Evaluation of a New System for Collection, Stabilization, and Purification of Circulating Tumor DNA

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Introduction

The current preanalytical workflows for circulating tumor DNA (ctDNA) analysis have limitations that affect the accurate detection and quantification of these plasma cancer biomarkers. Release of genomic DNA (gDNA) from white blood cells (WBCs) due to cell lysis or apoptosis during whole blood storage in EDTA tubes creates higher gDNA background levels, affecting the sensitivity of ctDNA assays. Current tubes that stabilize WBCs often contain crosslinking reagents, which may have negative effects on the performance of sensitive downstream assays, including methylation-based assays.

The new PAXgene® Blood ccfDNA System, consisting of a blood collection tube with unique, non-crosslinking chemistry and an automated circulating cell-free DNA (ccfDNA) extraction kit, was evaluated in 3 research studies for performance with ctDNA assays.

PAXgene Blood ccfDNA System

Tube features:

- Effective stabilization of:
  - White blood cells – Helps prevent release of gDNA
  - Red blood cells – Helps minimize hemolysis
- Helps maximize ccfDNA yield from plasma, minimize background gDNA
- Non-crosslinking, no DNA modification
- BD Vacutainer® plastic tube with BD Hemogard™ safety closure
  - Helps minimize risk of tube breakage
  - Enhances safety for healthcare and lab personnel
  - Helps minimize contamination between samples
  - Consistent blood draw volume

PAXgene Blood ccfDNA Tube (RUO)

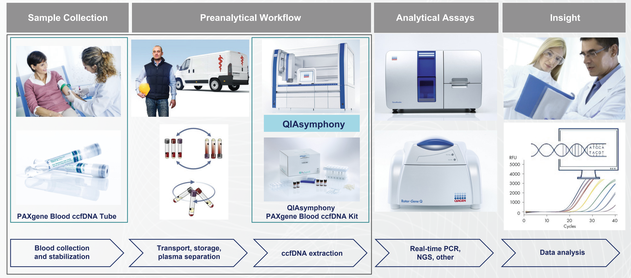
QIAsymphony® PAXgene Blood ccfDNA Kit (RUO)

Kit features:

- Dedicated ccfDNA isolation technology for use with the PAXgene Blood ccfDNA Tube
  - Binding chemistry optimized for use with PAXgene ccfDNA Tube reagent
  - Optimized input volumes to accommodate higher volume plasma
- Automated ccfDNA extraction
  - Standard protocol for preferred extraction of small fragments (≤ 500 bp) with 60 µl elution volume
  - Large fragment protocol for co-isolation of large fragments (> 500 bp) with flexible elution volume (60, 100, 150 µl)
- 2.4 ml or 4.8 ml plasma processing options
- Prefilled cartridges are ready to use

PAXgene Blood ccfDNA System Workflow

PAXgene Blood ccfDNA Tube and Automated QIAsymphony Kit: Integrated Collection-Stabilization-Preparation (CSP) System



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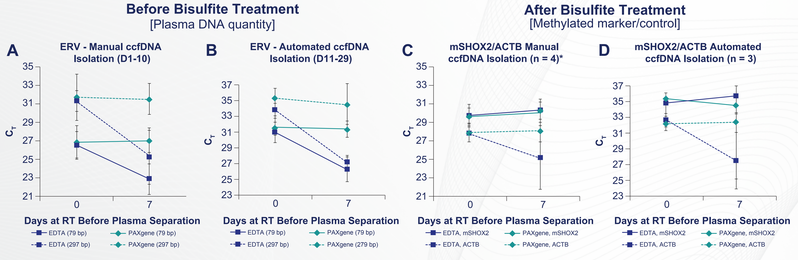
Study 1: Performance with Methylated ccfDNA Lung Cancer Biomarkers

Study Design and Methods

Blood from 29 consented lung cancer patients under treatment was collected in 2 K$_3$EDTA and 2 PAXgene Blood ccfDNA Tubes. Plasma was either separated from the blood cells shortly after blood collection or the blood was stored for 7 days at room temperature (RT) before plasma separation. ccfDNA was isolated manually from plasma samples of donor 1–10 by using the QIAamp® Circulating Nucleic Acid Kit (QIAGEN). An automated ccfDNA isolation procedure was used with plasma samples from donors 11–29 either using the QIAsymphony Circulating DNA Kit (QIAGEN) for EDTA plasma or using the QIAsymphony PAXgene Blood ccfDNA Kit (PreAnalytiX) for plasma generated from blood collected in PAXgene Blood ccfDNA Tubes. Due to swelling of blood cells during prolonged sample storage in EDTA tubes, the plasma yield decreased substantially. Therefore less plasma had to be used for the automated compared to the manual method. The difference in plasma input volume, along with variation between donor groups, is reflected by generally increased C_T levels of eluates generated with the automated preparation. Isolated ccfDNA was quantified by real-time PCR for the amount of the ERV (endogenous retrovirus) sequence as a measure of the total plasma DNA quantity and after bisulfite treatment for methylated SHOX2 as a marker for ctDNA and ACTB (β-actin) as unmethylated control sequence.

Figure 1: Change of target C_T over storage time for ccfDNA from plasma generated from EDTA and PAXgene Blood ccfDNA Tubes



Real-time PCR assays amplifying 2 fragments of the single copy ERV sequence were used to measure DNA content of original eluates after manual (A) and automated (B) ccfDNA isolation. After bisulfite treatment assays detecting methylated SHOX2 and unmethylated ACTB (control amplicon) were used to characterize these eluates using manual (C) and automated (D) ccfDNA isolation. 4 out of 10 samples processed using the manual method and 3 out of 19 samples processed using the automated method gave valid signals for methylated SHOX2 (C_T mSHOX2 < 37) and are shown here.

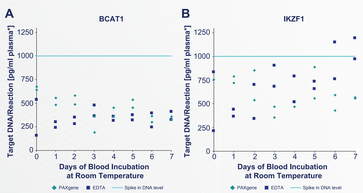
Study 2: Performance with Methylated ccfDNA Colorectal Cancer Biomarkers

Study Design and Methods

Whole blood was obtained from consented healthy donors in both K$_3$EDTA and PAXgene Blood ccfDNA Tubes. Blood from each tube type was pooled separately and spiked with 1000 pg/ml methylated DNA, transferred back into the respective tubes and left at room temperature for up to 7 days. Tubes were gently inverted twice every day to simulate tubes in transit. Two tubes, one of each type, were taken each day and centrifuged to generate plasma. ccfDNA was isolated from EDTA plasma using the QIAsymphony DSP Virus/Pathogen Kit (QIAGEN) or from PAXgene Blood ccfDNA Tube plasma using the QIAsymphony PAXgene Blood ccfDNA Kit (PreAnalytiX). Isolated ccfDNA fragments were bisulfite-converted using the EpiTect® FAST Kit (QIAGEN) and analyzed with the GEMINI® assay (Clinical Genomics) for 2 methylated targets (BCAT1 and IKZF1) and 1 control target (ACTB).

In a similar approach, pooled blood from 10 consented healthy donors was not spiked with methylated DNA to analyze the occurrence of false positive callings over time. Four blood samples of each tube type per day were processed with the same procedure as described above and tested for positivity with 3 PCR reaction replicates per target.

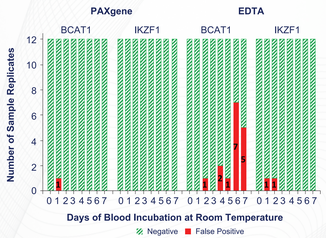
Figure 2: Change of target signal over storage time for plasma ccfDNA generated from blood collected and stored in EDTA and PAXgene Blood ccfDNA Tubes



After bisulfite treatment, a triplex real-time PCR assay was used to characterize the eluates: Two methylated target sequences BCAT (A) and IKZF1 (B) were amplified.

*Data were corrected for the reduced plasma volume. High losses from plasma recovery resulted in low target concentrations for the EDTA tube. This led to more variable and less accurate concentration determination.

Figure 3: False positive callings over storage time



Three PCR replicates per target sequence were analyzed with eluates generated from blood of 4 donors collected either in both EDTA and PAXgene Blood ccfDNA Tubes. Blood was stored for up to 7 days at room temperature (25°C).

Table 1: Overall GEMINI positivity

	EDTA	PAXgene	Total
POS	13 [41%]	1 [3%]	14
NEG	19	31	50
Total	32	32	64

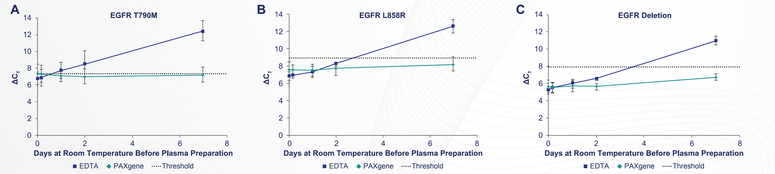
At least 1 of all 6 PCR reactions has to give a signal to be regarded as positive.

Study 3: Performance with EGFR ccfDNA Biomarkers

Study Design and Methods

Blood from 7 healthy donors was collected in 7 EDTA and 7 PAXgene Blood ccfDNA Tubes and spiked with restriction enzyme treated EGFR DNA containing exon 19 deletions and exon 20 and 21 substitutions (T790M, L858R). Plasma was either separated from the blood cells shortly after blood collection or the blood was stored for up to 7 days at room temperature before plasma separation. ccfDNA was isolated from plasma samples using an automated ccfDNA isolation procedure either with the QIAsymphony Circulating DNA Kit (QIAGEN) for EDTA plasma or using the QIAsymphony PAXgene Blood ccfDNA Kit (PreAnalytiX) for plasma generated from blood collected in PAXgene Blood ccfDNA Tubes. Isolated ccfDNA was tested with the QIAGEN *therascreen*® EGFR Plasma RGQ PCR Kit (RUO).

Figure 4: Change of ΔC_T values of target versus control signal over storage time for ccfDNA from plasma generated from EDTA and PAXgene Blood ccfDNA Tubes



The relative signals of the 3 mutations covered by the *therascreen* EGFR Plasma RGQ PCR assay are shown: (A) point mutation T790M, (B) L858R, and (C) deletion in exon 19. To calculate the ΔC_T value the average C_T of each mutant was subtracted by the average C_T of the wildtype sub-assay. The average of all donors is shown in the figure for all test time points. If the sample ΔC_T value is higher than the threshold, it is classed as "mutation not detected" or beyond the limits of detection of the kit. If the sample value is at or below the threshold, the sample is considered positive for a mutation detected by that assay.

Results

Both studies 1 and 2 demonstrated constant levels of the methylation ctDNA markers, *SHOX2, BCAT1* and *IKZF1*, in PAXgene Blood ccfDNA Tubes over the investigated time course. There was no significant release of gDNA in the PAXgene Blood ccfDNA Tube whereas a significant release of gDNA was detected in EDTA samples. The change in the ratio between target and control signal was significantly lower in ccfDNA from PAXgene compared to EDTA samples. For EDTA blood accessible plasma volume strongly decreased while hemolysis increased drastically over time compared to blood collected in PAXgene Blood ccfDNA Tubes where only small changes in these parameters were observed. In study 2 the percentage of false positive samples was 41% for EDTA and 3% for PAXgene samples.

Likewise, study 3 showed constant EGFR C_T values in the PAXgene system with reliable mutation detection, whereas the high DNA concentration from the EDTA system resulted in false-negative callings.

Conclusions

The new system allows researchers to accurately detect and quantify plasma cancer biomarkers from blood samples that have been stored in the tube for up to 7 days at RT. This includes challenging assays based on methylated ctDNA. The system provides the required assay sensitivity to allow the correct assay interpretation beyond the typical 3–6 hour storage limit for EDTA tubes.

Disclaimer: The PAXgene Blood ccfDNA System is For Research Use Only. Not for use in diagnostic procedures.