July 2016

QIAseq™ cfDNA All-in-One Kit Handbook

For combined cell-free DNA (cfDNA) extraction and library preparation for next generation sequencing (NGS) applications that use Illumina® instruments

Sample to Insight

QIAGEN

Contents

Kit Contents

QIAseq cfDNA All-in-One Kit Catalog no. Number of reactions		(24) 180023 24	(96) 180025 96
Component	**Tube cap color**	**Quantity in kit**	**Quantity in kit**
QIAGEN Mini columns		24	96
Tube Extenders (20 ml)		1 x 25	4 x 25
Collection Tubes (2.0 ml)		1 x 50	2 x 50
Elution Tubes (1.5 ml)		1 x 50	2 x 50
VacConnectors		1 x 50	2 x 50
Buffer ACL*		1 x 110 ml	2 x 220 ml
Buffer ACB* (concentrate)		3 x 60 ml	2 x 300 ml
Buffer ACW1* (concentrate)		1 x 19 ml	2 x 19 ml
Buffer ACW2† (concentrate)		1 x 13 ml	2 x 13 ml
Buffer AVE†	Violet	2 x 2 ml	10 x 2 ml
QIAGEN Proteinase K		2 x 7 ml	8 x 7 ml
Quickstart protocol		1	1
End-Polishing Enzyme Mix	Violet	2 tubes	1 tube
End-Polishing Buffer, 10x	Blue	1 tube	1 tube
cfDNA Ligase	Orange	1 tube	1 tube
cfDNA Ligation Buffer, 4x	Yellow	1 tube	3 tubes
HiFi PCR Master Mix, 2x	Green	2 tubes	2 tubes
Primer Mix Illumina Libr. Amp	Clear	2 tubes	1 tube
RNase-Free Water (1.9ml)	Clear	4 tubes	4 tubes
Adapter Plate 96-plex Illumina		./.	1 plate
Adapter Plate 24-plex Illumina		1 plate	./.
Quick-Start Protocol		1	1

* Adapters contain chaotropic salt. See page 6 for safety information.

† Contains sodium azide as a preservative.

QIAseq cfDNA Library Kit **Catalog no.** **Number of reactions**		**(96)** **180015** **96**
Component	**Tube cap color**	**Quantity in kit**
End-Polishing Enzyme Mix	Violet	1 tube
End-Polishing Buffer, 10x	Blue	1 tube
cfDNA Ligase	Orange	1 tube
cfDNA Ligation Buffer, 4x	Yellow	3 tubes
HiFi PCR Master Mix, 2x	Green	2 tubes
Primer Mix Illumina Libr. Amp	Clear	1 tube
RNase-Free Water (1.9 ml)	Clear	4 tubes
Adapter Plate 96-plex Illumina	N/A	1 plate
Adapter Plate 24-plex Illumina	N/A	./.
Quick-Start Protocol	N/A	1

Shipping and Storage

The QIAseq cfDNA All-in-One Kit comes in two boxes; the QIAseq cfDNA Library Kit is shipped on dry ice and should be stored immediately upon receipt at –30°C to –15°C in a constant-temperature freezer, the QIAseq cfDNA Extraction Kit is shipped and stored at ambient temperature and the QIAamp Mini columns should be stored at 4°C upon arrival.

Intended Use

QIAseq All-in-One 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 a 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.

CAUTION: DO NOT add bleach or acidic solution directly to waste containing Buffer ACL, Buffer ACB or Buffer ACW1.

Buffer ACL, Buffer ACB and Buffer ACW1 contain guanidine salts, which can form highly reactive compounds when combined with bleach. If liquid containing these buffers is spilt, clean with suitable laboratory detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then clean with 1% (v/v) sodium hypochlorite.

The following risk and safety phrases apply to components of the QIAamp Circulating Nucleic Acid Kit.

Buffer ACL and Buffer ACB

Contains guanidine thiocyanate: harmful. Risk and safety phrases:* R20/21/22-32, S13-26-36/37/39-46

Buffer ACW1

Contains guanidine hydrochloride: harmful, irritant. Risk and safety phrases:* R22-36/38, S13-26-36-46

QIAGEN Proteinase K

Contains proteinase K: sensitizer, irritant. Risk and safety phrases:* R36/37/38-42/43, S23-24-26-36/37

* R20/21/22: Harmful by inhalation, in contact with skin and if swallowed; R22: Harmful if swallowed; R32: Contact with acids liberates very toxic gas; R36/37/38: Irritating to eyes, respiratory system and skin; R36/38: Irritating to eyes and skin; R42/43: May cause sensitization by inhalation and skin contact; S13: Keep away from food, drink and animal feeding items; S23: Do not breathe vapor; S24: Avoid contact with the skin; S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice; S36: Wear suitable protective clothing; S36/37: Wear suitable protective clothing and gloves; S36/37/39: Wear suitable protective clothing, gloves and eye/face protection; S46: If swallowed, seek medical advice immediately and show container or label.

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center (Mainz, Germany), Tel: +49-6131-19240

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of QIAseq cfDNA All-in-One Kit is tested against predetermined specifications to ensure consistent product quality.

Introduction

Free-circulating nucleic acids, such as tumor-specific extracellular DNA fragments in the blood or fetal nucleic acids in maternal blood, are present in plasma usually as short fragments <500 bp (DNA). The concentration of free-circulating DNA (cfDNA) in plasma is usually low and can range considerably from 1–100 ng/ml among different individuals.

Next-generation sequencing (NGS) is a driving force for numerous new and exciting applications, including cancer research and non-invasive prenatal testing (NIPT). While NGS technology is continuously improving, library preparation particularly from cfDNA remains a process bottleneck for many labs.

QIAGEN QIAseq cfDNA All-in-One Kits combine sample extraction and library prep modules that have been fully adapted to each other to maximize success in NGS analysis from plasma samples.

The QIAseq cfDNA Extraction Kits enable efficient purification of cfDNA from human plasma. Samples can be either fresh or frozen (provided that they have not been frozen and thawed more than once). Extension tubes and vacuum processing on the QIAvac 24 Plus enable starting sample volumes of up to 5 ml, and flexible elution volumes between 20 µl and 100 µl allow concentration of nucleic acid species that are present in low amounts in the sample material. Free-circulating cell-free DNA is eluted in Buffer AVE, ready for use in subsequent library preparation as well as amplification reactions or storage at –15 to –30°C. Purified nucleic acids are free of proteins, nucleases and other impurities.

The QIAseq cfDNA Library Kits have been designed to be the definitive solution for generating high quality libraries from free-circulating DNA samples. Intended for NGS researchers who seek a unique library preparation to enable new insights by maximizing performance for any NGS-based liquid biopsy, one can generate NGS libraries from extracted cfDNA in less than 2.5 hours.

Principle and Procedure

QIAseq cfDNA Extraction Kits

The QIAseq cfDNA extraction procedure comprises 4 steps (lyse, bind, wash, elute) and is carried out using QIAamp Mini columns on a vacuum manifold. The robust procedure helps to eliminate sample-to-sample cross-contamination, which is particularly important for NGS applications, and increases user safety when handling potentially infectious samples. The simple procedure, which is highly suited for simultaneous processing of multiple samples, provides pure nucleic acids in less than 2 hours for 24 samples.

Sample volumes

QIAamp Mini columns can bind fragmented nucleic acids that are as short as 20 bases, but yield depends on the sample volume and the concentration of circulating nucleic acids in the sample. The QIAseq cfDNA Extraction procedure has been optimized for large sample volumes of up to 5 ml.

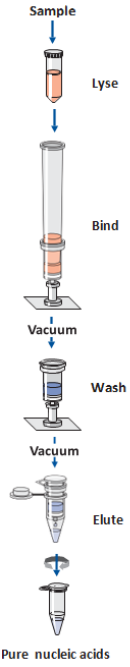


Figure 1: Procedure of column-based manual, cell-free DNA extraction.

Lysing samples

Free-circulating nucleic acids in biological fluids are usually bound to proteins or enveloped in vesicles, requiring an efficient lysis step in order to release nucleic acids for selective binding to the QIAamp Mini column. Hence, samples are lysed under highly denaturing conditions at elevated temperatures in the presence of Proteinase K and Buffer ACL, which

together ensure inactivation of DNases and RNases and complete release of nucleic acids from bound proteins, lipids and vesicles.

Adsorption to the QIAamp Mini membrane

Binding conditions are adjusted by adding Buffer ACB to allow optimal binding of the circulating nucleic acids to the silica membrane. Lysates are then transferred onto a QIAamp Mini column, and circulating nucleic acids are adsorbed from a large volume onto the small silica membrane as the lysate is drawn through by vacuum pressure. Salt and pH conditions ensure that proteins and other contaminants, which can inhibit enzymatic reactions within the library preparation procedure, are not retained on the QIAamp Mini membrane. A vacuum manifold (e.g., the QIAvac 24 Plus with the QIAvac Connecting System) and a vacuum pump capable of producing a vacuum of –800 to –900 mbar (e.g., QIAGEN Vacuum Pump) are required for the protocol. A vacuum regulator should be used for easy monitoring of vacuum pressures and convenient vacuum release.

Removal of residual contaminants

Nucleic acids remain bound to the membrane, while contaminants are efficiently washed away during three wash steps. In a single step, highly pure circulating nucleic acids are eluted in Buffer AVE and equilibrated to room temperature.

Elution of pure nucleic acids

Elution is performed using Buffer AVE. The elution volume can be as low as 40 µl. The eluate volume recovered can be up to 5 µl less than the volume of elution buffer applied to the column; for example, an elution volume of 50 µl results in >45 µl final eluate. Eluted cfDNA is collected in 1.5 ml microcentrifuge tubes (provided). If the purified cfDNA are to be stored for up to 24 hours, storage at 2–8°C is recommended. For periods of storage longer than 24 hours, storage at –15°C to –30°C is recommended.

QIAseq cfDNA Library Kits

Starting from very clean naturally fragmented double-stranded cfDNA, QIAseq cfDNA Library Kits use an optimized end-polishing reaction protocol and a new ligation formulation that optimally fit to the All-in-One sample preparation procedure. Together with QIAGEN's proprietary HiFi PCR Master Mix, this combination maximizes the conversion rate of sample cfDNA into NGS library while efficiently and evenly amplifying even high and low GC content regions of the genome. This protocol enables the highest possible yield of a sequencing library free of adapter dimer contamination starting from 1–100 ng cfDNA input. Due to the kit's flexible protocol, the same kit can also be used for higher DNA input amounts. Highly efficient adapter ligation chemistries enable PCR-free library preparation from as little as 10 ng.

Barcoded sequencing adapter plates

Dual-barcoded, pre-platted adapters are included with both 24- and 96-reaction size QIAseq cfDNA Library Kits. Each well in the 96-plex adapter plate contains a single-use dual-barcoded adapter; the adapters in the 24-plex kit also come in a 96-well plate where only the first three columns are used. The adapters consist of a unique combination of two eight-nucleotide identification barcodes. By combining one D5 barcode and one D7 barcode in each ready-to-use adapter, the 96-reaction QIAseq cfDNA All-in-One Kit supports up to 96-plex pooling of libraries prior to sequencing (see Appendix B for adapter barcode sequencing information).

Bead-based cleanup

QIAGEN's QIAseq cfDNA Library Kit, with its innovative buffer and enzyme formulations, provides an optimized solution to efficiently construct Illumina libraries from as little as 1 ng input DNA. Following adapter ligation and library amplification steps, reaction cleanup and removal of residual adapter dimers can be achieved by using Agencourt AMPure XP beads, which enable easy automation on various high throughput automation platforms.

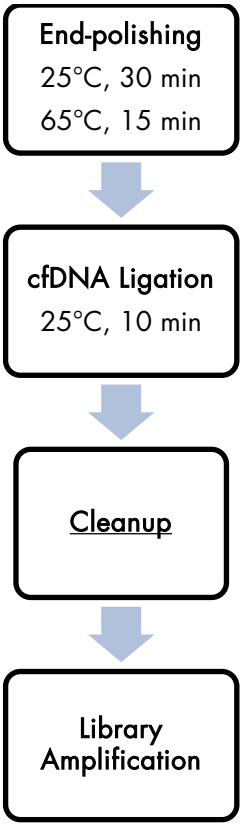


Figure 2: QIAseq cfDNA Library preparation procedure.

Automation

Automated QIAseq sample preparation on the QIAcube

Purification of cfDNA using the QIAseq cfDNA Extraction Kit can be semi-automated on the QIAcube. Perform the lysis and vacuum steps prior to use of the QIAcube. Due to the high sample input volume of up to 5 ml, only the three wash steps and the elution step can be automated on the QIAcube. The innovative QIAcube uses advanced technology to process QIAGEN spin columns, enabling seamless integration of automated, low-throughput sample prep into your laboratory workflow. Sample preparation using the QIAcube follows the same steps as the manual procedure (i.e., wash, dry and elute), enabling you to continue using the QIAseq cfDNA Extraction Kit for purification of high-quality cfDNA. For more information about the automated procedure, see the relevant protocol sheet on cfDNA extraction using QIAamp Mini columns available at www.qiagen.com/MyQIAcube.

PCR setup on QIAgility

QIAgility provides rapid, high-precision PCR setup in almost all plate and tube formats and can thus be used for PCR setup of the library enrichment PCR or qPCR setup for the quality assessment of generated libraries. The easy-to-use software is plug-and-play and requires no special programming, and optimized protocols assure fast startup and immediate results.

Automated library construction

QIAseq cfDNA Library Preparation protocols can be easily automated on the commonly used liquid handling platforms, as they offer: a highly streamlined, one-tube protocol; automation-friendly, single-use barcode adapter plates; and magnetic beads-based size selection and purification.

Description of Protocols

Sample preparation

Two sample preparation protocols are provided in this handbook for different volumes of plasma: one protocol for ≤3 ml plasma and one for processing 4–5 ml plasma.

Library preparation

The library preparation described in this handbook contains two subsequent protocols for generation of cfDNA libraries that are for use on NGS platforms from Illumina. The first protocol describes end polishing, adapter ligation, cleanup and size selection of cfDNA – to generate libraries for next-generation sequencing. The second protocol describes an optional, high-fidelity amplification step that can be used to ensure high amounts of cfDNA library from as little as 1 ng of starting material.

Starting materials

- Plasma samples for combined sample and library prep workflow
- Cell-free DNA (cfDNA) isolated with QIAsymphony SP or any other automated cfDNA extraction protocol for NGS library prep

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.

For sample preparation

- Sterile pipette tips (pipette tips with aerosol barriers are recommended to help prevent cross-contamination) and pipettes
- Water bath or heating block capable of holding 50 ml centrifuge tubes at 60°C
- Heating block or similar at 56°C (capable of holding 2 ml collection tubes)
- Microcentrifuge
- 50 ml centrifuge tubes
- QIAvac 24 Plus vacuum manifold (cat. no. 19413)
- QIAvac Connecting System (cat. no. 19419) or equivalent
- Vacuum Pump (cat. no. 84010 [USA and Canada], 84000 [Japan] or 84020 [rest of world]) or equivalent pump capable of producing a vacuum of –800 to –900 mbar
- Ethanol (96–100%)*
- Crushed ice
- Some samples may require dilution with phosphate-buffered saline (PBS)
- Optional: VacValves (cat. no. 19408)

* Do not use denatured alcohol, which contains other substances such as methanol or methyl ethyl ketone.

For library preparation

- Sterile pipette tips and pipettes

- Agencourt AMPure XP beads (Beckman Coulter Inc., cat. no. A63880, A63881) for bead-based library purification
- 100% ethanol (ACS grade)
- Nuclease-free water
- Buffer EB (QIAGEN, cat. no. 19086)
- PCR tubes or plates
- Vortexer
- Microcentrifuge
- Thermocycler
- Magnetic stand
- Capillary electrophoresis device (e.g., QIAGEN QIAxcel, Agilent Bioanalyzer or similar method to assess the quality of DNA library)
- Rotorgene Q qPCR instrument and QIAseq Library Quant Array Kit (QIAGEN, cat. no. 333304) or a similar Real-time PCR cycler and method for qPCR-based library quantitation

Important Notes

Preparation of Buffers and Reagents

Buffer ACB

Before use, add 200 ml isopropanol (100%) to 300 ml buffer ACB concentrate to obtain 500 ml Buffer ACB. Mix well after adding isopropanol.

Buffer ACW1

Before use, add 25 ml ethanol (96–100%) to 19 ml buffer ACW1 concentrate to obtain 44 ml Buffer ACW1. Mix well after adding ethanol.

Buffer ACW2

Before use, add 30 ml ethanol (96–100%) to 13 ml buffer ACW2 concentrate to obtain 43 ml Buffer ACW2. Mix well after adding ethanol.

The QIAvac 24 Plus

The QIAvac 24 Plus is designed for fast and efficient vacuum processing of up to 24 QIAGEN spin columns in parallel. Samples and wash solutions are drawn through the column membranes by vacuum instead of centrifugation, providing greater speed and reduced hands-on time in purification procedures.

In combination with the QIAvac Connecting System, the QIAvac 24 Plus can be used as a flow-through system. The sample flow-through is collected in a separate waste bottle.

For maintenance of the QIAvac 24 Plus, refer to the handling guidelines in the QIAvac 24 Plus Handbook.

Processing QIAamp Mini columns on the QIAvac 24 Plus

QIAamp Mini columns are processed on the QIAvac 24 Plus using disposable VacConnectors and reusable VacValves. VacValves (optional) are inserted directly into the luer slots of the QIAvac 24 Plus manifold and ensure a steady flow rate, facilitating parallel processing of samples of different natures (e.g., blood and body fluids), volumes or viscosities. They should be used if sample flow rates differ significantly in order to ensure consistent vacuum. VacConnectors are disposable connectors that fit between QIAamp Mini columns and VacValves or between the QIAamp Mini columns and the luer slots of the QIAvac 24 Plus. They prevent direct contact between the spin column and VacValve during purification, thereby avoiding any cross-contamination between samples. VacConnectors are

discarded after a single use. Due to the large solution volumes used, the QIAvac Connecting System (or similar setup with waste bottles) is required (see Figure 3).

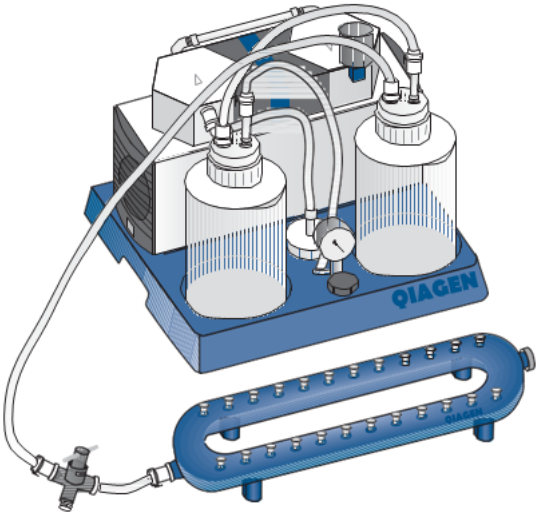


Figure 3: QIAcac 24 Plus, QIAvac Connecting System and Vacuum Pump

Handling guidelines for the QIAvac 24 Plus

- Always place the QIAvac 24 Plus on a secure bench top or work area. If dropped, the QIAvac 24 Plus manifold may crack.
- Always store the QIAvac 24 Plus clean and dry. For cleaning procedures see the QIAvac 24 Plus Handbook.
- The components of the QIAvac 24 Plus are not resistant to certain solvents (Table 1). If these solvents are spilt on the unit, rinse unit thoroughly with water.
- To ensure consistent performance, do not apply silicone or vacuum grease to any part of the QIAvac 24 Plus manifold.

- Always use caution and wear safety glasses when working near a vacuum manifold under pressure.
- Contact QIAGEN Technical Services or your local distributor for information concerning spare or replacement parts.
- The vacuum pressure is the pressure differential between the inside of the vacuum manifold and the atmosphere (standard atmospheric pressure 1013 millibar or 760 mm Hg) and can be measured using the QIAvac Connecting System or a vacuum regulator. The protocols require a vacuum pump capable of producing a vacuum of –800 to –900 mbar (e.g., QIAGEN, Vacuum Pump). Higher vacuum pressures must be avoided. Use of vacuum pressures lower than recommended may reduce nucleic acid yield and purity and increase the risk of clogged membranes.

Table 1. Chemical resistance properties of QIAvac 24 Plus

Resistant to		
Acetic acid	Chaotropic salts	Chlorine bleach
Chromic acid	Concentrated alcohols	Hydrochloric acid
SDS	Sodium Chloride	Sodium hydroxide
Tween® 20	Urea	
Not resistant to		
Benzene	Chloroform	Ethers
Phenol	Toluene	

Setup of the QIAvac 24 Plus vacuum manifold

1. Connect the QIAvac 24 Plus to a vacuum source. If using the QIAvac Connecting System, connect the system to the manifold and vacuum source as described in Appendix A of the QIAvac 24 Plus Handbook.
2. Insert a VacValve (optional) into each luer slot of the QIAvac 24 Plus that is to be used (see Figure 4). Close unused luer slots with luer plugs or close the inserted VacValve.
3. VacValves should be used if flow rates of samples differ significantly to ensure consistent vacuum.

4. Insert a VacConnector into each VacValve (see Figure 3).
5. Perform this step directly before starting the purification to avoid exposure of VacConnectors to potential contaminants in the air.
6. Place the QIAamp Mini columns into the VacConnectors on the manifold (see Figure 4).

   Note: Save the collection tube from the plastic bag for use in the purification protocol.
7. Insert a tube extender (20 ml) into each QIAamp Mini column (see Figure 4).

   Note: Make sure that the tube extender is firmly inserted into the QIAamp Mini column in order to avoid leakage of sample.
8. For nucleic acid purification, follow the instructions in the protocols. Discard the VacConnectors appropriately after use.
9. Leave the lid of the QIAamp Mini column open while applying vacuum.
10. Switch off the vacuum between steps to ensure that a consistent, even vacuum is applied during processing. For faster vacuum release, a vacuum regulator should be used.

    Note: Each VacValve can be closed individually when the sample is completely drawn through the spin column, allowing parallel processing of samples of different volumes or viscosities.
11. After processing samples, clean the QIAvac 24 Plus (see "Cleaning and Decontaminating the QIAvac 24 Plus" in the QIAvac 24 Plus Handbook).

    Note: Buffers ACL, ACB and ACW1 are not compatible with disinfecting agents containing bleach. See page 6 for safety information.

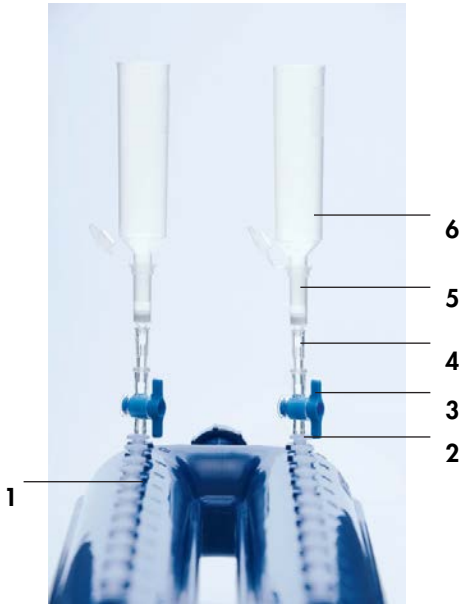


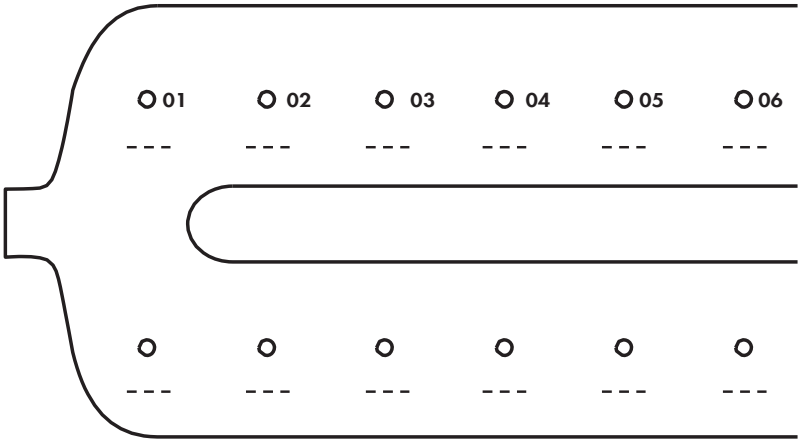
Figure 4: Setting up the QIAvac 24 Plus with QIAamp Mini columns using VacValves, VacConnectors and Tube Extenders. (1: QIAvac 24 Plus vacuum manifold, 2: Luer slot of the QIAvac 24 Plus (closed with luer plug), 3: QIAamp Mini column, 4: VacConnector, VacValce (must be purchased separately), 6: Tube Extender).

We recommend labeling the tubes and the QIAamp Mini columns for use on the QIAvac 24 Plus vacuum system according to the scheme in Figure 5 in order to avoid the mix-up of samples. This figure can be photocopied and labeled with the names of the samples.

Date: _____

Operator: _____

Run ID: _____



.

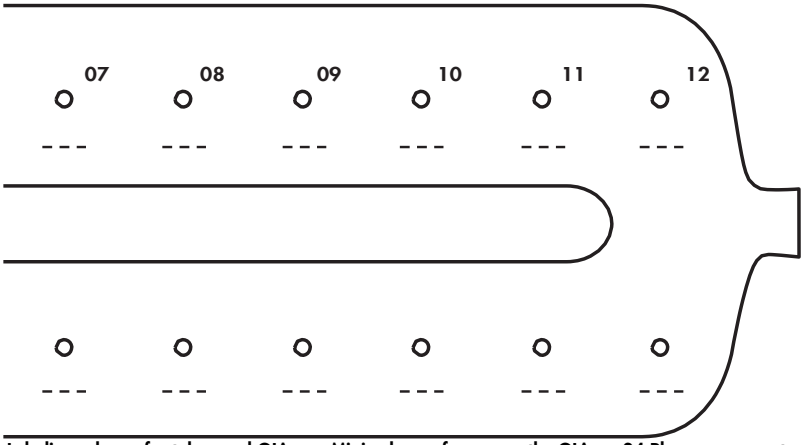


Figure 5: Labeling scheme for tubes and QIAamp Mini columns for use on the QIAvac 24 Plus vacuum system

Recommended library quantification method

We recommend final library quantification by qPCR using primers complementary to the platform-specific adapters. This allows measurement of only complete library molecules, which are the only molecules able to perform bridge PCR on Illumina instruments. QIAGEN's QIAseq Library Quant Array Kit (cat. no. 333304) is highly recommended for accurate qPCR quantification of the prepared library. The QIAseq Library Quant Array Kit is compatible with all major NGS platforms and qPCR instruments, and includes pre-dispensed, sequentially diluted DNA standard to eliminate manual titration steps.

For the most accurate qPCR analysis using the QIAseq Library Quant Array Kit Excel sheet, the library fragment size distribution should be as narrow as possible, with a known mean value in base pairs. Wide fragment distributions are more difficult to accurately quantitate by qPCR, and the smallest library fragments will often be overrepresented in NGS data due to their higher efficiency of amplification during bridge PCR. The double AMPure XP bead cleanup included in the QIAseq cfDNA Library Kit protocol helps to ensure a narrow fragment size distribution for accurate qPCR and even sequencing coverage of all library molecules.

Protocol: cfDNA Sample Extraction

Protocol: Purification of Circulating Nucleic Acids from 1 ml, 2 ml or 3 ml Plasma

Important points before starting

- Prepare Green (marked with a ■) denotes ■ sample volumes of 1 ml plasma; blue (marked with a ◆) denotes ◆ sample volumes of 2 ml plasma; red (marked with an e) denotes e sample volumes of 3 ml plasma.
- All centrifugation steps are carried out at room temperature (15–25°C).
- Switch off vacuum between steps to ensure that a consistent, even vacuum is applied during protocol steps.

Things to do before starting

- Equilibrate samples to room temperature.
- If samples are ■ <1 ml, ◆ <2 ml or e <3 ml, bring the volumes up to ■ 1 ml, ◆ 2 ml or e 3 ml with phosphate-buffered saline.
- Set up the QIAvac 24 Plus as described on pages 19–22.
- Heat a heating block to 56°C for use with 2 ml collection tubes in step 14.
- Equilibrate Buffer AVE to room temperature for elution in step 15.
- Ensure that Buffer ACB, Buffer ACW1 and Buffer ACW2 have been prepared according to the instructions on pages 18–19.

Procedure

1. Pipet ■ 100 µl, ◆ 200 µl or ● 300 µl QIAGEN Proteinase K into a 50 ml centrifuge tube (not provided).
2. Add ■ 1 ml, ◆ 2 ml or ● 3 ml of plasma to the 50 ml tube.
3. Add ■ 0.8 ml, ◆ 1.6 ml or ● 2.4 ml Buffer ACL. Close the cap and mix by pulse-vortexing for 30 s.

   Make sure that a visible vortex forms in the tube. In order to ensure efficient lysis, it is essential that the sample and Buffer ACL are mixed thoroughly to yield a homogeneous solution.

   Note: Do not interrupt the procedure at this time. Proceed immediately to step 4 to start the lysis incubation.
4. Incubate at 60°C for 30 min.
5. Place the tube back on the lab bench and unscrew the cap.
6. Add ■ 1.8 ml, ◆ 3.6 ml or ● 5.4 ml Buffer ACB to the lysate in the tube. Close the cap and mix thoroughly by pulse-vortexing for 15–30 s.
7. Incubate the lysate-Buffer ACB mixture in the tube for 5 min on ice.
8. Insert the QIAamp Mini column into the VacConnector on the QIAvac 24 Plus. Insert a 20 ml tube extender into the open QIAamp Mini column. Make sure that the tube extender is firmly inserted into the QIAamp Mini column in order to avoid leakage of sample.

   Note: Keep the collection tube for the dry spin in step 13.
9. Carefully apply the lysate-Buffer ACB mixture from step 7 into the tube extender of the QIAamp Mini column. Switch on the vacuum pump. When all lysates have been drawn through the columns completely, switch off the vacuum pump and release the pressure to 0 mbar. Carefully remove and discard the tube extender. Please note that large sample lysate volumes (about 11 ml when starting with 3 ml sample) may need up to 10 minutes to pass through the QIAamp Mini membrane by vacuum force. For fast and convenient

release of the vacuum pressure, the Vacuum Regulator should be used (part of the QIAvac Connecting System).

Note: To avoid cross-contamination, be careful not to move the tube extenders over neighboring QIAamp Mini columns.

10. Apply 600 µl Buffer ACW1 to the QIAamp Mini column. Leave the lid of the column open, and switch on the vacuum pump. After all of Buffer ACW1 has been drawn through the QIAamp Mini column, switch off the vacuum pump and release the pressure to 0 mbar.
11. Apply 750 µl Buffer ACW2 to the QIAamp Mini column. Leave the lid of the column open, and switch on the vacuum pump. After all of Buffer ACW2 has been drawn through the QIAamp Mini column, switch off the vacuum pump and release the pressure to 0 mbar.
12. Apply 750 µl of ethanol (96–100%) to the QIAamp Mini column. Leave the lid of the column open, and switch on the vacuum pump. After all of ethanol has been drawn through the spin column, switch off the vacuum pump and release the pressure to 0 mbar.
13. Close the lid of the QIAamp Mini column. Remove it from the vacuum manifold and discard the VacConnector. Place the QIAamp Mini column in a clean 2 ml collection tube, and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min.
14. Place the QIAamp Mini column into a new 2 ml collection tube. Open the lid, and incubate the assembly at 56°C for 10 min to dry the membrane completely.
15. Place the QIAamp Mini column in a clean 1.5 ml elution tube (provided) and discard the 2 ml collection tube from step 14. Carefully apply 40–60 µl of Buffer AVE to the center of the QIAamp Mini membrane. Close the lid and incubate at room temperature for 3 min.

    Important: Ensure that the elution buffer AVE is equilibrated to room temperature (15–25°C). If elution is done in small volumes (<50 µl), the elution buffer has to be dispensed onto the center of the membrane for complete elution of bound DNA. Elution volume is flexible and can be adapted according to the requirements of downstream applications.

The recovered eluate volume will be up to 5 µl less than the elution volume applied to the QIAamp Mini column.

16. Centrifuge in a microcentrifuge at full speed (20,000 x g; 14,000 rpm) for 1 min to elute the nucleic acids.

Protocol: Purification of Circulating Nucleic Acids from 4 ml or 5 ml Plasma

Important points before starting

- Prepare Blue (marked with a ◆) denotes ◆ sample volumes of 4 ml plasma; red (marked with an e) denotes e sample volumes of 5 ml plasma.
- All centrifugation steps are carried out at room temperature (15–25°C).
- Switch off vacuum between steps to ensure that a consistent, even vacuum is applied during protocol steps.

Things to do before starting

- Equilibrate samples to room temperature.
- If samples are ◆ <4 ml or e < 5 ml, bring the volumes up to ◆ 4 ml or e 5 ml with phosphate-buffered saline.
- Set up the QIAvac 24 Plus as described on pages 19–22.
- Heat a water bath or heating block to 60°C for use with 50 ml centrifuge tubes in step 4.
- Heat a heating block to 56°C for use with 2 ml collection tubes in step 14.
- Equilibrate Buffer AVE to room temperature for elution in step 15.
- Ensure that Buffer ACB, Buffer ACW1, and Buffer ACW2 have been prepared according to the instructions on page 18–19.

Procedure

1. Pipet ◆ 400 µl or e 500 µl QIAGEN Proteinase K into a 50 ml centrifuge tube (not provided).
2. Add ◆ 4 ml or e 5 ml of plasma to the tube.
3. Add ◆ 3.2 ml or e 4.0 ml Buffer ACL. Close the cap and mix by pulse-vortexing for 30 s. Make sure that a visible vortex forms in the tube. To ensure efficient lysis, it is

essential that the sample and Buffer ACL are mixed thoroughly to yield a homogeneous solution.

Note: Do not interrupt the procedure at this time. Proceed immediately to step 4 to start the lysis incubation.

4. Incubate at 60°C for 30 min.
5. Place the tube back on the lab bench and unscrew the cap.
6. Add 7.2 ml or 9 ml Buffer ACB to the lysate in the tube. Close the cap and mix thoroughly by pulse-vortexing for 15–30 s.
7. Incubate the lysate-Buffer ACB mixture in the tube for 5 min on ice.
8. Insert the QIAamp Mini column into the VacConnector on the QIAvac 24 Plus. Insert a 20 ml tube extender into the open QIAamp Mini column. Make sure that the tube extender is firmly inserted into the QIAamp Mini column in order to avoid leakage of sample.

   Note: Keep the collection tube for the dry spin in step 13.
9. Carefully apply the lysate-Buffer ACB mixture from step 7 into the tube extender of the QIAamp Mini column. Switch on the vacuum pump. When all lysates have been drawn through the columns completely, switch off the vacuum pump and release the pressure to 0 mbar. Carefully remove and discard the tube extender.

   Please note that large sample lysate volumes (about 20 ml when starting with 5 ml sample) may need up to 15 minutes to pass through the QIAamp Mini membrane by vacuum force. For fast and convenient release of the vacuum pressure, the Vacuum Regulator should be used (part of the QIAvac Connecting System).

   Note: To avoid cross-contamination, be careful not to move the tube extenders over neighboring QIAamp Mini columns.
10. Apply 600 µl Buffer ACW1 to the QIAamp Mini column. Leave the lid of the column open, and switch on the vacuum pump. After all of Buffer ACW1 has been drawn through the QIAamp Mini column, switch off the vacuum pump and release the pressure to 0 mbar.

11. Apply 750 µl Buffer ACW2 to the QIAamp Mini column. Leave the lid of the column open, and switch on the vacuum pump. After all of Buffer ACW2 has been drawn through the QIAamp Mini column, switch off the vacuum pump and release the pressure to 0 mbar.
12. Apply 750 µl of ethanol (96–100%) to the QIAamp Mini column. Leave the lid of the column open, and switch on the vacuum pump. After all of ethanol has been drawn through the spin column, switch off the vacuum pump and release the pressure to 0 mbar.
13. Close the lid of the QIAamp Mini column. Remove it from the vacuum manifold and discard the VacConnector. Place the QIAamp Mini column in a clean 2 ml collection tube, and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min.
14. Place the QIAamp Mini Column into a new 2 ml collection tube. Open the lid, and incubate the assembly at 56°C for 10 min to dry the membrane completely.
15. Place the QIAamp Mini column in a clean 1.5 ml elution tube (provided) and discard the 2 ml collection tube from step 14. Carefully apply 40–60 µl of Buffer AVE to the center of the QIAamp Mini membrane. Close the lid and incubate at room temperature for 3 min.

    Important: Ensure that the elution buffer AVE is equilibrated to room temperature (15–25°C). If elution is done in small volumes (<50 µl), the elution buffer has to be dispensed onto the center of the membrane for complete elution of bound DNA. Elution volume is flexible and can be adapted according to the requirements of downstream applications. The recovered eluate volume will be up to 5 µl less than the elution volume applied to the QIAamp Mini column.
16. Centrifuge in a microcentrifuge at full speed (20,000 x g; 14,000 rpm) for 1 min to elute the nucleic acids.

Protocol: cfDNA Library Preparation

Important points before starting

- This protocol is for constructing sequencing libraries from 1–100 ng of cfDNA input for Illumina NGS platforms.
- QIAseq sequencing adapters are dissolved in duplex buffer and are ready to use.
- Adapters are fully compatible with all Illumina instruments, including MiniSeq, MiSeq, NextSeq and HiSeq instruments. A PCR step is not required to complete the adapter sequences – they are full length and ready for sequencing following the ligation step.
- The majority of circulating cell-free DNA (cfDNA) has the size of around 170 bp and does not require further fragmentation prior to library step.
- Do not use a heated lid during the adapter ligation step.

Protocol: End-Polishing and Adapter Ligation

This protocol describes end repair, A-addition, adapter ligation and library cleanup, generating libraries that are ready to quantify and use in next-generation sequencing on instruments from Illumina.

Things to do before starting

- Thaw frozen reagents on ice. Once thawed, buffers should be mixed thoroughly by quick vortexing to avoid any localized concentrations. Briefly spin down vortexed reagents before use.
- Sample cfDNA should be dissolved in AVE, EB/Tris buffer or H2O before starting.
- The majority of circulating cell-free DNA (cfDNA) has the size of around 170 bp and does not require further fragmentation prior to library preparation.
- Prepare 80% ethanol

Procedure

Table 2. End-polishing and adapter ligation thermal cycling conditions

Program	Temperature	Time	Additional Comments
End-Polishing	25°C	30 min	Polishing the ends of cfDNA fragments
	65°C	15 min	Inactivation of end-polishing enzymes; A-tailing
	4°C	∞	Hold
Ligation	25°C	10 min	Ligation of the adapters to the cfDNA fragments
	4°C	∞	Hold

End-polishing

1. Set up the End-polishing Reaction Mix on ice according to Table 3.

Table 3. End-polishing reaction setup

Component	Volume/reaction (µl)
Input DNA (cfDNA)	Variable
End-Polishing Buffer 10X	5
End-Polishing Enzyme Mix	2
Nuclease-free water	Variable
Total reaction volume	**50**

2. Mix by gently pipetting. Do not vortex.
3. Load into the thermal cycler and start end-polishing program (Table 2). Proceed to the next step.

Table 4. Ligation reaction setup

Component	Volume/reaction (µl)
End-polished cfDNA	50
cfDNA Ligation Buffer, 4X	25
cfDNA Ligase	5
QIAseq Adapter (96-plex or 24-plex plate)	1
Nuclease-free water	19
Total reaction volume	**100**

Adapter Ligation

4. During the end-polishing reaction, vortex and spin down the thawed adapter plate. Remove the protective adapter plate lid.
5. When the thermal cycler reaches the 4°C hold, stop the program.
6. Remove reactions from the thermal cycler and add ligation components on ice according to Table 4. Pierce foil seal of the individual adapter plate well with pipette tip used for adapter addition to minimize risk of cross contamination. Return any unused adapter to –20°C.
7. Mix adapter ligation reactions by pipetting 5–6 times.
8. Incubate at 25°C for 10 min.

   Important: Do not use a thermocycler with a heated lid.
9. When the program is complete, proceed immediately to adapter ligation cleanup.
10. Add 80 µl resuspended Agencourt® AMPure® XP beads to each sample and mix.
11. Incubate for 5 min at room temperature.
12. Pellet the beads on a magnetic stand and carefully discard the supernatant.
13. Add 200 µl fresh 80% ethanol to each pellet. Pellet the beads on the magnetic stand and carefully discard the supernatant.
14. Repeat step 13 for a total of 2 ethanol washes. Remove excess ethanol.

15. Incubate on the magnetic stand for 5–10 min or until the beads are dry. Overdrying may result in lower DNA recovery. Remove from the magnetic stand.
16. Elute by resuspending in 52.5 µl Buffer EB. Pellet beads on the magnetic stand. Carefully transfer 50 µl supernatant to a new PCR plate.
17. Add 50 µl of resuspended Agencourt AMPure XP beads to each sample.
18. Follow steps 11–15 above. Elute by resuspending in 26 µl Buffer EB. Pellet the beads on the magnetic stand. Carefully transfer 23.5 µl of supernatant into a new PCR plate or tube.
19. If the library will not be amplified, store the DNA at –20°C until ready for QC, quantification and sequencing. If amplifying the library, proceed with the protocol "Amplification of cfDNA Library."
20. Assess the library quality using a capillary electrophoresis method. The median library size will be the fragment size plus 120 bp for the adapters. Libraries can be quantified with qPCR using a QIAseq Library Quant Array (QIAGEN, cat. no. 333304).

Note: Libraries generated from less than 10 ng input DNA may not be visible by capillary electrophoresis prior to library amplification.

Protocol: Amplification of cfDNA Library

PCR-based library amplification is normally required if the input cfDNA amount is below 10 ng, or if a higher amount of final library is needed (e.g., for hybrid capture experiments). This protocol is for optional, high-fidelity amplification of the cfDNA library using the QIAseq HiFi PCR Master Mix that is included in the kit.

Things to do before starting

- Prepare library cfDNA using the protocol "End-Polishing and Adapter Ligation"

- Thaw frozen reagents on ice. Once thawed, buffers should be mixed thoroughly by quick vortexing to avoid any localized concentrations. Briefly spin down vortexed reagents before use.

Table 5. Library amplification cycling conditions

Incubation time	Temperature	Number of cycles
2 min	98°C	1
20 s 30 s 30 s	98°C 60°C 72°C	Variable depending on DNA input*: 5 (100 ng input); 8 (10 ng input); 10 (1 ng input)
1 min	72°C	1
∞	4°C	Hold

* Number of PCR cycles depends on input DNA amount and quality. Sufficient library amounts for sequencing can be achieved from 10 ng of input without PCR.

Table 6. Amplification reaction setup

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

1. Program a thermal cycler with heated lid according to Table 5.
2. Prepare reactions on ice according to Table 6. Pipet 6–8 times to mix.
3. Transfer the PCR plate to the thermocycler and start the program.
4. When the program is complete, remove the plate and add 50 µl of resuspended Agencourt AMPure XP beads to each amplified library.
5. Follow steps 11–15 above. Elute by resuspending in 25 µl nuclease-free water or Buffer EB. Pellet the beads on the magnetic stand. Carefully transfer supernatants to new DNA LoBind tubes for storage.

6. The purified library can be safely stored at –20°C until ready for sequencing or hybrid capture.

Library QC and quantification

1. Assess the library quality using a capillary electrophoresis device such as QIAGEN QIAxcel or Agilent BioAnalyzer. Check for expected size distribution and the absence of adapters or adapter dimers around 120 bp (see Figure 6).

   Note: The median library size will be the fragment size plus 120 bp for the adapters. Libraries can be quantified with qPCR using a QIAseq Library Quant Array (sold separately).

   Note: The median fragment size can be used for subsequent qPCR-based quantification methods to quantify the library concentration (step 2).

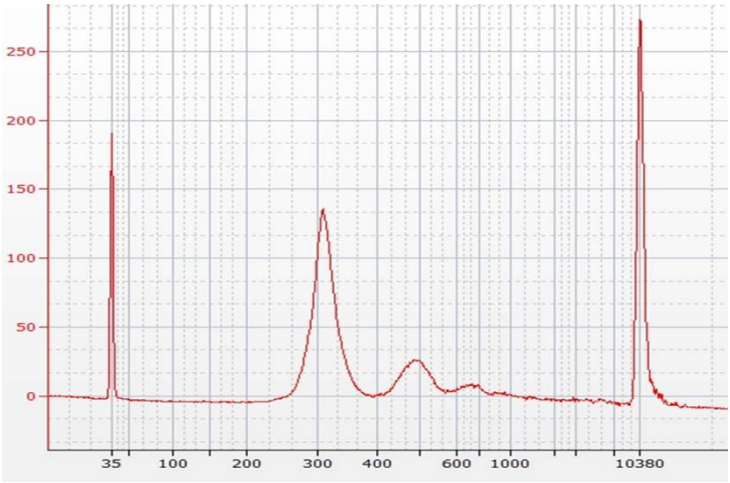


Figure 6. Capillary electrophoresis device trace data showing the correct size distribution of library fragments from cfDNA and the absence of adapters or adapter dimers.

2. Quantify the library using the QIAseq Library Quant Array Kit or a comparable qPCR-based method.

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**).

QIAseq cfDNA Extraction Kit

		Comments and suggestions
Little or no nucleic acids in the eluate		
a)	Primary blood tube contains an anticoagulant other than EDTA	Anticoagulants other than EDTA may lead to accelerated DNA degradation compared to EDTA blood. Repeat the purification procedure with new samples.
b)	Extended time between blood draw and plasma preparation	Blood cells may disintegrate and release genomic DNA into the plasma, diluting the target nucleic acid.
c)	Samples frozen and thawed more than once	Repeated freezing and thawing should be avoided as this may lead to DNA degradation. Always use fresh samples or samples thawed only once.
d)	Low concentration of target DNA in the samples	Samples were left standing at room temperature for too long. Repeat the purification procedure with new samples.
e)	Inefficient sample lysis in Buffer ACL	If QIAGEN Proteinase K was subjected to elevated temperature for a prolonged time, it can lose activity. Repeat the procedure using new samples and fresh QIAGEN Proteinase K.
f)	Low-percentage ethanol used instead of 96–100%	Repeat the purification procedure with new samples and 96–100% ethanol. Do not use denatured alcohol, which may contain methanol or methyl ethyl ketone.
g)	Buffer ACB prepared incorrectly	Check that Buffer ACB concentrate was reconstituted with the correct volume of isopropanol (not ethanol, see page 18).

		Comments and suggestions
h)	Buffer ACW1 or Buffer ACW2 prepared incorrectly	Check that Buffer ACW1 and Buffer ACW2 concentrates were diluted with the correct volume of ethanol (see page 19). Repeat the purification procedure with new samples.
i)	Buffer ACW1 or Buffer ACW2 prepared with 70% ethanol	Check that Buffer ACW1 and Buffer ACW2 concentrates were diluted with 96–100% ethanol (see page 19). Repeat the purification procedure with new samples.

		Comments and suggestions
DNA does not perform well in downstream library preparation		
a)	Little or no DNA in the eluate	See "Little or no nucleic acids in the eluate" above for possible reasons. Increase the amount of eluate added to the reaction if possible.
b)	Inappropriate elution volume used	Using elution volumes of 40-60µl is optimal for downstream library preparation protocol. Small elution volumes lead to high percentage of sample loss on extraction column, too high elution volumes may lead to highly diluted DNA and thereby limit the percentage of DNA sample that can be used for library generation.
c)	Buffers not mixed thoroughly	Salt and ethanol components of wash Buffer ACW2 may have separated out after being left for a long period between runs. Always mix buffers thoroughly before each run.
General handling		
a)	Clogged QIAamp Mini column	Close the VacValve, if used, and carefully remove the whole assembly consisting of tube extender, QIAamp Mini column, VacConnector and VacValve from the QIAvac 24 Plus manifold. Carefully transfer the remaining sample lysate from the tube extender to a new 50 ml tube. Remove the QIAamp Mini column from the assembly (see above), place it in a 2 ml collection tube and spin it at full speed for 1 minute or until sample has completely passed through the membrane. Re-assemble the QIAamp Mini column with Tube Extender, VacConnector and (optional) VacValve. Transfer the remaining sample lysate into the Tube Extender, switch on the vacuum pump, open the VacValve, and pass the remaining lysate through the QIAamp Mini column. Repeat the above procedure if the QIAamp Mini column continues to clog. Cryoprecipitates may have formed in plasma due to repeated freezing and thawing. These can block the QIAamp Mini column. Do not use plasma that has been frozen and thawed more than once.

b)	Little or no DNA in the eluate	Different samples can affect the volume of the final eluate. The recovered eluate volume will be up to 5 µl less than the elution volume applied to the QIAamp Mini column.
c)	Presence of larger library fragments prior to library enrichment.	The vacuum manifold is not tightly closed. Press down on the lid of the vacuum manifold after the vacuum is switched on. Check if vacuum pressure is reached.
		Gasket of QIAvac lid has worn out. Check the seal of the manifold visually and replace it if necessary. VacValves have worn out. Remove all VacValves and insert VacConnectors directly into the luer extensions. Insert QIAamp Mini columns into VacConnectors, close the lid of the columns and switch on vacuum. Check if vacuum pressure is reached. Replace VacValves if necessary.

QIAseq cfDNA Library Kit

		Comments and suggestions
Low library yields		
a)	Suboptimal reaction conditions due to low DNA quality	Make sure to use the highest quality sample DNA available to ensure optimal activity of the library enzymes.
b)	Insufficient amount of starting DNA for direct sequencing without library amplification	Typically, 100 ng of fragmented genomic 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 or applications (eg., hybrid capture), a library amplification step can be performed.
c)	Insufficient amount of starting DNA for direct sequencing without library amplification	RNA from the sample material can be co-purified with genomic DNA. This contaminating RNA will affect the accuracy of DNA quantification. To remove RNA during the sample preparation protocol, it is recommended to perform RNase A treatment of the DNA.
d)	Overdrying of the Ampure beads during clean-up steps.	Overdrying of the Ampure beads can make it difficult to elute the DNA off the beads. Do not dry beads for more than 10 min at room temperature.
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 Illumina flow cell and will be sequenced, this will reduce the capacity of the flow cell for the library fragments. A low ratio of adapter dimers versus library will not be a problem. Please make sure the correct volume of the AMPure beads is used for the cleanup steps.
b)	Presence of larger library fragments after library enrichment	If the fragment population shifts higher 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. Make sure to use as few amplification cycles as possible to avoid this effect.
c)	Presence of larger library fragments after library enrichment	If the fragment population shifts higher than expected, this can also be due to the carry-over of the AMPure beads. Make sure not to aspirate beads while taking supernatant during the clean-up steps.
d)	Incorrect library fragment size after adapter ligation	During library preparation, adapters of approximately 60 bp are ligated to both ends of the DNA library fragments. This should be reflected on a capillary electrophoresis device by a shift in size of all library fragments of 120 bp. If using library adapters from other suppliers, please refer to the size information given in the respective documentation.

Appendix A: Recommendation for Plasma Separation and Storage

In order to isolate circulating, cell-free nucleic acids from blood samples, we recommend following this protocol which includes a high g-force centrifugation step in order to remove cellular debris and thereby reduces the amount of cellular or genomic DNA in the sample.

1. Place whole EDTA blood in BD Vacutainer™ tubes (or other primary blood tubes containing EDTA as anti-coagulant) in centrifuge with swinging-out rotor and appropriate buckets.
2. Centrifuge blood samples for 10 min at 1900 x g [3000 rpm] and +4°C temperature setting.
3. Carefully aspirate plasma supernatant without disturbing the buffy coat layer. About 4–5 ml plasma can be obtained from one 10 ml primary blood tube.

   Note: Plasma can be used for circulating nucleic acid extraction at this stage.
4. However, the following high-speed centrifugation will remove additional cellular debris and contamination of the circulating nucleic acids by gDNA and RNA derived from damaged blood cells.
5. Aspirated plasma is transferred into fresh 15 ml centrifuge tubes with conical bottom.
6. Centrifuge plasma samples for 10 min at 16,000 x g [in fixed-angle rotor] and +4°C temperature setting. This will remove additional cellular nucleic acids attached to cell debris.
7. Carefully remove supernatant to a new tube with a pipette without disturbing the pellet.
8. If plasma will be used for nucleic acid extraction on the same day, store at 2–8°C until further processing. For longer storage, keep plasma frozen at –80°C. Before using the plasma for circulating nucleic acid extraction, thaw plasma tubes at room temperature.

In case of cryoprecipitates, follow these two steps:

- To remove cryoprecipitates, centrifuge plasma sample for 5 min at 16,000 x g [in fixed angle rotor] and 4°C temperature setting.
- Remove supernatant to a new tube, and then begin with the nucleic acid extraction protocol.

Appendix B: Adapter Barcodes for the 96-plex and 24-plex Adapter Plates

The barcode sequences used in the QIAseq cfDNA Library Kit 96-plex and 24-plex adapter plates are listed in Table 7. Indices 501–508 and 701–712 correspond to the respective Illumina adapter barcodes. The layout of the 96-plex and 24-plex single use adapter plates are displayed in Figures 7 and 8.

Table 7. Adapter barcodes used in the QIAseq cfDNA Library Kit (96) Adapter Plate

Codes for entry on sample sheet				
D50X barcode name	**i5 bases for entry on sample sheet (MiSeq, HiSeq 2000/ 2500)**	**i5 bases for entry on sample sheet (MiniSeq, NextSeq, HiSeq 3000/ 4000)***	**D50X barcode name**	**i7 bases for entry on sample sheet**
D501	TATAGCCT	AGGCTATA	D701	ATTACTCG
D502	ATAGAGGC	GCCTCTAT	D702	TCCGGAGA
D503	CCTATCCT	AGGATAGG	D703	CGCTCATT
D504	GGCTCTGA	TCAGAGCC	D704	GAGATTCC
D505	AGGCGAAG	CTTCGCCT	D705	ATTCAGAA
D506	TAATCTTA	TAAGATTA	D706	GAATTCGT
D507	CAGGACGT	ACGTCCTG	D707	CTGAAGCT
D508	GTACTGAC	GTCAGTAC	D708	TAATGCGC
			D709	CGGCTATG
			D710	TCCGCGAA
			D711	TCTCGCGC
			D712	AGCGATAG

* Note: Sequencing on the MiniSeq, NextSeq and HiSeq 3000/4000 systems follow a different dual-indexing workflow than other Illumina systems, which requires the reverse complement of the i5 index adapter sequence.

	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 7. 96-plex Adapter Plate layout.

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

Figure 8: 24-plex Adapter Plate layout.

Appendix C: Library Quantification and Quality Control

Quality control for the library construction process can be performed using QIAGEN's QIAseq Library Quant Array Kit (cat. no. 333304). With this assay, the correct dilution of the library can also be determined for sequencing. Please refer to the corresponding handbook for library quantification and quality control.

Ordering Information

Product	Contents	Cat. no.
QIAseq cfDNA All-in-One Kit (96)	For 96 reactions on Illumina sequencers: QIAamp Mini columns, tubes, reagents and buffers for cfDNA extraction for NGS. Enzymes and buffers for cfDNA library prep, Illumina Adapter Plate 96-plex, Illumina Library Amplification Primer and PCR Master Mix	180025
QIAseq cfDNA All-in-One Kit (24)	For 24 reactions on Illumina sequencers: QIAamp Mini columns, tubes, reagents and buffers for cfDNA extraction for NGS. Enzymes and buffers for cfDNA library prep, Illumina Adapter Plate 24-plex, Illumina Library Amplification Primer and PCR Master Mix	180023
QIAseq cfDNA Library Kit (96)	For 96 reactions on Illumina sequencers: enzymes and buffers for cfDNA library prep, Illumina Adapter Plate 96-plex, Illumina Library Amplification Primer and PCR Master Mix	180015
Related products		
QIAseq Library Quant Array Kit	Laboratory-verified forward and reverse primers for 500 x 25 µl reactions (500 µl); DNA Standard	333304

	(100 µl); Dilution Buffer (30 ml); (1.35 ml x 5) GeneRead qPCR SYBR® Green Mastermix

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.

1103581 HB-2178-001 07/2016