# Cell-free DNA Quality and Quantity Assessment – A Method Comparison

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## Introduction

Workflow standardization is required for successful use of circulating cell-free DNA (ccfDNA) in cancer research. The new ISO standard for isolation of ccfDNA from plasma (ISO 20186-3:2019) focuses on preanalytical factors like preventing cellular DNA release by using blood stabilization tubes and dedicated isolation procedures. Furthermore, accurate determination of ccfDNA quality and quantity is required for downstream analyses like NGS, quantitative or digital PCR (qPCR/dPCR).

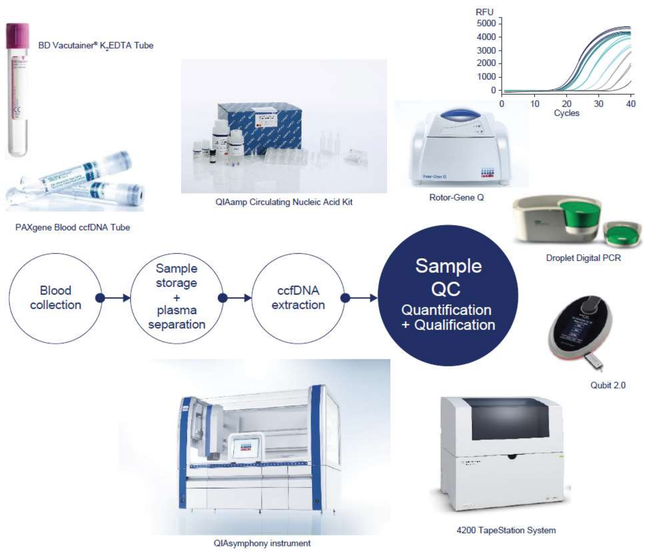
## Methods

Blood from eight healthy donors was collected in EDTA (BD) and PAXgene® Blood ccfDNA Tubes†+* (PreAnalytiX). Paired tubes were processed directly (T0) or after storage for 7 days (T7d) at 25°C. CcfDNA extraction was performed manually using the QIAamp Circulating Nucleic Acid Kit (QA QIAGEN) or automated on the QIAsymphony® instrument (QS) using tube-specific kits and protocols. All 64 samples were analyzed for ccfDNA quality and/or quantity with QC methods based on fluorescence, automated electrophoresis and PCR according to Table 1.

| Method Category | Assay Name | Instrument | Supplier |
|---|---|---|---|
| Fluorometric Measurement | Qubit™ dsDNA HS Assay Kit (10x) | Qubit 2.0 | Thermo Fisher Scientific |
| | Qubit 1x dsDNA HS Assay Kit* - premixed kit | | |
| Automated electrophoresis | Cell-free DNA ScreenTape assay | 4200 TapeStation System | Agilent Technologies |
| qPCR | Investigator® Quantiplex Pro* (91 bp amplicon) | Rotor-Gene® Q | QIAGEN |
| dPCR | 18S rDNA 66bp (QIAGEN in-house assay) | QX200™ Droplet Digital PCRTM System | (QIAGEN) BioRad |

## Study Design

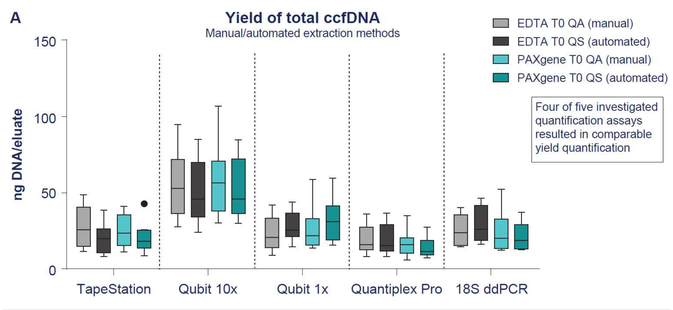
### ccfDNA QC Workflow

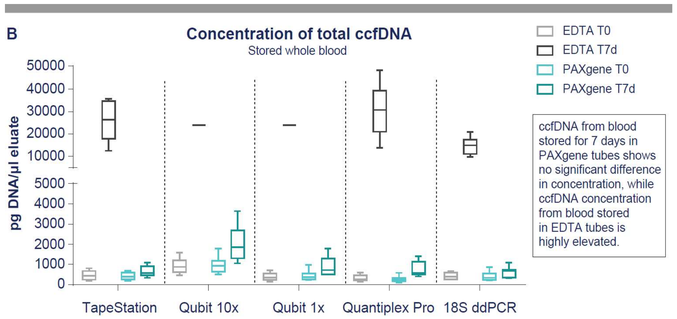



## Results

### Total ccfDNA Quantification

- Manual and automated ccfDNA extraction methods enable high workflow consistency and comparable ccfDNA yields quantified with the TapeStation system, Qubit 1x, Quantiplex Pro and 18S ddPCR assays.
- Storage of unstabilized blood in EDTA tubes for 7 days at 25°C results in up to 80-fold increase of total DNA in plasma.
- Stabilization for consistent ccfDNA quantification is needed
- Electrophoretic and PCR-based DNA quantification methods show comparable results when analyzing low and high ccfDNA concentrations.
- Fluorometric assays (Qubit 1x and 10x) show higher variation when measuring ccfDNA. For high concentration ranges both Qubit assays have limited measuring capacity.




### Comparison of quantification methods for ccfDNA samples of plasma from stored and unstored whole venous blood collected in EDTA and PAXgene Blood ccfDNA Tubes.

(A) Total DNA yield quantification (ng DNA/eluate) of ccfDNA eluates isolated with QIAamp (QA) and QIAsymphony (QS) from unstored EDTA and PAXgene samples (T0), n = 8.
(B) Concentration of total ccfDNA (pg DNA/µl eluate) extracted from plasma of unstored (T0) and stored (T7d) EDTA and PAXgene samples using QIAamp, n =8.

Data presented as box plots with median and upper and lower quartiles, whiskers with maximum 1.5 Interquartile ranges (Tukey method). Outliers are shown as dots, extremes were excluded from analysis.

## Disclaimer

*For Research Use Only. Not for use in diagnostic procedures.

†This research was conducted using the PAXgene Blood ccfDNA Tube (RUO) which is available in the United States and other parts of the world outside of Europe.

⁺For up-to-date licensing information and product specific disclaimers, see the respective QIAGEN or PreAnalytiX kit handbook or user manual. QIAGEN and PreAnalytiX kit handbooks and user manuals are available at www.qiagen.com or www.preanalytix.com or can be requested from QIAGEN Technical Services or your local distributor.

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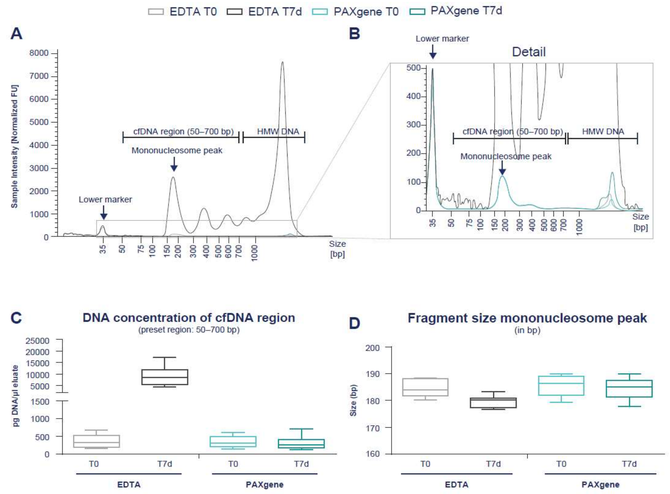
## Results Continued

### ccfDNA qualification and quantification

- PAXgene Blood ccfDNA stabilization ensures reliable and consistent ccfDNA quantification and qualification of unstored and stored blood samples.
- Blood storage in EDTA tubes leads to gDNA release resulting in increase of high molecular weight (HMW) DNA and ccfDNA (degraded gDNA) concentration.
- The new Cell-free DNA ScreenTape assay enables total quantification as well as quantification of a predefined cfDNA region (from 50 to 700 bp) without an upper marker.




### ccfDNA qualification and region quantification on the TapeStation system (TS) using the Cell-free DNA ScreenTape assay

(A) Overlay of ccfDNA electropherogram profiles from one exemplary donor. Quantification of ccfDNA is evaluated by the assay using a pre-set region from 50-700 bp. Indicated are the lower marker, mononucleosomal peak and HMW DNA.
(B) Detail of indicated region from figure A.
(C) ccfDNA concentration in the pre-set region of 50-700 bp (in pg DNA/µl eluate), n = 8.
(D) Main peak size of mononucleosomal peak (in bp) as evaluated by the TapeStation Analysis software (n = 8).

Data presented as box plots with median and upper and lower quartiles, whiskers with maximum 1.5 Interquartile ranges (Tukey method).

## Conclusions

- The ccfDNA workflow using manual and automated QIAGEN/PreAnalytiX extraction kits is compatible with quantification instruments including Qubit and TapeStation as well as PCR-based methods resulting in comparable yield quantification across different methods.
- The yield of smaller ccfDNA fragments (50-700 bp) and ccfDNA fragment size is consistent in plasma from blood stored for 7 days in PAXgene Blood ccfDNA Tubes and comparable to EDTA T0 (control). It can be analyzed with the new Cell-free DNA ScreenTape assay.
- The release of genomic DNA in EDTA tubes (as HMW and cell-free DNA) over time indicates the need for stabilization of blood in PAXgene Blood ccfDNA tubes and an accurate quantification method for optimal workflow control.



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