Performance Evaluation of a New Integrated Blood Collection and Sample Preparation System for the Stabilization and Extraction of Circulating Cell-Free DNA (ccfDNA)

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Background

Introduction: The extraction of the low abundance and highly fragmented circulating cell-free DNA (ccfDNA) for cancer research is technically challenging. PreAnalytiX has developed the PAXgene® Blood ccfDNA System*, consisting of the PAXgene Blood ccfDNA Tube*, a plastic BD Vacutainer® tube with a unique, non-crosslinking chemistry preserving extracellular levels of ccfDNA and preventing the release of intracellular DNA from cells into the plasma, and the QIAsymphony® PAXgene Blood ccfDNA Kit for automated ccfDNA extraction.

This research evaluated the performance of the PAXgene Blood ccfDNA System in comparison to EDTA and Streck Cell-Free DNA BCT® tubes for ccfDNA applications. Five experiments were conducted:

- **Study 1:** Proof of principle of the PAXgene Blood ccfDNA stabilization reagent effectiveness vs. EDTA and Streck
- **Study 2A:** Performance with manual and automated ccfDNA extraction
- **Study 2B**: Evaluation of the PAXgene Blood ccfDNA System performance vs. EDTA
- **Study 3:** Stabilization of ccfDNA during transport simulation and storage vs. EDTA
- **Study 4:** Research study using blood samples with spiked-in mutation-containing DNA to evaluate cancer biomarker preservation vs. EDTA

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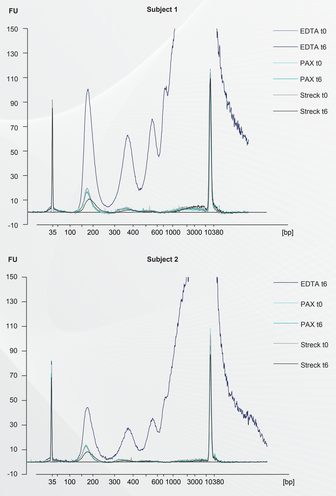
Study 1: Proof of Principle of the PAXgene Blood ccfDNA Stabilization Reagent Effectiveness vs. EDTA and Streck

Materials and Methods: Whole blood from 2 subjects was collected and either left unstabilized (EDTA) or stabilized with PAXgene Blood ccfDNA reagent (PAX) or Streck Cell-Free DNA BCT (Streck). The whole blood specimens were immediately processed (t0) or stored for 6 days at room temperature (t6). Plasma was generated by double centrifugation and ccfDNA was extracted from plasma using the PAXgene Blood ccfDNA QIAsymphony Kit. 1 µl eluate was analyzed using the Agilent High Sensitivity DNA Kit.

Results:

- Results for both subjects are shown in the electropherograms **(A)**.
- Fragment length of the main peak for the PAX- and Streck-stabilized samples are shown in table **(B)**.
- Plasma generated from whole blood stored for 6 days in EDTA tubes shows an increase of apoptotic DNA fragments.
- Release of gDNA into the plasma is prevented by stabilizing blood with PAXgene Blood ccfDNA stabilization reagent.
- Plasma generated from whole blood stored for 6 days in Streck tubes shows a shift of the main ccfDNA peak towards larger fragments, indicating DNA modification.

A.



B.

Size (bp) of main peak	PAX t0	PAX t6	Streck t0	Streck t6
Subject 1	173	176	175	182
Subject 2	171	174	173	181

PAXgene Blood ccfDNA System Workflow

PAXgene Blood ccfDNA Tube and automated QIAsymphony kit: Integrated collection-stabilization-preparation (CSP) system

PAXgene Blood ccfDNA System

PAXgene Blood ccfDNA Tube

QIAsymphony PAXgene Blood ccfDNA Kit

Tube features:

- PAXgene Blood ccfDNA non-crosslinking stabilization reagent in a plastic BD Vacutainer Tube
- Plastic material mitigates risk of tube breakage during transport and centrifugation
- BD Hemogard™ safety closure helps protecting laboratory personnel from contact with blood
- PAXgene Blood ccfDNA Tube allows distinct separation of plasma from buffy coat

Kit features:

- Optimized binding chemistry and input volumes for plasma from PAXgene Blood ccfDNA Tubes
- 2.4 ml or 4.8 ml plasma processing
- Two protocol lines (standard and large fragment protocols)
- Prefilled cartridges are ready to use

QIAsymphony protocol features:

- Flexibility to process between 1 and 96 samples per run
- Onboard measures to prevent cross-contamination (drop catchers, UV lamp, segregation of used consumables)
- Eluate cooling enabling overnight runs
- Run report documents run information including user ID, sample ID and expiration dates

QIAsymphony Protocols

Standard Protocol Line (STA)
(Small fragments preferred)
Plasma volume: 2.4 or 4.8 ml

Binding / Proteinase K digestion step with Buffer BCD1
↓
Wash step 1
↓
Wash step 2
↓
Wash step 3
↓
Elution 60 µl

Large Fragment Protocol Line (LAF)
(Efficient co-isolation of large fragments and unbiased isolation of small fragments)
Plasma input volume: 2.4 or 4.8 ml

Binding / Proteinase K digestion step with Buffer BCD2
↓
Wash step 1
↓
Wash step 2
↓
Wash step 3
↓
Elution 60 / 100 / 150 µl

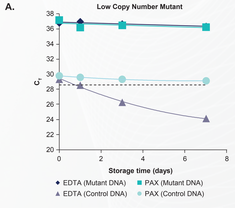
Study 4: Research Study Using Blood Samples With Spiked-in Mutation-Containing DNA to Evaluate Cancer Biomarker Preservation vs. EDTA

Materials and Methods: Whole blood from 6 subjects was collected into EDTA and PAXgene Blood ccfDNA Tubes (PAX) and either low (50) or higher (500) copy numbers of restriction-enzyme digested DNA containing an established mutation was spiked in. Plasma was generated at the indicated time points after blood storage. ccfDNA was extracted from plasma using the QIAamp Circulating Nucleic Acid Kit and ccfDNA was analyzed for wild-type control and mutant DNA by real-time PCR.

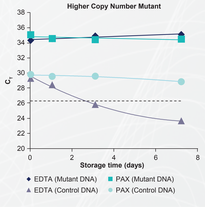
Results:

- Shown is the average C_t value of the control and mutant genes for samples with spiked in low copy number mutant DNA (**Figure A**) and a higher copy number mutant DNA (**Figure B**). The assay ΔC_t cut-off ($C_{t\ mutant} - C_{t\ control} \leq 8$) is represented as a dashed black line for illustration purposes.
- Blood stabilized with PAXgene Blood ccfDNA stabilization reagent can be used for real-time PCR based cancer biomarker assays.
- After 1 to 3 days of blood storage, control assay C_t values for ccfDNA from EDTA blood falls below the cut-off due to the release of additional cellular DNA, generating false negative results.

A.



B.



Studies 2 and 3: Efficient Isolation of ccfDNA With Manual or Automated Extraction Methods and Stabilization of Whole Blood During Transport and Storage

Study 2:

Overview of results from two studies that evaluated product performance specifications

Materials and Methods:

- Blood collection tubes: EDTA tube and PAXgene Blood ccfDNA Tube
- Extraction kits: QIAGEN QIAamp Circulating Nucleic Acid Kit, QIAsymphony PAXgene Blood ccfDNA Kit
- Instruments: QIAGEN QIAsymphony SP instrument
- Assays: 18S rDNA real-time PCR (66 bp amplicon), DYS14 real-time PCR (Y chromosome specific)

Study 2A	
Comparison of PAXgene automated kit vs. manual procedure[1]	
Study Parameters	
Samples tested	825
Subjects	189
Runs	42
Instruments	6
Study Results	
ccfDNA yield	104%
Portion of dsDNA[2]	> 96%
Total failures	0
Study 2B	
Evaluation of PAXgene system performance[3]	
Study Parameters	
Samples tested	1636
Subjects	204
Runs	71
Instruments	6
Study Results	
Yield compared to EDTA plasma[4]	No significant difference
ccfDNA stability at room temperature[5]	7 days
Cross-contamination	No evidence
Eluate freeze/thaw cycles	3
Sample throughput per day[6]	192
Open reagent cartridge stability	6 weeks
Runtime for 96 samples	5 h 40 min

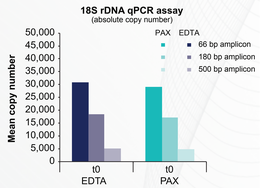
[1]QIAamp Circulating Nucleic Acid Kit; [2]Tested with restriction enzyme based assay for subset of eluates; QIAamp circulating Nucleic Acid Kit eluates with comparable portion of dsDNA; [3]Performance evaluated against RUO product specifications; [4]Tested with 60 samples from 20 subjects, directly after blood collection; [5]Tested with 60 samples from 20 subjects; [6]Includes plasma processing and overnight runs

Study 3: Stabilization of ccfDNA during transport simulation and storage vs. EDTA

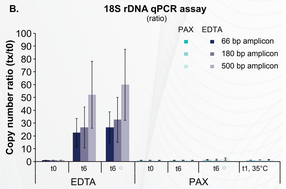
Materials and Methods: Whole blood from 8 subjects was collected and either left unstabilized (EDTA) or stabilized with PAXgene Blood ccfDNA reagent (PAX). All blood samples were stored for one day at 35°C (t1, 35°C) or for 6 days at room temperature (t6). For transport simulation, samples were continually inverted for 5 hours, then stored horizontally for 6 days at room temperature (t6o). ccfDNA was extracted from plasma using the PAXgene Blood ccfDNA QIAsymphony protocol and ccfDNA yield was quantified by real-time PCR (18S rDNA gene, 66 bp/180 bp/ 500 bp amplicons).

Results: Shown are the average copy number at day of blood collection (n = 8) **(A)** and the average ratio of copy numbers at day of storage to t0 with standard deviation (n = 8) **(B)**.

A.



B.



Conclusion

- The PAXgene Blood ccfDNA Tube stabilizes blood cells for the efficient extraction of ccfDNA from plasma using the QIAsymphony PAXgene Blood ccfDNA Kit.
- The ccfDNA yield from blood stabilized with PAXgene Blood ccfDNA reagent was comparable to blood drawn into EDTA tubes when plasma was generated immediately after blood draw, with the added benefit of 7 day room temperature stability of ccfDNA in whole blood.
- Storage and transport of blood stabilized with PAXgene Blood ccfDNA reagent has no impact on ccfDNA quality (fragment size) or quantity (yield).
- The fully automated QIAsymphony PAXgene Blood ccfDNA Kit workflow offers two protocols for purification of predominately small ccfDNA fragments or co-isolation of small and large ccfDNA fragments to provide optimal sample conditions for various research applications.
- The PAXgene Blood ccfDNA System provides accurate detection and quantification of cancer biomarkers from ccfDNA isolated from plasma of stored blood for blood samples stored for up to 7 days at room temperature.

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