Performance Evaluation of Collection/Stabilization/Purification Systems for Liquid Biopsy Cancer Biomarker Applications

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Background

There is a growing need for collection, stabilization and purification systems for circulating tumor DNA (ctDNA) in liquid biopsies for clinical oncology research. Workflow challenges for ctDNA detection and analysis include release of genomic DNA from white blood cells (WBCs). Also, tubes that stabilize WBCs often contain reagents that can have negative effects on sensitive downstream assays.

In this study, the performance of collection, stabilization and automated extraction systems was evaluated using unstabilized blood samples and blood samples stabilized with either non-crosslinking or crosslinking (formaldehyde releasing substances) on sensitive downstream assays including quantitative PCR (qPCR) and next generation sequencing (NGS).

Methods

Blood samples from healthy donors were collected into spray-dried EDTA tubes, PAXgene® Blood ccfDNA Tubes* and Streck Cell-Free DNA BCT® tubes. DNA containing EGFR exon 20 and 21 substitutions (T790M, L858R), sheared with restriction enzymes, was spiked into a subset of samples after blood draw. Paired tubes were stored for up to 14 days at temperatures ranging from 5 to 35°C. Automated ccfDNA extraction was performed on the QIAsymphony® instrument (QIAGEN) using tube-specific kits and protocols. ccfDNA profiles were analyzed on the Agilent Bioanalyzer using the Agilent High Sensitivity DNA Kit. Formaldehyde concentration in reagents and plasma were measured with MQuant™ Formaldehyde Test (Merck). ccfDNA yield and stability were determined by qPCR. EGFR mutations were analyzed by qPCR and sequenced using the GeneReader™ instrument (QIAGEN) with the GeneRead™ QIAact Actionable Insights Tumor (AIT) Panel (QIAGEN).

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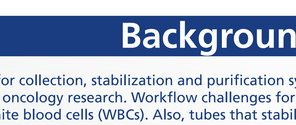
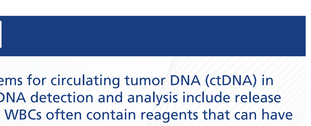
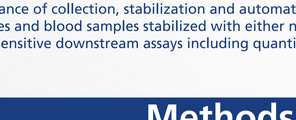
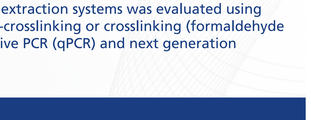
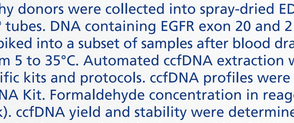
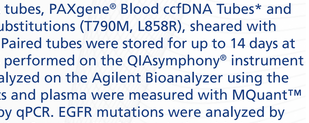
Current stability claims for the PAXgene Blood ccfDNA Tube are 2–25°C for up to 7 days and 35°C for up to 1 day. These studies represent ongoing data generation to explore the performance limits of stabilization and purification technologies.

Detection of Formaldehyde

PAXgene Blood ccfDNA Tube reagent and plasma is free of formaldehyde or formaldehyde releasing substances.

	Reagent	Plasma
Control **Left:** Water **Right:** EDTA plasma undiluted	 0 g/L - formaldehyde-free	 0 g/L - formaldehyde-free
PAXgene **Left:** Reagent undiluted **Right:** Plasma undiluted	 0 g/L - formaldehyde-free	 0 g/L - formaldehyde-free
Streck **Left:** Reagent 1:200 **Right:** Plasma undiluted	 12 g/L ~ 1.2% formaldehyde	 0.1 g/L ~ 0.01% formaldehyde
Formalin (NBF) **Left:** Reagent 1:1000 **Right:** Plasma (10 ml EDTA blood + 200 µl NBF) 1:10	 4 g/L ~ 4% formaldehyde	 0.6 g/L ~ 0.06% formaldehyde

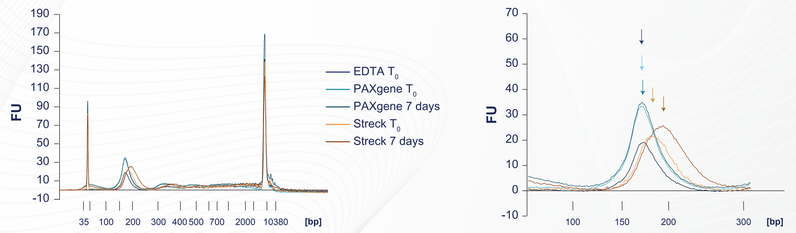
Formaldehyde concentration in reagents and plasma measured with MQuant Formaldehyde Test (Merck, Product number 110036). Formaldehyde (HCHO) reacts with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole to form a purple-red tetrazine. The formaldehyde concentration is measured semi quantitatively by visual comparison of the reaction zone of the test strip with the fields of a color scale. Formalin = Formalin Solution, 10% Neutral Buffered, containing 4% formaldehyde (w/v) (Sigma-Aldrich).

Plasma samples were generated within 2 h after blood collection. For fixation with formaldehyde 200 µl NBF were added to K_2EDTA blood directly after blood collection.

ccfDNA Profiles

Storage of blood in PAXgene Blood ccfDNA Tubes does not change integrity due to chemical modification and crosslinking.

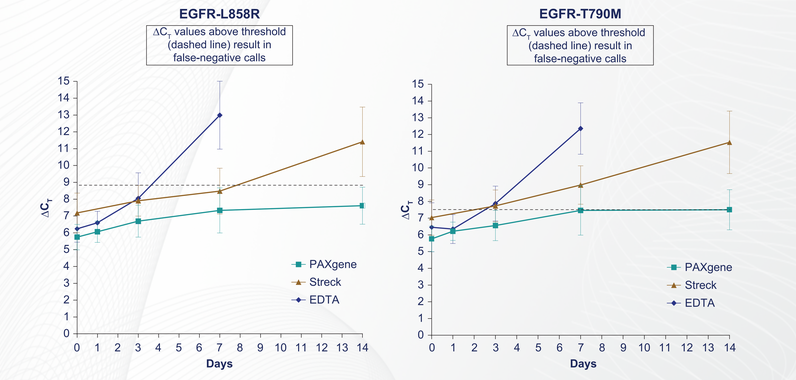
Blood sample	EDTA T_0	PAX T_0	PAX 7d	Streck T_0	Streck 7d
Size (bp) of main ccfDNA peak	173	170	172	181	196



ccfDNA profiles from blood of one donor drawn into K_2EDTA, PAXgene Blood ccfDNA and Streck Cell-Free DNA BCT tubes. Plasma was processed directly after blood draw (T_0), or after blood storage for 7 days at 25°C (7*d*). DNA analyzed on the Agilent Bioanalyzer using the Agilent High Sensitivity DNA Kit.

EGFR Mutation Detection with qPCR

Improved sensitivity with PAXgene Blood ccfDNA System using QIAGEN *therascreen*® EGFR Plasma RGQ PCR Kit.

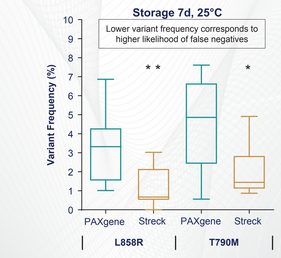


EGFR mutation analysis by qPCR: DNA containing 500 copies of the EGFR mutation L858R and T790M spiked in blood tubes directly after blood draw. ccfDNA extracted after storage at room temperature. Mutation analysis for EGFR L858R and T790M variants with the *therascreen* EGFR Plasma RGQ PCR Kit (CE-IVD); ΔC_T values were calculated by subtracting C_T of the wildtype assay from C_T for the mutation; ΔC_T values above threshold (dashed line) result in false-negative calls.

Number of donors per tube type included: PAXgene T_0 *n* = 29, 1d *n* = 5, 3d *n* = 25, 7d *n* = 45, 14d *n* = 40; Streck T_0 *n* = 18, 3d *n* = 15, 7d *n* = 23, 14d *n* = 17; EDTA T_0 *n* = 45, 1d *n* = 4, 3d *n* = 6, 7d *n* = 27. Average and standard deviation shown.

EGFR Mutation Detection with NGS

Improved sensitivity with PAXgene Blood ccfDNA System using GeneReader NGS workflow.



EGFR mutation analysis by NGS: DNA containing 500 copies of the EGFR mutation spiked in blood tubes directly after blood draw.

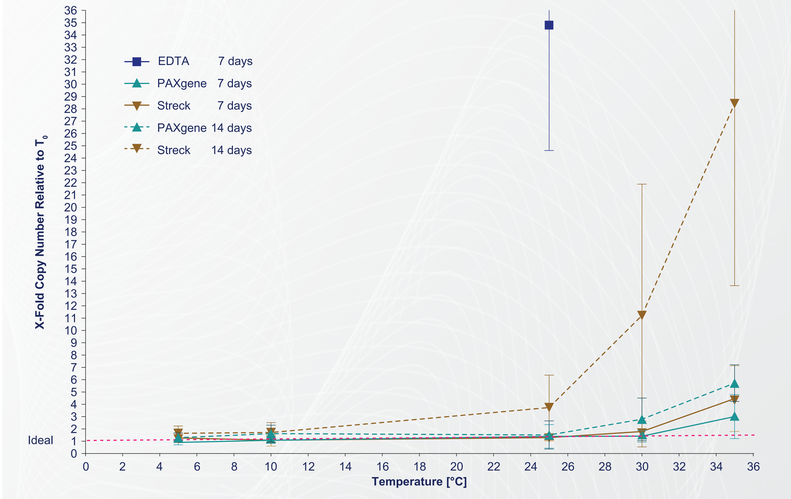
ccfDNA extracted after storage for 7 days at 25°C. Variant frequencies detection for EGFR mutations L858R and T790M with the GeneReader NGS AIT Panel.

Data presented as percentage variant calls in wildtype background reported from QIAGEN QCI™ analysis; box plots with median, upper and lower quartiles, whiskers with min. and max., and outliers (*).

Matched donors *n* = 12.

Temperature-dependent ccfDNA In Situ Stability

The PAXgene Blood ccfDNA System enables reliable stabilization of ccfDNA level over a broad temperature range



ccfDNA stability: copy numbers of 18S rDNA 66 bp fragment measured by Primer/Probe qPCR assay. Relative copy numbers in plasma from EDTA, PAXgene and Streck tubes stored at different temperatures for 7 and 14 days compared to directly after blood draw (T_0).

Dashed line indicating ideal: i.e., no change versus T_0. Average and standard deviation shown.

Time (d)	Temp (°C)	# matched donors	Median x-fold copy number relative to T_0		
			PAXgene	Streck	EDTA
7	2–8	34	0.9	1.2	n/a
	10	9	1.1	1.1	n/a
	25	28	1.1	1.3	28
	30	42	1.2	1.6	121
	35	8	3.2	3.1	n/a
14	2–8	9	1.2	1.9	n/a
	10	9	1.4	1.4	n/a
	25	28	1.2	3.2	98
	30	17	2.5	7.8	131
	35	4	5.1	27.0	n/a

Conclusions

- Stabilization of whole blood for sensitive ccfDNA applications like qPCR and NGS is mandatory.
- The PAXgene Blood ccfDNA Tube stabilizes ccfDNA over a broad range of temperatures.
- The preservative in the PAXgene Blood ccfDNA Tube does not contain the crosslinking agent formaldehyde or formaldehyde-releasing substances.
- The profile of ccfDNA isolated using the PAXgene Blood ccfDNA System is preserved without any evidence of DNA modification.
- ccfDNA isolated with the PAXgene Blood ccfDNA System can be used for high sensitivity mutation detection.
- Fixative-mediated stabilization with chemical modification of ccfDNA may increase the risk of interfering with the sensitivity of downstream assays.

Disclaimer

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Handbooks and user manuals are available at **www.qiagen.com** and **www.preanalytix.com** or can be requested from QIAGEN Technical Services or your local distributor.

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www.gov.uk/government/organisations/innovate-uk; http://gtr.rcuk.ac.uk/projects?ref=101867