Thoroughly mix the Normalizer Reagent by vortexing for 60 seconds. 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 1.5/2.0 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.
- 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 1.5/2.0 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 seconds. 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 Normalization 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 the 1.5 mL tube containing Normalization Reagent. Mix well by pipetting or vortexing.
6. Incubate for 10 minutes 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 minutes 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 minutes.
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 minutes 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 minutes 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. Proceed to "Guidelines: Pooling and sequencing of normalized libraries for use with all QIAseq Normalizer Kits". Alternatively, the normalized libraries can be stored at –20°C for up to 3 months.

# Protocol: (Plate format) QIAseq Library Normalization for use with all QIAseq Normalizer Kits

This protocol describes the normalization of previously modified Illumina libraries in 96-well plate format. Only use libraries that have been modified/amplified in presence of the Normalizer Primer Mix (Workflow A or B). Using non-modified libraries will lead to normalization failure. QIAseq Normalization will yield double-stranded libraries at a concentration of approx. 4 nmol/L. Normalized libraries can be pooled for sequencing at equal volumes without further quantification.

## Things to do before starting

- Modify libraries to be normalized following Workflow A or Workflow B. Do not use non-modified libraries. See page 10 for further guidance.
- Be sure your libraries and preparation workflow meet the recommendations for use with QIAseq Normalizer (pages 14–15)
- For normalization use libraries that consistently reach concentrations of at least 15-20 nmol/L or more.
- Thoroughly mix the Normalizer Reagent by vortexing for 60 seconds. 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. Then prepare an aliquot of wash buffer in 15 mL or 50 mL Falcon tubes and preheat to 55°C using a water bath or a heating block compatible with the receptacle. Prepare 400 µL of wash buffer x number of reactions. E.g. for 72 reactions, prepare 28.8 mL.
- 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. Then prepare an aliquot of wash buffer in 15 mL or 50 mL Falcon tubes and preheat to 55°C using a water bath or a heating block compatible with the receptacle. Prepare 400 µL of wash buffer x number of reactions. E.g. for 72 reactions, prepare 28.8 mL. Leave the wash buffer at 55°C until use.
2. Thoroughly mix the Normalizer Reagent by vortexing for 60 seconds. Make sure the bead pellet 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. Transfer 5 µL of homogenized Normalizer Reagent to a fresh PCR plate using a 10 µL pipette or compatible repetitive pipette. Prepare 1 well for every library to be normalized.
5. Transfer 15 µL of the modified library to be normalized to the PCR plate.
6. Seal the plate using a sealing foil and vortex to mix. Then, gently tap the plate on the benchtop to collect most liquid.
7. Incubate for 10 minutes at room temperature to bind library fragments to the beads.
8. After the incubation is complete, pulse spin the plate to collect all liquid at the bottom. Then carefully remove the foil seal.
9. Add 180 µL pre-warmed Normalizer Wash Buffer to each well.
10. Pellet the beads on a plate-format magnetic stand for 2 minutes or until the solution is clear.

    **Important**: Make sure all beads have pelletized. If you are uncertain, leave the tubes on the magnet for 5 minutes.
11. Using a multichannel pipette, carefully discard the supernatant without disturbing the pellet. Leave the plate on the magnet.

12. Add 200 µL pre-warmed Normalizer Wash Buffer to each well.
13. Move the plate to switch positions on the magnet and force the beads to the opposite side of the tube. Switch the plate position 3 times to thoroughly wash the beads. Then 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 minutes.
14. Using a multichannel pipette set to 150 µL, carefully discard the supernatant without disturbing the pellet. Then, using the same pipette, remove as much remaining liquid as possible. Proceed to the next step immediately.

    **Note**: Drying the beads is not required.
15. Remove the plate from the magnet. Then, add 25 µL Normalizer Elution Buffer to each pellet using a repetitive pipette.
16. Seal the plate using a sealing foil and mix well by vortexing. Make sure the pellet is completely dissolved.
17. Pulse spin the plate at low force.

    **Important**: If a compact bead pellet has formed after pulse spin, vortex again to mix and pulse spin with smaller force.
18. Place the plate in a thermal cycler and incubate for 5 minutes at 55°C with a heated lid.
19. Remove the plate from the thermal cycler and pulse spin to collect all liquid at the bottom, then pellet the beads on a magnetic stand for 2 minutes and wait until the solution is clear.
20. Carefully transfer 22 µL of the supernatant to a new plate using a multichannel pipette without aspirating beads.

    **Important**: Do not discard the supernatant. The supernatant contains the ready-to-sequence library.
21. Proceed to protocol: Library Pooling and Sequencing. Alternatively, the normalized libraries can be stored at –20°C for up to 3 months.

# Guidelines: Pooling and sequencing of normalized libraries for use with all QIAseq Normalizer Kits

The following guidelines describe the pooling and sequencing of normalized libraries for Illumina instruments. Libraries normalized using the QIAseq Library Normalizer Kits or QIAseq Universal Normalizer Kits are double-stranded and have a concentration of approx. 4 nmol/L. Quantification of the library pool prior to sequencing is optional, but can be done by electrophoresis or colorimetric assays. QIAseq normalized libraries are compatible for sequencing on Illumina instruments using patterned or non-patterned flow cells such as the NovaSeq® 6000, NextSeq® 1000/2000 or the NextSeq 550/500, MiSeq® and others.

## Pooling

- 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 approx. 4 nmol/L.
- For joint sequencing, only combine libraries that have different indexes to allow complete demultiplexing of sequence data.
- For optimal pooling results we recommend to only pool libraries of the same type. For example, we do not recommend pooling normalized QIAseq FX DNA Libraries with QIAseq Stranded RNA Libraries. Even though both are compatible for sequencing, combining different library types may result in a less balanced library representation.

## Library pool qualification and optional quantification

- QIAseq Normalizer cannot remove adapter dimer contamination from libraries. If the employed library preparation method tends to yield libraries with strong adapter dimer contamination, we recommend to qualify the library pool by gel electrophoresis prior to sequencing.
- 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.
- Quantificaiton using colorimetric assays (e.g. Qubit) or a photospectrometer (e.g. Nanodrop) 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 5 lists the expected concentration (in ng/µL) for typical library fragment sizes.

**Table 5. Theoretical library concentrations (ng/µL) 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

- Decide on either single-end or paired-end sequencing as well as well as the read length based on the requirements of your type of library.
- The QIAseq normalization procedure does not interfere with the use of custom read or index sequencing primers. If your original library type requires custom primers for sequencing, it will still do so after normalization.
- 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 approx. 4 nmol/L. Follow the system-specific Illumina documents for denaturing and loading - starting from a concentration of 4 nmol/L.

# 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 |
|---|---|---|
| **No detection of clusters on Illumina instruments and/or abort of the sequencing run** | | |
| a) | Too little functional library loaded to sequencer | Perform quantitative PCR and/or gel electrophoresis to assay whether your library pool contains functional library. If there is no functional library detectable or the concentration is very low, please see "The concentration of the normalized libraries is much smaller than 4 nmol/L" in the Troubleshooting section. |
| b) | Too much functional library loaded to sequencer | Perform quantitative PCR and/or gel electrophoresis to assay whether your library pool contains functional library. Make sure your library pool is free of adapter dimers. If the library concentration is very high, please see "The concentration of the normalized libraries is much greater than 4 nmol/L" in the Troubleshooting section. |
| c) | Inappropriate Read1 sequencing primer was used | Use of a Read1 sequencing primer compatible with your library type is essential for cluster definition. Make sure to either use the default or a custom Read1 sequencing primer in accordance with your library type. |
| d) | Loaded libraries were not appropriately denatured | Make sure to denature libraries using an appropriate concentration of NaOH and follow the instrument-specific loading and denaturation guidelines provided in the Illumina documentation. |
| e) | Sequencing instrument failure | Run system diagnostics on your sequencing instrument and/or contact Illumina customer support. |

| | | Comments and suggestions |
|---|---|---|
| **The concentration of the normalized libraries is much greater than 4 nmol/L causing overly high cluster densities and/or sequencing failure,** | | |
| a) | Adapter dimer contamination | Perform gel electrophoresis of your library pool to assay for peaks between 60-120 bp. These peaks represent library adapters and adapter-dimers that occur due to ineffective adapter depletion after library preparation. QIAseq Normalizer cannot remove adapter-dimers from libraries. 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 or may in the worst case lead to sequencing abort. |
| b) | Presence of larger library fragments after library enrichment | Perform gel electrophoresis of your library pool. Test if the fragment population shifts to higher sizes than expected after adapter ligation and library amplification (e.g., more than the expected 120 bp shift). This can be a PCR artifact due to over-amplification of the DNA library. Over-amplified libraries tend to increase cluster densities after normalization. Try to reduce library amplification by 1 or 2 cycles to counteract this effect. |
| c) | Inappropriate washing conditions during normalization | Omitting washing steps or not prewarming the Normalizer Wash Buffer to target temperature may lead to failure in washing away excess of library molecules during normalization. Make sure to conduct washing steps using wash buffer prewarmed to 55°C. |
| **The concentration of the normalized libraries is much smaller than 4 nmol/L causing suboptimal cluster densities or failure to detect clusters** | | |
| a) | Primers other than Normalizer Primer Mix were used for library amplification | Amplifying libraries using the Normalizer Primer Mix is always required prior to normalization. Only modified library molecules can bind to beads contained in the Normalizer Reagent. Using unmodified libraries for normalization will result in complete library loss. Make sure to always use Normalizer Primer Mix to amplify/modify libraries prior to normalization. |
| b) | Input library concentrations were too low | Libraries subjected to normalization have a minimum concentration requirement of 15-20 nmol/L. If the majority of libraries in a sequencing pool had concentrations below that threshold, the post-normalization concentration will likely be below 4 nmol/L. Try to increase the library yield to reach optimal conditions for normalization. Alternatively, adjust the dilution factor when loading the sequencing instrument to compensate. |
| c) | A buffer other than Normalizer Elution buffer was used to elute libraries | Typical nucleic acid elution buffers such as EB, TE or similar will fail to elute normalized libraries from the beads contained in the Normalizer Reagent. Make sure to only use Normalizer Elution Buffer to elute normalized libraries. |

| | | Comments and suggestions |
|---|---|---|
| d) | Elution temperature too low or elution time too short | Normalizer Elution Buffer requires 5 minutes at 55°C to effectively elute libraries from the beads contained in the Normalizer Reagent. Too short incubation times or temperatures less than 55°C may result in incomplete elution and thus in decreased concentrations after normalization. |
| e) | Wash buffer temperature during normalization was too high | Normalizer Wash Buffer at temperatures larger than 55°C may wash off a fraction of bead-bound library molecules resulting in decreased concentrations after normalization. Make sure to prewarm the Normalizer Wash Buffer to 55°C. When applying the wash buffer to the pellet, leave the tube/plate at room temperature. Do not incubate in a heating block or thermal cycler during the washing step. |
| f) | Inappropriate storage conditions | The beads contained in the Normalizer Reagent specifically bind modified library molecules by nature of the bead's surface. Freezing the Normalizer Reagent may damage the beads and impair the binding performance. Always store the Normalizer Reagent and all other components of the QIAseq Normalizer Reagent Kit at 2 – 8 °C. Do not freeze the Normalizer Reagent. |
| **The libraries in the pool or the sequence reads are not homogeneously distributed** | | |
| a) | Different library types were sequenced together | Different types of libraries may form clusters with different efficiencies, which can lead to unbalanced library representation. For optimal sequence distribution we recommend to only pool libraries of the same type. |
| b) | Pooled libraries have different average fragment sizes | Variable average fragment sizes of libraries may lead to variable clustering efficiencies. This can cause unbalanced library representation, even though single libraries have the same concentration after normalization. For optimal balancing, we recommend to only pool libraries with similar fragment size distributions. E.g. libraries generated from high molecular weight DNA and libraries from strongly degraded formalin-compromised DNA will possibly have different fragment size distributions and should not be pooled. |
| c) | Some of the input libraries had very low concentrations | Libraries subjected to normalization have a minimum concentration requirement of 15-20 nmol/L. Libraries within a pool that had lower concentrations before normalization, will likely be underrepresented. For successful normalization we recommend to employ a library preparation workflow with established parameters that consistently yield libraries at concentrations higher than 15-20 nmol/L in 15 µL volume. |

| | | Comments and suggestions |
|---|---|---|
| d) | Use of non-homogenized Normalization Reagent | The beads contained in the Normalizer Reagent bind and release a defined amount of library molecules in a tightly controlled fashion. Therefore, the amount of beads per reaction is pivotal for success. Improper homogenization of the Normalizer Reagent will cause too large or too small amounts of beads to be present per normalization reaction. This will impact the library concentration after normalization proportionally. Make sure the Normalizer Reagent is well homogenized directly before use. |
| e) | Loss of beads during the normalization procedure | The beads contained in the Normalizer Reagent bind and release a defined amount of library molecules in a tightly controlled fashion. Therefore, the amount of beads per reaction is pivotal for success. If beads are lost during binding and washing steps of the normalization workflow, the library concentration after normalization will decrease proportionally. Be sure to carefully aspirate the supernatant during binding and washing steps. Wait sufficiently long for the beads to form a pellet before aspirating the supernatant. |

# Ordering Information

| Product | Contents | Cat. no. |
|---|---|---|
| **QIAseq Normalizer Kits** | | |
| QIAseq Library Normalizer Kit (96) | For 96 reactions: Reagents for the normalization of libraries using QIAseq CDI/UDI Y-Adapters or compatible. | 180605 |
| QIAseq Library Normalizer Kit (24) | For 24 reactions: Reagents for the normalization of libraries using QIAseq CDI/UDI Y-Adapters or compatible. | 180603 |
| QIAseq Universal Normalizer Kit (96) | For 96 reactions: Reagents for the normalization of libraries using indexed library amplification primers. | 180615 |
| QIAseq Universal Normalizer Kit (24) | For 24 reactions: Reagents for the normalization of libraries using indexed library amplification primers. | 180613 |
| **QIAseq Y-Adapter Kits for Illumina** | | |
| QIAseq CDI Y-Adapter Kit (24) | Combinatorial Dual-Index Adapters for Illumina | 180301 |
| QIAseq CDI Y-Adapter Kit (96) | Combinatorial Dual-Index Adapters for Illumina | 180303 |
| QIAseq UDI Y-Adapter Kit (24) | Unique Dual-Index Adapters for Illumina (1–24) | 180310 |
| QIAseq UDI Y-Adapter Kit A (96) | Unique Dual-Index Adapters for Illumina (1–96) | 180312 |
| QIAseq UDI Y-Adapter Kit B (96) | Unique Dual-Index Adapters for Illumina (97–192) | 180314 |

| | | |
|---|---|---|
| QIAseq UDI Y-Adapter Kit C (96) | Unique Dual-Index Adapters for Illumina (193–288) | 180316 |
| QIAseq UDI Y-Adapter Kit D (96) | Unique Dual-Index Adapters for Illumina (289–384) | 180318 |

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.

# Document Revision History

| Revision | Description |
|---|---|
| 04/2023 | Initial release |
| 07/2023 | Updated “Kit Contents” to include RNase Free Water under QIAseq Normalizer PCR Kit |

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