Precision and Robustness of the PAXgene Blood ccfDNA Workflow

[1]Tomasz Krenz, [1]Daniel Groelz, [1]Andrea Ullius, [1]Thorsten Voss and [1]Eric Provencher
[1]PreAnalytiX GmbH, Hombrechtikon, Switzerland

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Background

Knowing the precision and robustness of a workflow to analyze circulating cell-free DNA (ccfDNA) is key for its successful application to liquid biopsy in research settings. This study investigated characteristics of the PAXgene® Blood ccfDNA workflow, including blood collection into PAXgene Blood ccfDNA Tubes*, sample storage, automated ccfDNA extraction on the QIAsymphony® instrument and qPCR analysis. The goals were to determine the workflow precision and the effects of sub-optimal blood collection conditions such as tube underfilling, variation in tube inversion and endogenous interfering substances on analysis performance.

Methods

Precision was evaluated using 10 ml venous whole blood drawn from 34 healthy consented donors. The tubes were inverted 8 times after draw, plasma was separated by centrifugation (15 min at 1,900 × *g* followed by 10 min at 1,900 × *g*), and automated ccfDNA extraction from 2.4 ml plasma was performed using the QIAsymphony PAXgene Blood ccfDNA Kit.* Variations were included by using different instruments, kit lots, samples and operators.

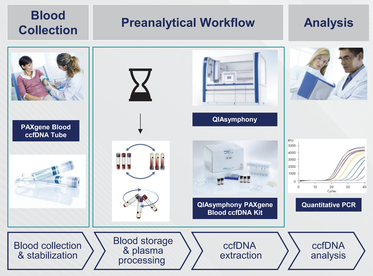
For analysis of the impact of tube inversion, completely filled tubes were either inverted 8× directly after blood draw as indicated in the handbook, minimally inverted (0–2 inversions) or vortexed. For underfilling studies, tubes were filled completely with 10 ml as described in the handbook, or blood draw was interrupted after 5–9 ml. Endogenous potentially interfering substances were tested by spiking different amounts of hemoglobin, bilirubin, cholesterol, triglycerides, albumin and glucose into blood. Blood was processed directly after phlebotomy or stored for up to 10 days at varying temperatures. ccfDNA was analyzed with a validated 18s rDNA qPCR assay.

Results

PAXgene Blood ccfDNA workflow & precision

- PAXgene Blood ccfDNA workflow precision, measured as coefficient of variation, ranged from 9.1–17.6% for the determination of absolute ccfDNA yield
- No major differences were observed for different instruments, operators or ccfDNA samples

A



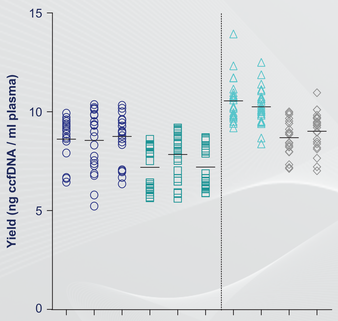
Blood draw:
Blood from 34 healthy donors was drawn into 6 PAXgene Blood ccfDNA Tubes. Samples were either processed within 2 hours (samples 1–3) or stored for 7 days at 30°C (samples 4–6).

Plasma:
Blood was centrifuged at ambient temperature for 15 min at 1,900 × *g*. The plasma was removed by pipetting without disturbing the cellular fraction and transferred into a fresh tube. In a second round of centrifugation, plasma samples were centrifuged for 10 min at 1,900 × *g*. From each tube, 4 ml of plasma was transferred and plasma from 17 individual donors was combined to generate pool 1 and pool 2, respectively. Each plasma pool was generated independently by three different operators (A, B, C).

ccfDNA purification:
For all samples, automated ccfDNA extraction from plasma was performed on different QIAsymphony SP instruments using the QIAsymphony PAXgene Blood ccfDNA Kit and 2.4 ml protocol with 60 µl elution. In total, 24 replicates from each pool were extracted in a single QIAsymphony run.

ccfDNA analysis:
Analysis of ccfDNA eluates from different QIAsymphony runs and different plasma pools was performed by using a quantitative PCR assay for detection of a 18s rDNA target. For total yield calculation a standard curve with genomic DNA of known concentration was included.

B



Instrument run	1	2	3	1	2	3	4	5	4	5
Operator	A	B	C	A	B	C	A	C	A	C
ccfDNA sample	1	1	1	2	2	2	3*	3*	4*	4*
Mean yield (ng)	8.6	8.6	8.8	7.2	7.8	7.2	10.6	10.3	8.7	9.0
σ	1.1	1.5	1.1	1.1	1.1	1.1	1.1	0.9	0.8	0.9
% CV	**13.1**	**17.6**	**13.0**	**15.1**	**14.5**	**14.7**	**10.1**	**9.1**	**9.7**	**10.5**

*Operator B was excluded due to user error

Tube underfilling & inversion

- The PAXgene blood ccfDNA workflow is robust against tube underfilling and variable tube inversion conditions
- ccfDNA yield was reduced when reduced blood volumes were drawn; however, underfilling did not affect the ccfDNA stabilization properties and yield was stable after blood storage even when the tube was only half-filled (5 ml, i.e., 50%)
- Less inversions (0×, 2×) or more stringent mixing (vortexing) than the recommended tube inversion conditions (8×) did not impact ccfDNA yield when blood was processed directly after blood draw nor when samples were stored prior to processing

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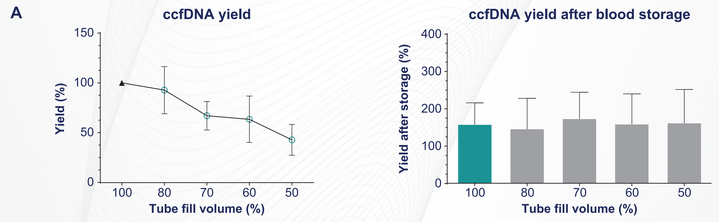

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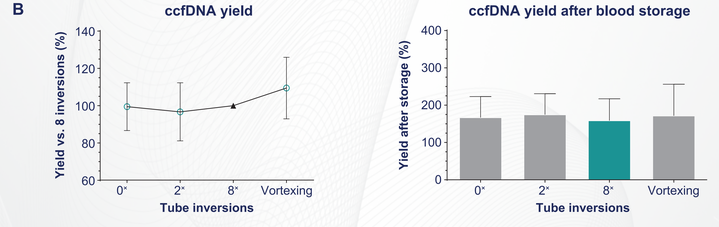

Effect of blood draw volumes on ccfDNA yield from the PAXgene Blood ccfDNA Tube.
Tubes were either filled completely with 10 ml venous blood (reference), or incompletely with ~5 ml, ~6 ml, ~7 ml or ~9 ml blood. ccfDNA was extracted within 2 hours of draw and after blood storage for 7 days at 30°C. The effect is shown on relative ccfDNA yield at day 0 compared to the reference (left) and as yield after blood storage comparing each condition to day 0 (right). The values are means ± SD from n = 10 individual donors.

Effect of tube inversions on ccfDNA yield from the PAXgene Blood ccfDNA Tube.
Immediately after blood draw, the tube was either inverted 8× (reference), 0×, 2×, or vortexed. ccfDNA was extracted within 2 hours of draw and after blood storage for 7 days at 30°C. The effect is shown on ccfDNA yield compared to the reference (left) and as yield after blood storage compared to day 0 (right). The values are means ± SD from n = 10 individual donors.

Interference

- The PAXgene Blood ccfDNA workflow is robust against elevated levels of endogenous substances in blood such as fat, protein and sugar
- ccfDNA yield was stable for different types and concentrations of interfering substances
- Variability in ccfDNA yield increased for the highest concentrations of hemoglobin, triglycerides and albumin

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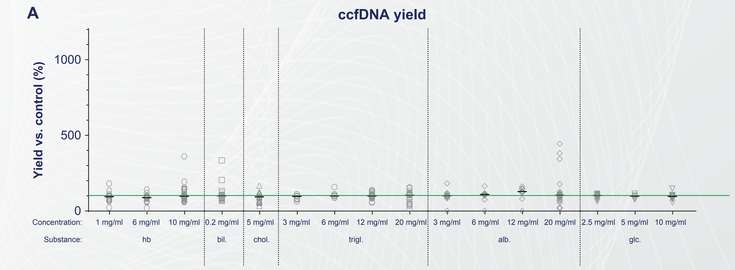

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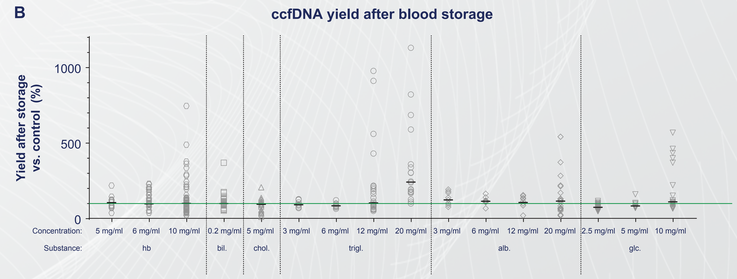

Effect of elevated level of physiological substances on ccfDNA yield from the PAXgene Blood ccfDNA Tube.
Results from multiple experiments are shown, in which hemoglobin (hb) bilirubin (bil.), cholesterol (chol.), triglycerides (trigl.), albumin (alb.) and glucose (glc.) were spiked into blood (or plasma in the case of hb) from PAXgene Blood ccfDNA Tubes in exogenous concentrations ranging from 0.2–20 mg/ml. Data is shown as aligned dot plots including the median. Each data point represents a single blood donor. The green line represents an optimal 100% ccfDNA yield. **A:** Effect on ccfDNA yield compared to control without spiked-in substances. **B:** Effect on ccfDNA yield after blood storage (7 days at 30°C or 10 days at 2–8°C) compared to control at day 0.

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Conclusions

- The PAXgene Blood ccfDNA workflow, including blood collection, storage, plasma generation and ccfDNA extraction has low variability and therefore good precision
- The workflow is robust against tube underfilling, improper mixing and interferents as shown for ccfDNA yield with and without blood storage
- This study demonstrates the reliability and robustness of the PAXgene Blood ccfDNA workflow for research using liquid biopsies