Preanalytical considerations

QIAGEN

Prepare for successful biomarker profiling from cell-free DNA

Sample to Insight

Contents

Cell-free DNA as a biomarker

Cell-free DNA (cfDNA) shed into the bloodstream or body fluids of healthy or disease-affected individuals is an important analyte in liquid biopsy. These circulating DNA fragments can reveal various alterations such as single nucleotide variants, insertions and deletions and larger chromosomal abnormalities, including copy translocations. Addtional information, including structural variants or modifications, such as DNA methylation, would complete cfDNA assessment. All of these have potential use as biomarkers.

cfDNA holds great promise to improve clinical practice in several ways:

- By advancing basic disease research and improving our understanding of cfDNA release mechanisms and tissues of origin
- As a companion diagnostic for identifying cancer treatment options through analysis of a defined set of mutations or chromosomal aberrations
- For early disease detection in cancer, the identification of the tissue of origin, therapy monitoring and the assessment of emerging resistance or minimal residual disease monitoring
- For non-invasive prenatal testing (NIPT), enabling the detection of fetal chromosomal abnormalities by analyzing fetal DNA in maternal blood
- As a surrogate marker for post-transplant monitoring

These technical guidelines compile our expert scientists' best practice recommendations and advice to support the standardization of methods for preanalytical cfDNA sample collection and preparation.

Biology of cfDNA

In healthy individuals, most cfDNA comes from cells of hematopoietic origin (1), primarily through DNA shedding during apoptosis. cfDNA of apoptotic origin typically circulates in fragments ranging from 120–220 base pairs (bp), with a predominant fragment size of around 166–170 bp. This corresponds to the length of DNA that can wrap around a nucleosome (147 bp), plus an additional stretch of DNA to link two nucleosome cores.

Apoptosis also can produce longer cfDNA fragments that correspond to di-, tri- or poly-nucleosomes. (2) Besides apoptosis, cfDNA can be generated by releasing genomic or mitochondrial DNA from any cell type via various mechanisms, including necrosis, active secretion, pyroptosis, autophagy, mitotic catastrophes and NETosis.

The half-life of cfDNA in blood ranges from 15 minutes to 2.5 hours (3), and when it originates from tumor cells, we call it circulating tumor DNA (ctDNA).

cfDNA typically circulates in fragments that range from 120–220 base pairs, with a predominant fragment size of 170 base pairs.

Challenges in cfDNA analysis

The molecular analysis of cfDNA in biofluid samples is complicated by various challenges. First, the analyte is only available in very low and highly variable concentrations, ranging from 1–50 ng/mL in healthy individuals.
cfDNA is also highly fragmented and often degraded, affecting downstream molecular analyses. Furthermore, the target cfDNA molecules for analysis are typically diluted by a higher-abundance background of wild-type or maternal cfDNA and genomic DNA, which makes it difficult to detect the marker of interest.

The potential for experimental bias through contamination with cellular DNA or substances that inhibit downstream analyses is high, so it is crucial to minimize this risk. During the storage and transport of blood samples, cells can lyse and release DNA, making it appear that the sample has a higher level of cfDNA than it actually does. The type of anticoagulant and crosslinking reagents in blood collection tubes can impact the quality of cfDNA and the end results (4).

Preanalytical handling

Various biofluids, including blood serum or plasma and urine, can be used for cfDNA analysis. The choice of sample type depends on the research objective and disease context.

Analysis of cfDNA in serum and plasma samples is vital to many research applications, including the evaluation of the fetal DNA fraction in maternal blood or the examination of circulating tumor DNA.

Five key pitfalls to avoid in cfDNA purification:

- Lack of stabilization during sample collection
- Inadequate storage or transportation concept
- Suboptimal centrifugation parameters
- Cumbersome or inappropriate cfDNA purification method
- Suboptimal methods for quality control or yield quantification

Blood serum and plasma

Avoiding cellular DNA contamination

Obtaining accurate results requires appropriate collection and isolation methods that ensure preservation of the sample's actual cfDNA profile. Since cfDNA is only present in blood in low amounts, its level can dramatically change if it becomes diluted with genomic DNA from apoptotic or lysed cells from improper handling.

Therefore, you should take measures to prevent lysis of cells in all steps, from collecting whole blood to preparing serum and plasma. Failure to do so may prevent the subsequent detection of low-abundance cfDNA.

Cellular DNA contamination will influence all quantifications of cfDNA yield, except for capillary electrophoresis analysis and similar methods that assess DNA fragment sizes. Cellular contamination can be indirectly quantified by quantitative PCR using both a short and a long target amplicon, where the long assay will only detect cellular DNA (5).

Serum or plasma – which is preferred?

Both serum and plasma samples have been used successfully for biomarker discovery. However, with some serum preparation tubes, significant contamination of the cfDNA fraction with genomic DNA can occur.

Therefore, plasma is the preferred sample type for cfDNA analysis, and most ctDNA studies to date have also been carried out with plasma samples (6).

Collection and stabilization

Using dedicated, high-quality blood collection tubes minimizes negative impacts on downstream experiments. At QIAGEN, we recommend PAXgene Blood ccfDNA Tubes, which stabilize cfDNA in whole blood samples and reduce hemolysis and variability from different shipping or storage conditions (Figures 1–2).

PAXgene Blood ccfDNA Tubes are compatible with our offering of cfDNA purification kits to enable a seamless and standardized preanalytical workflow.

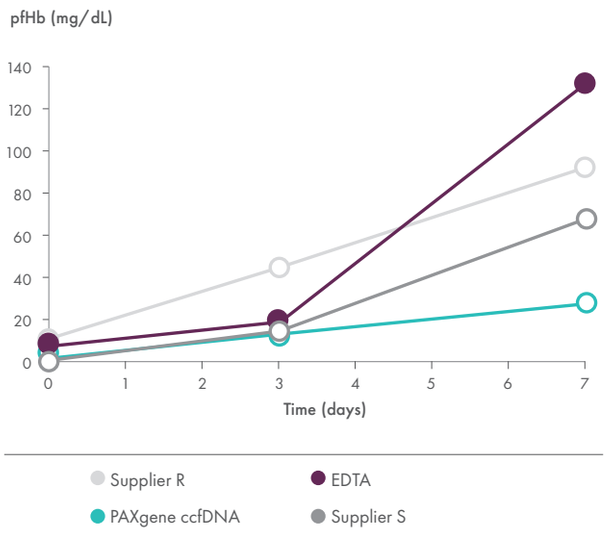


Figure 1. PAXgene Blood ccfDNA stabilization minimizes hemolysis compared to alternative solutions.
Whole blood from 20 subjects was collected into PAXgene Blood ccfDNA Tubes, EDTA tubes and blood collection tubes designed for ccfDNA stabilization from two other suppliers. Plasma was separated directly after blood draw and after storage at room temperature (15–25°C). An increase in sample hemolysis during sample storage at room temperature was minimized in PAXgene Blood ccfDNA Tubes compared to the other blood collection tubes.

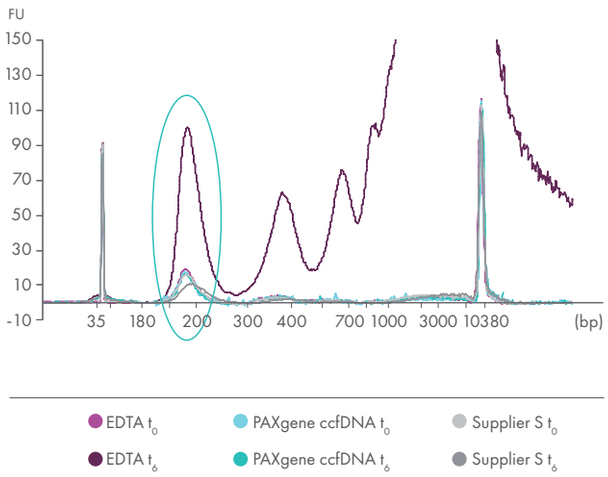


Figure 2. PAXgene Blood ccfDNA stabilization reagent helps prevent release of gDNA into plasma and leaves cfDNA chemically unmodified.
Whole blood was stored in EDTA tubes, PAXgene Blood ccfDNA Tubes or tubes from supplier S. cfDNA was extracted from the plasma immediately following blood collection (t_0) or after 6 days storage (t_6) at room temperature. Plasma from EDTA tubes showed an increase in apoptotic gDNA fragments. Plasma from supplier S showed a broadening and shifting of the main cfDNA peak towards larger fragments, indicating DNA modifications. Plasma from PAXgene Blood ccfDNA Tubes showed a cfDNA profile comparable to day 0.

Sample storage and transportation

Blood samples should be kept within the optimal temperature range from the time of collection through to cfDNA purification. However, temperature control throughout transport is often impossible, and fluctuations can occur, even with cooling packs.

When using PAXgene Blood ccfDNA Tubes, blood samples can be stored for up to 10 days at temperatures up to 25°C, seven days at temperatures up to 30°C or three days at temperatures up to 37°C. Do not store blood-filled tubes below 2°C.

Sample handling and pretreatment

It is essential to handle all samples consistently to avoid introducing any technical variation. The blood draw is a critical first step in plasma preparation, and hemolysis can even occur at the time of phlebotomy. Therefore, blood should only be collected by those highly experienced in venipuncture and according to a standardized procedure. Where possible, samples should also be collected at the same time.

To achieve optimal results from archived samples, select only samples collected and processed according to the same protocol. For multi-center studies, bear in mind that different institutions may use different equipment and procedures for sample collection. The sampling method must be consistent throughout the study to minimize any preanalytical variables.

Plasma preparation

Whole blood should be processed immediately after collection into either serum or plasma. If the whole blood is not centrifuged immediately following collection (or after the clotting time, in the case of serum), the samples should be stored at 2–8°C for no longer than 4 hours. Prolonged storage may result in lysis of thrombocytes and contamination of the cfDNA profile.

Standard plasma preparation generates three fractions: red blood cells, an intermediate buffy coat layer containing white blood cells, and plasma. The optimal centrifugation speed, time and temperature depend on the blood collection tube used. We recommend a second high-speed centrifugation step to reduce cellular contamination (7).

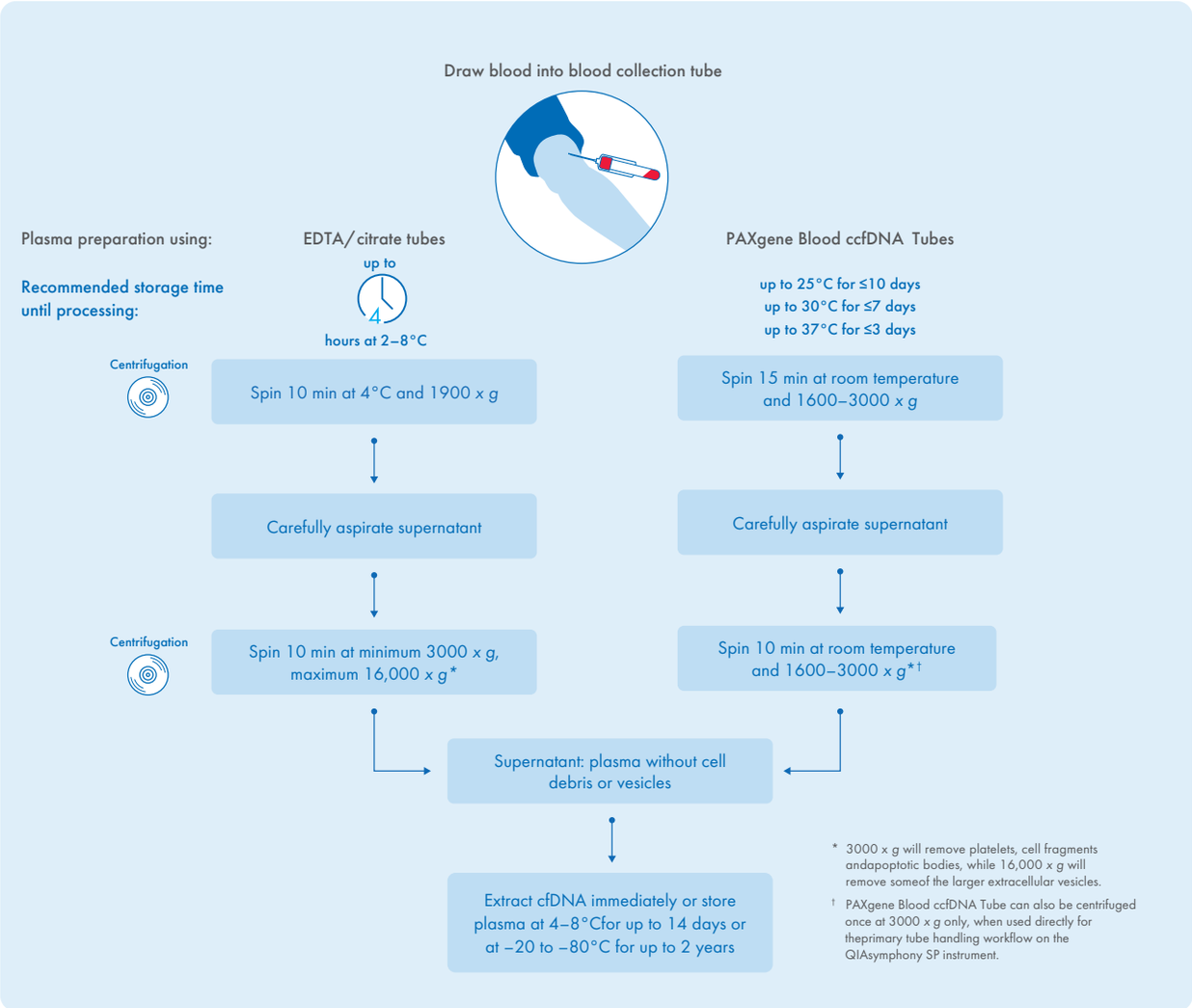


Figure 3. Plasma preparation for cfDNA analysis. For more information about PAXgene products, see www.preanalytix.com.

Our recommended plasma pre-processing workflow is summarized in Figure 3. The second centrifugation step can be performed at 3000 *x g* for 15–20 minutes, which is generally sufficient to remove residual cells, platelets, apoptotic bodies and other cell fragments.

The centrifugation step can also be performed at 10,000–16,000 x *g* to remove larger extracellular vesicles (sometimes called microvesicles or microparticles) in the 200–500 nm size range, as they may also contain cfDNA.

Screening for hemolysis

A quick and low-cost method to screen serum and plasma samples for hemolysis is to perform spectrophotometric measurements of oxyhemoglobin absorbance at 414 nm. Usually, optical density is scanned from around 200 to 700 nm, and you can use the presence of distinct absorbance peaks at 414 nm to disqualify samples affected by hemolysis from further analysis.

Our recommendations for blood plasma or serum samples:

- ✔ Only use blood collected by highly trained staff
- ✔ Use appropriate blood collection tubes, such as PAXgene Blood ccfDNA Tubes
- ✔ Minimize the time between blood draw and serum or plasma generation
- ✔ Keep sampling methods consistent for multi-center studies or when using archived samples
- ✔ Maintain proper and consistent shipping and storage conditions
- ✔ Screen for hemolysis

Urine

Urine is an interesting sample material for cfDNA analysis. In contrast to blood plasma, samples are easier to obtain and sample volume is not usually limiting.

You can use larger volumes of urine for cfDNA extraction increasing the amount of cfDNA available for downstream assayss. But, keep in mind that urine has the risk of containing concentrated metabolites that could interfere with PCR- or NGS-based analysis. Stabilization of urine samples after collection is also important.

Urine sample pre-processing for cfDNA analysis

When collecting urine from animals, such as for preclinical studies, consider possible sources of contamination and variation due to sample collection procedures. We recommend standardizing sample handling and storage protocols.

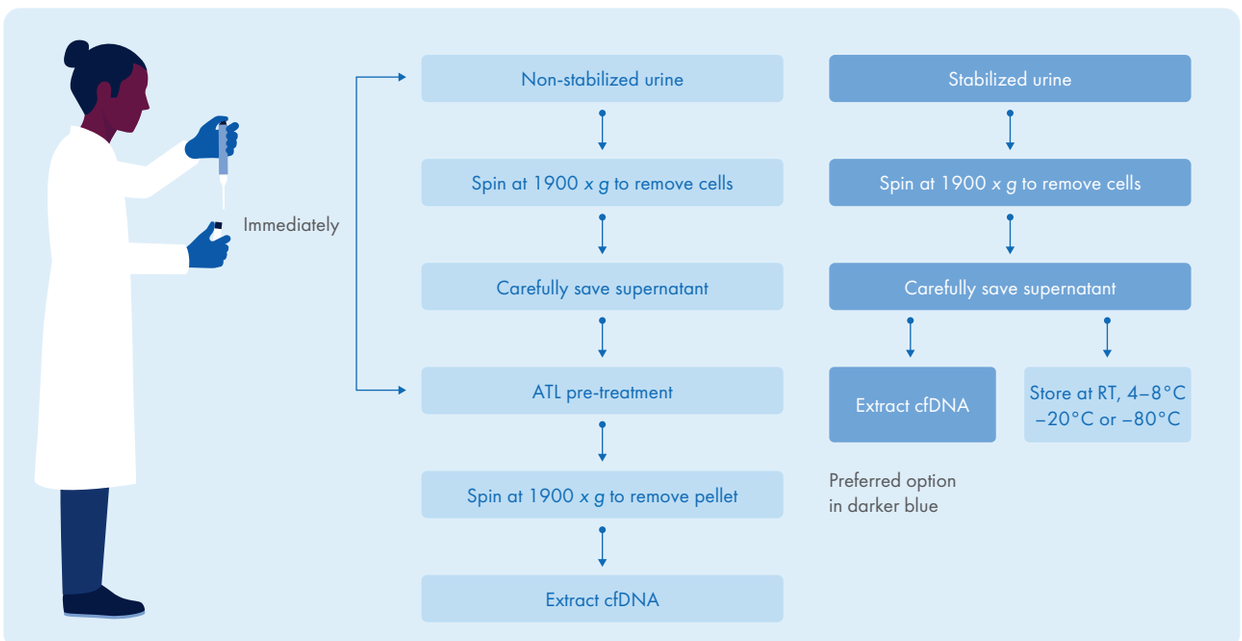


Figure 4. Urine pre-processing for cfDNA analysis.

The number of different cell types present in urine may vary between individuals or disease states, or following certain medical treatments, and this can affect the cfDNA analysis of whole urine samples. Therefore, urine samples should be centrifuged to remove cells and other debris.

The resulting supernatant can be used directly for cfDNA extraction or stored at –80°C. It is essential to perform the centrifugation step before freezing, as freezing the urine sample first will lead to lysis of cells and the release of genomic DNA upon thawing.

To analyze cfDNA from urine, stabilize samples immediately after collection using appropriate collection devices – to prevent DNase activity and release of gDNA from cells.

If no stabilizing agent is available, centrifuge the cells immediately after sample collection to prevent genomic DNA release. We recommend adding Buffer ATL, which contains SDS, to the saved supernatant at a ratio of 1:10 to eliminate DNase activity. After a second low-speed centrifugation step, the cell-free supernatant can be used for cfDNA extraction.

cfDNA purification

You can use either silica membrane-based spin columns or magnetic bead technology for cfDNA purification. Regardless of the method, we recommend using a cfDNA purification kit with a lysis step that releases nucleic acids that are bound to proteins and lipids and contained in vesicles (8).

Some purification kits include the option of adding carrier RNA before extraction to increase the yield of cfDNA, but carrier RNA may also negatively affect downstream analysis (9).

The input volume can vary from 1 to 10 mL, depending on the target DNA, the application, and the downstream assay's sensitivity. Typically, input volumes of 4 mL are used, which corresponds to the total plasma volume from one blood collection tube.

Typically, 1 mL of plasma from a healthy individual can contain 1–50 ng of cfDNA.

Recommended purification kits

We provide a range of cfDNA purification kits that offer fast workflows and unmatched cfDNA concentrations from varying sample inputs for high sensitivity in downstream NGS and PCR analyses. The QIAamp Circulating Nucleic Acid Kit has come to be recognized by the scientific community as the gold standard for cfDNA isolation, while the QIAamp MinElute ccfDNA Midi Kit is the second generation product that allows processing of up to 10 mL serum or plasma and can be partially automated on QIAcube Connect.

Automated cfDNA purification

Automated cfDNA purification methods reduce variability and increase throughput while reducing hands-on time. In contrast to basic research applications, automated cfDNA purification is often the preferred choice in translational research and in the diagnostic routine, as it ensures a standardized workflow with high traceability and LIMS compatibility, operator-independent reliability and optimal use of time and resources

A fully automated workflow also offers the advantages of using a dedicated cfDNA purification kit, including:

- Optimized purification chemistry
- Prefilled reagent cartridges with bar codes for safety and ease of use
- Reduced risk of exposure and sample mix-up
- Highest reproducibility

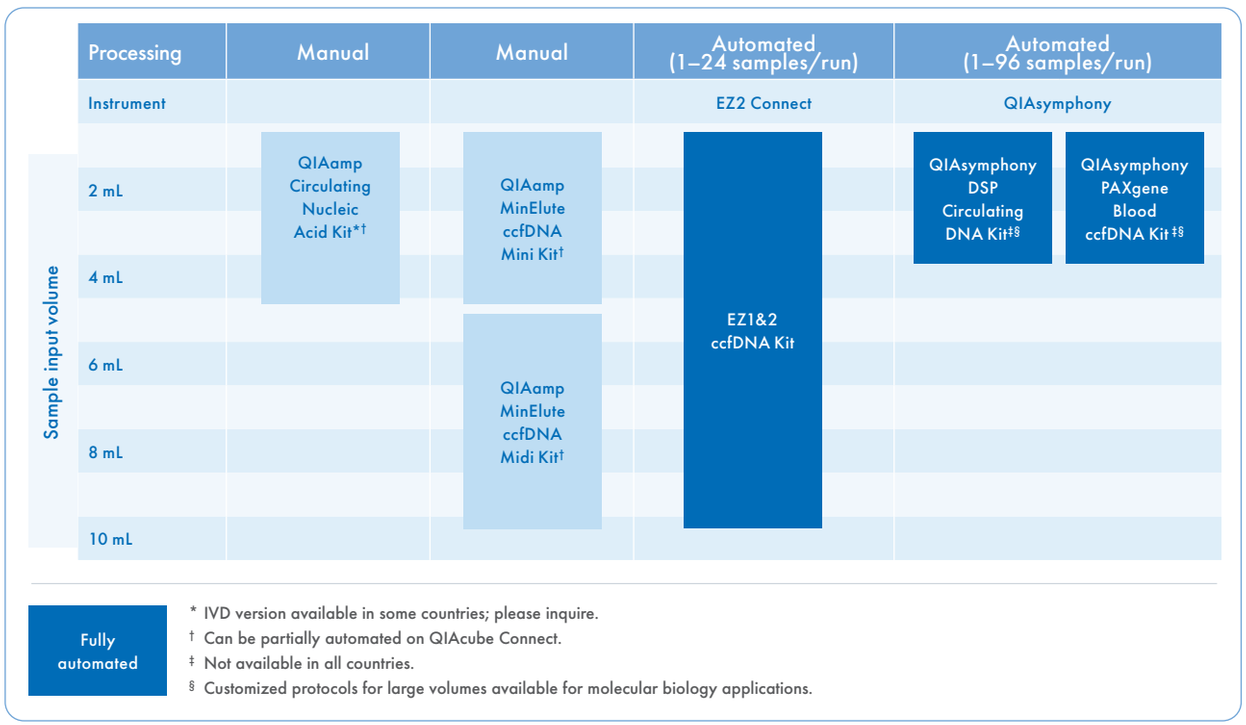


Figure 5. Overview of our cfDNA solutions for various sample throughputs and input volumes.

Determining background

When profiling very low-abundance targets such as biofluid cfDNA, it is important to ensure the signals you are measuring are reliably above any background signal. A blank purification, for example, with water added in place of a biofluid sample in the cfDNA purification, can be used as a negative control to measure any background signal.

cfDNA storage

Store purified cfDNA at –15 to –30°C or –65 to –90°C in RNase-free water or manufacturer-supplied elution buffer. Under these conditions, no degradation of DNA is detectable after one year.

cfDNA quality control

Consider the following parameters when assessing the quality of cfDNA purified from cell-free biofluids:

- The efficiency of the cfDNA purification and the yield
- The size pattern of the extracted cfDNA
- The absence of any inhibitors of downstream enzymatic processes such as PCR or NGS library preparation
- The absence of nucleases
- The presence or absence of gDNA from cellular contamination or hemolysis

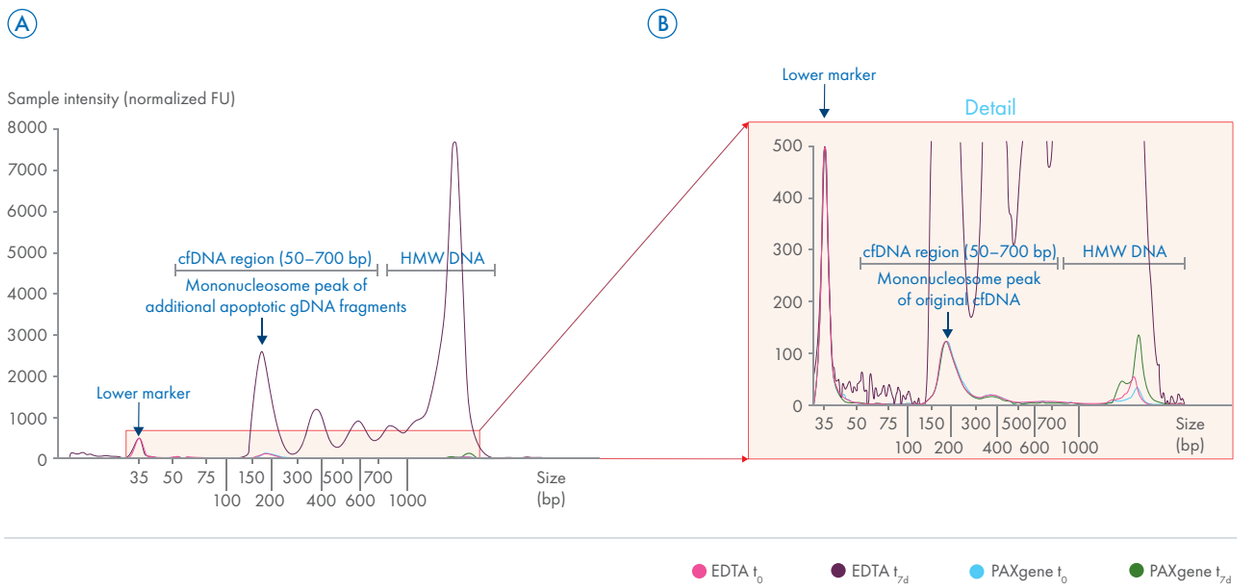


Figure 6. cfDNA qualification by electrophoresis.
Blood was collected in EDTA and PAXgene Blood ccfDNA Tubes. Paired tubes were processed directly (t_0) or after storage for 7 days (t7d) at 25°C. cfDNA extraction was performed automated on the QIAsymphony instrument (QS) using tube-specific kits and protocols. (A) Plasma from stored EDTA tubes showed an increase in apoptotic gDNA fragments. Overlay of cfDNA electropherogram profiles from one exemplary donor. Quantification of cfDNA is evaluated by the assay using a pre-set region from 50–700 bp. Indicated are the lower marker, mononucleosomal peak and high molecular weight (HMW) DNA. (B) Detail of indicated region from panel A.

Basic QC for standard samples

The amount of cfDNA that can be purified and the amount of residual inhibitors that remain after purification can vary from sample to sample. When analyzing cfDNA from standard human serum or plasma samples, we recommend performing quality control assessments on all samples – to monitor the purification yield and absence of PCR inhibitors – and to identify any potential problems in the sample set before qPCR or NGS experiments. For studies involving a very large number of samples, at least a subset should be quality controlled.

You can confirm successful and high-quality urine collection and stabilization by analyzing the size of the extracted cfDNA. cfDNA will be visible on a profile at a fragment size of about 200 bp.

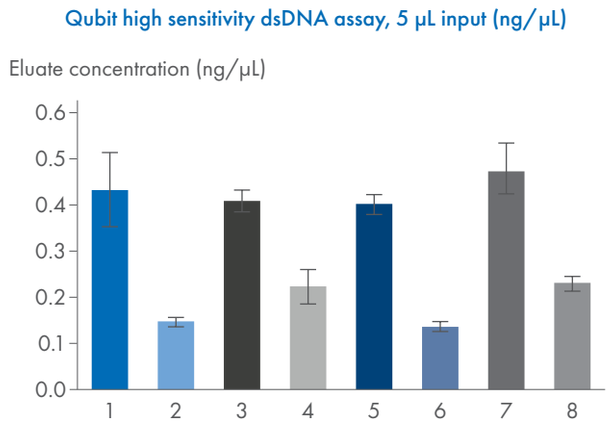
cfDNA quantification

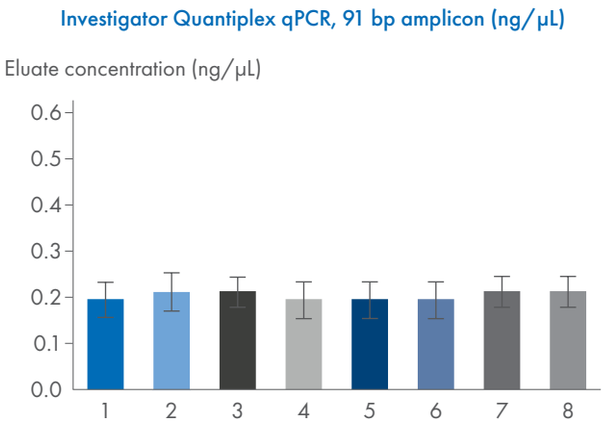
Quantitative, real-time PCR using a multi-copy target gene with a known and stable copy number per genome (e.g., GAPDH or ß-actin) is the most accurate method for determining the concentration of small amounts of cfDNA (Figure 7). Alternatively, you can use 18S rDNA as a target (10). We don't recommend spectrophotometric quantification (e.g., Nanodrop®) because the amount of cfDNA in plasma and urine is usually too low for reliable measurements.

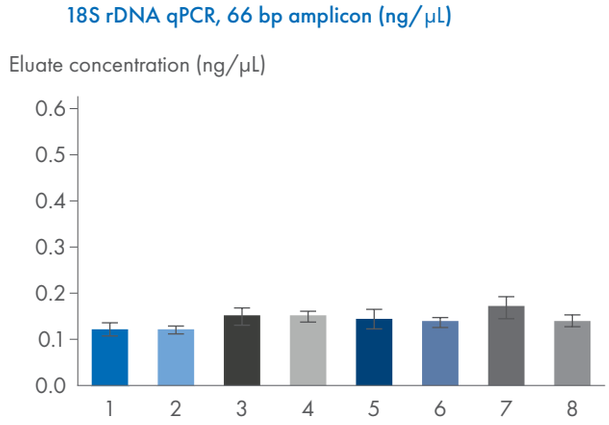
Fluorometric (e.g., Qubit®) or electrophoretic quantification can also be unreliable for cfDNA due to its short nucleic acid fragments. Use designated cfDNA assays for electrophoretic quantification.

Preferred cfDNA quantification methods

- ✔ Real-time qPCR: short amplicon (<100 bp) of housekeeping gene
- ✔ Digital PCR (e.g., QIAcuity®)

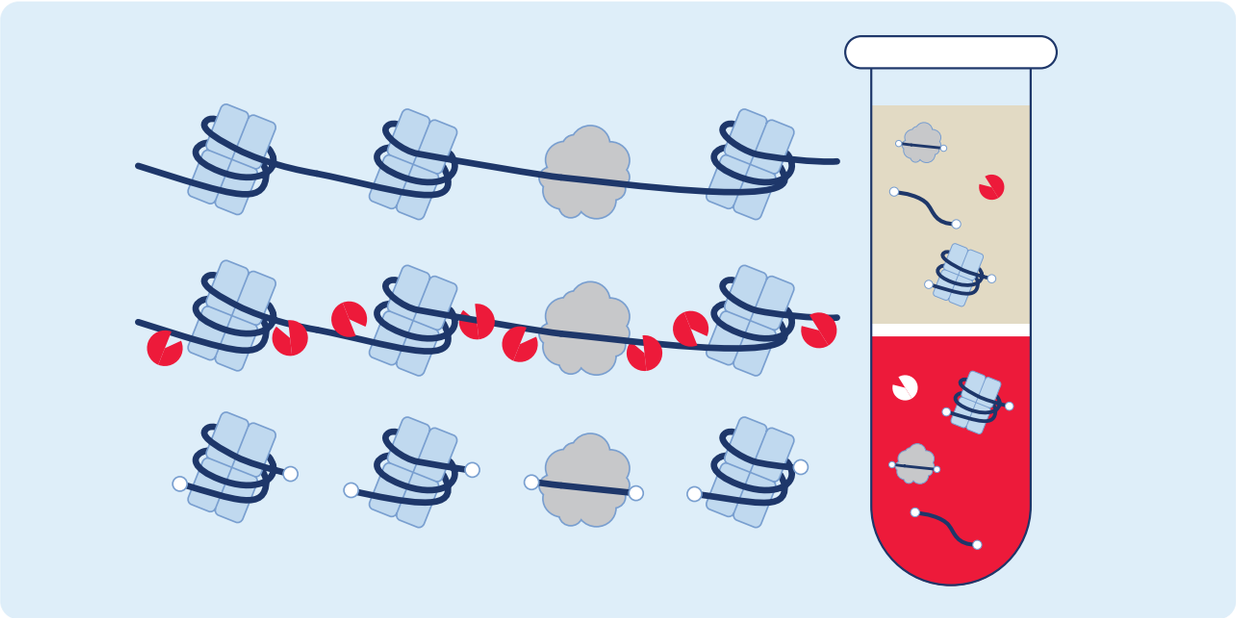






	Plasma used	Collection tube	Extraction kit
1	2 mL	Streck	QIAamp Circulating Nucleic Acid Kit
2			QIAsymphony DSP Circulating DNA Kit
3		EDTA	QIAamp Circulating Nucleic Acid Kit
4			QIAsymphony DSP Circulating DNA Kit
5		PAXgene Blood ccfDNA Tubes	QIAamp Circulating Nucleic Acid Kit
6			QIAsymphony DSP Circulating DNA Kit
7	2.4 mL		QIAamp Circulating Nucleic Acid Kit
8			QIAsymphony PAXgene Blood ccfDNA Kit

Figure 7. Comparison of methods for cfDNA quantification.
cfDNA was isolated from six donors using various sample collection tubes and cfDNA extraction methods (manual or automated on QIAsymphony). cfDNA quantification using qPCR methods (Investigator® Quantiplex® Pro Kit or an 18S rDNA qPCR assay) are reliably homogenous in contrast to cfDNA quantification by Qubit, which results in highly variable quantification results.



Method standardization

While cfDNA analysis may hold much promise for future clinical applications, standardization of preanalytical methods is a key prerequisite for its successful use in biobanks, biomarker research and translational research (15).

Various multinational initiatives, including the SPIDIA4P consortium led by QIAGEN, are driving the development of standardized methods in this field. This involves the creation and transfer of optimal preanalytical workflows and expert knowledge into CEN technical specifications and ISO standards. For more information about the SPIDIA4P consortium, visit www.spidia.eu.

Multianalyte liquid biopsy

While cfDNA from blood is widely used as an analyte in liquid biopsy research applications, circulating cell-free RNA (ccfRNA), circulating tumor cells (CTCs), exosomes and other extracellular vesicles (EVs), and their contents have also gained relevance for biomarker studies. The ability to combine insights from all of these analytes holds much promise for increasing our understanding of underlying molecular processes.

Further information, including a handy guide, science talks, references and a downloadable quick-start workflow poster, is available at: www.qiagen.com/multianalyte-liquid-biopsy.

For standardization of the pre-examination process refer to:
ISO 20186-3:2019 – Molecular in vitro diagnostic examinations – Specifications for pre-examination processes for venous whole blood – Part 3: Isolated circulating cell free DNA from plasma

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