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 European technical specification for isolation of ccfDNA (CEN/TS 16835-3:2015) and future ISO standard 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).

This research study compares common methods for quality control of ccfDNA from venous whole blood collected and stored for 7 days in EDTA and PAXgene® Blood ccfDNA Tubes* and processed with manual and automated preparation kits.

Methods

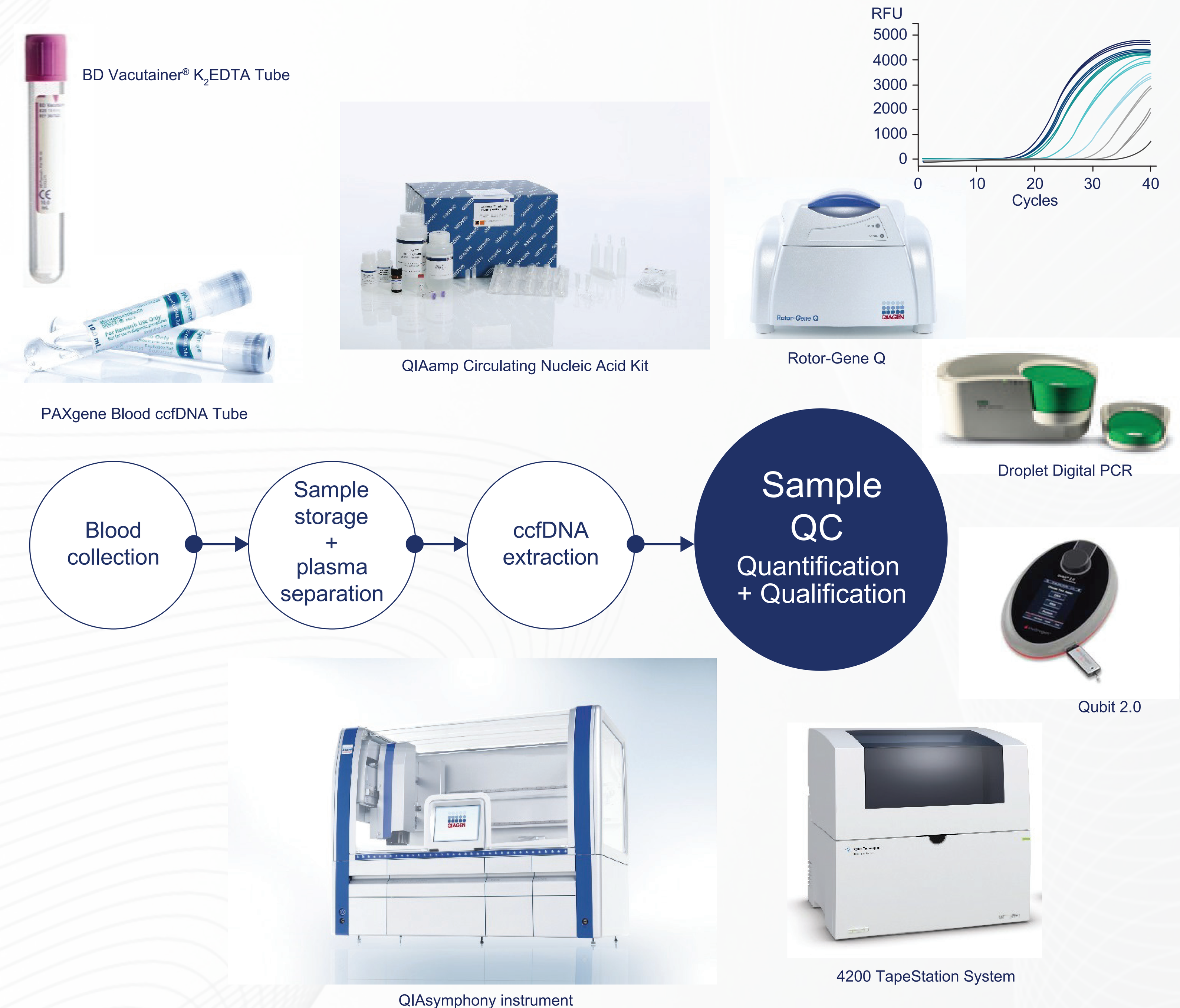
Blood from eight healthy donors was collected in EDTA (BD) and PAXgene Blood ccfDNA Tubes† (PreAnalytiX). Paired tubes were processed directly (T0) or after storage for 7 days (T7d) at 25°C. CcfDNA extraction was performed manually using the QIAamp Circulating Nucleic Acid Kit (QA, QIAGEN) or automated 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 66 bp (QIAGEN in-house assay)	QX200™ Droplet Digital PCRTM System	(QIAGEN)/BioRad

Table 1 - Methods used for ccfDNA quality and quantity assessment

Study Design

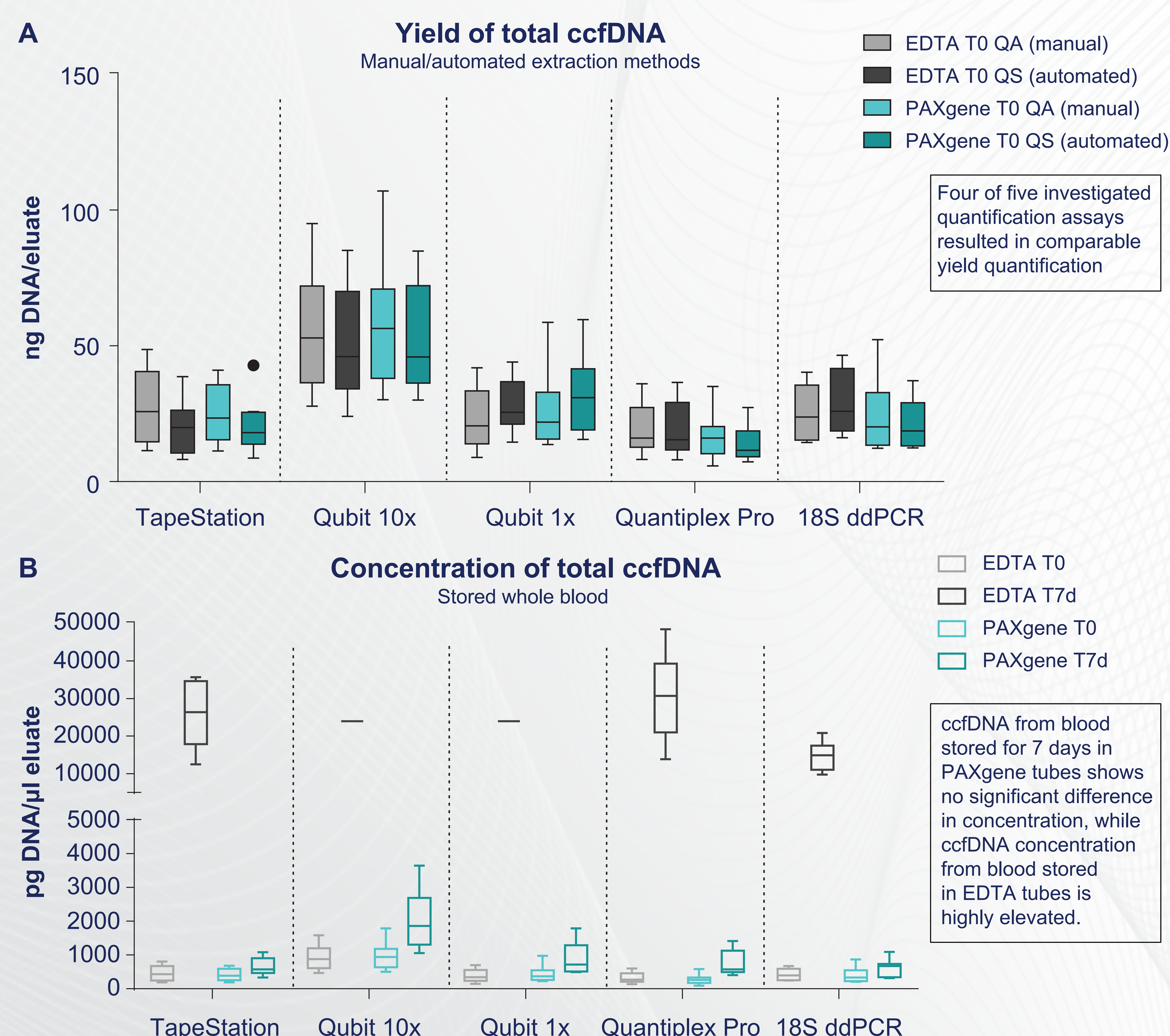
ccfDNA QC workflow



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.

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 an 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 quantfication on the TapeStation system (TS) using the Cell-free DNA ScreenTape assay

(A) 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 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 PAXgene Blood ccfDNA workflow using manual or automated ccfDNA extraction is compatible with quantification instruments including Qubit and TapeStation as well as PCR-based methods resulting in comparable 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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