White Paper

Does Stabilization Chemistry Matter? Performance Comparison of PAXgene® and Streck® Stabilization Blood Collection Tubes for ccfDNA Testing

Introduction

There is growing interest in using circulating cell-free DNA (ccfDNA) as a marker in liquid biopsy applications for non-invasive prenatal testing (NIPT), oncology, transplant medicine, and organ diseases. In most cases, only a small amount of ccfDNA is found in blood, requiring efficient isolation and highly sensitive downstream methods to detect these markers. The release of genomic DNA (gDNA) from lysed or apoptotic white blood cells (WBC) exacerbates the challenge by diluting the already miniscule amount of ccfDNA in whole blood. To minimize the risk of gDNA release from nucleated blood cells, blood collected into tubes without stabilization chemistry, such as EDTA tubes, must be processed to separate plasma within 4–6 hours of sample collection (1–3).

Plasma separation within a close timeframe may be difficult to achieve and track at collection sites, not to mention the equipment and supplies required for separation of plasma prior to shipment. Commercially available blood collection tubes that stabilize ccfDNA in whole blood for several days overcome this problem. These tubes are prefilled with liquid reagents that stabilize the blood sample immediately upon mixing the collected blood with additives in the tube. These additives prevent release of gDNA from nucleated blood cells, which is essential for stabilization of ccfDNA levels.

This study compares the performance of two blood collection tubes that use different liquid additive chemistries to stabilize ccfDNA: the PAXgene Blood ccfDNA Tube (PreAnalytiX GmbH, Switzerland) and the Streck Cell-Free DNA BCT® (Streck, NE, USA). In some studies the Cell-Free DNA Collection Tube (Roche® Sequencing, CA, USA) was also evaluated. The described methods and analyses are for Research Use Only, and not for in vitro diagnostic procedures.

Methods

Sample collection, processing and ccfDNA extraction

Blood samples from healthy donors were collected into BD Vacutainer® EDTA Tubes containing spray-dried K_2EDTA (EDTA tubes), PAXgene Blood ccfDNA Tubes (PAXgene tubes) and Streck Cell-Free DNA BCT (Streck tubes). Paired tubes were stored for up to 14 days at temperatures ranging from 2–8°C to 35°C. Tubes of each type were centrifuged to separate plasma within 2 hours of collection (day 0), and after storage for 3, 7 and 14 days (depending on the study). Centrifugation conditions used for plasma separation are presented in Table 1. Automated ccfDNA extraction was performed on the QIAsymphony® SP (QIAGEN®) using the QIAsymphony PAXgene Blood ccfDNA Kit (PreAnalytiX GmbH) to process PAXgene plasma samples, and QIAsymphony DSP Circulating DNA Kit (QIAGEN) to process EDTA and Streck plasma samples. The input plasma volume recommended by each corresponding kit handbook was used.

Tube type	1st Centrifugation	2nd Centrifugation
EDTA	15 min at 1900 × g	10 min at 16000 × g
PAXgene	15 min at 1900 × g	10 min at 1900 × g
Streck	15 min at 1600 × g	10 min at 16000 × g

Table 1. Centrifugation conditions for plasma separation

ccfDNA profile and in situ stability

ccfDNA eluates were analyzed on the Agilent® BioAnalyzer (Agilent Technologies, Santa Clara, CA) to obtain DNA electropherograms. Relative ccfDNA amount over the storage period was estimated indirectly by measuring the cellular release of the 18S rDNA 66 bp fragment using qPCR (4). ccfDNA stability was determined as the ratio of 18S rDNA copy number on day 7 and 14 to that on day 0. Ratio values closest to 1 represent the most effective ccfDNA preservation (i.e., no change over time).

Formaldehyde measurement in tube stabilization additives

Stabilization additives and plasma from the PAXgene and Streck tubes were subjected to the MQuant™ Formaldehyde Test (Merck®, Germany). In this method, formaldehyde or other aldehydes (e.g., acetaldehyde) present in a test solution react with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole on a test strip to form purple-red tetrazine. Formaldehyde concentration is then measured semi-quantitatively by visually comparing the color on the test strip with the test color scale. A formalin solution with 10% neutral buffered formalin (NBF) containing a mass fraction of 3.7% formaldehyde (Sigma-Aldrich) served as a positive control in this study. For another positive control, formalin was used as stabilization agent (5) and 200 µl NBF were added to 10 ml K_2EDTA blood directly after blood collection. Water and plasma from EDTA tubes were used as negative controls.

Hemolysis

For measurement of plasma free hemoglobin (pfHb) to evaluate hemolysis, blood samples from 20 healthy donors were collected into PAXgene, Roche, Streck and EDTA tubes. Plasma was separated at initial time point, within 2 hours after blood collection (day 0), and after 3 days, and after 7 days of whole blood storage at room temperature. Testing of plasma samples for pfHb levels was performed on Beckman Coulter® DxC/I chemistry analyzer.

Mutation detection technologies

Blood samples from 20 healthy donors were collected into EDTA, PAXgene and Streck tubes. The equivalent of 500 copies of EGFR exons 20 and 21 carrying T790M and L858R substitution mutations was spiked into blood samples after blood draw. The spike-in copy number 500 was selected to be near the cut-off point for ΔC_T when using the *therascreen*® EGFR Plasma RGQ PCR Kit (QIAGEN) for mutation detection. Prior to spiking, the EGFR DNA was sheared with restriction enzymes to mimic the size of natural ccfDNA fragments. For each donor, spiked blood samples were processed at 3 time points: on day 0, after 7 and after 14 days of storage at room temperature. Centrifugation conditions for plasma separation were as indicated in **Table 1.** Automated ccfDNA extraction from plasma samples was performed on the QIAsymphony SP using tube-specific kits and protocols: the QIAsymphony PAXgene Blood ccfDNA Kit protocol for PAXgene tubes and the QIAsymphony DSP Circulating DNA Kit protocol for EDTA and Streck tubes.

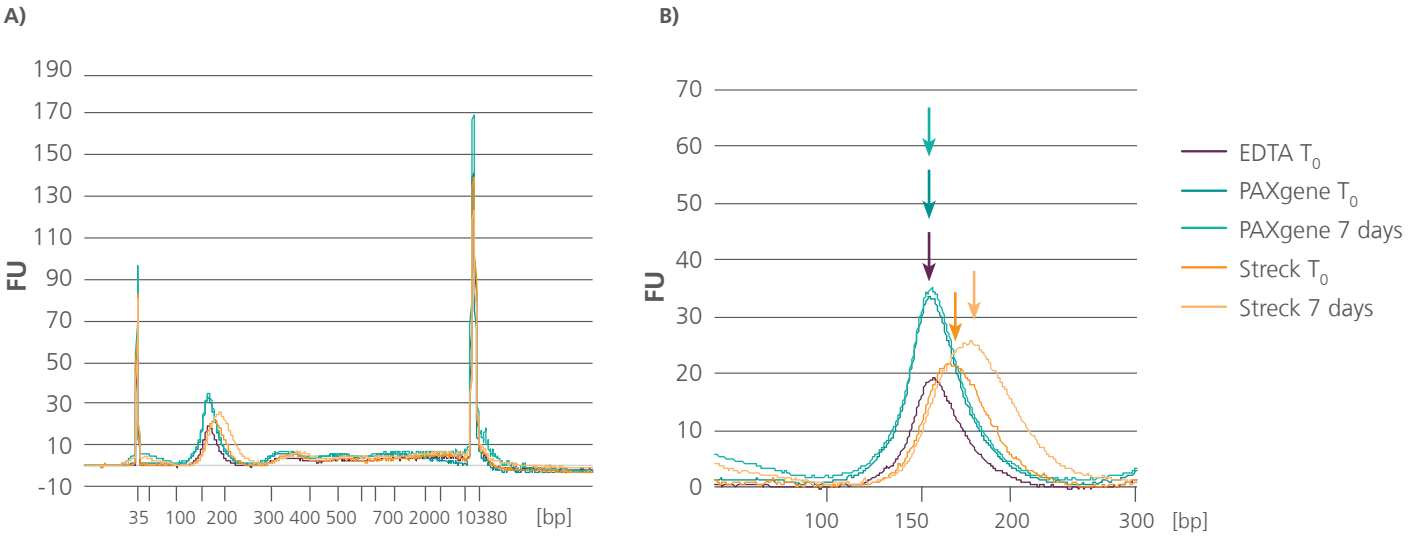
The *therascreen* EGFR Plasma RGQ PCR Kit was used to analyze the EGFR T790M mutation. This real-time qualitative PCR assay enables the detection of exon 19 deletions and exons 20 and 21 substitutions (T790M and L858R, respectively) in the EGFR oncogene against a background of wild type gDNA, using mutation-specific primers and probes to selectively amplify mutated DNA. ΔC_T values for EGFR mutation T790M were calculated by subtracting C_T of the wild type control assay from C_T for the mutation assays, as described in the handbook.

Variant detection by NGS was performed on the QIAGEN GeneReader® instrument using the GeneRead® QIAact Actionable Insights Tumor (AIT) Panel (QIAGEN). Out of the total 20 samples spiked with L858R and T790M mutations, 12 samples from day 7 were tested by NGS. The 12 samples were selected for having the largest ΔC_T difference between PAXgene and Streck tubes in the *therascreen* EGFR test.

Results

Stabilization chemistry

The profiles of ccfDNA isolated from EDTA, Streck and PAXgene plasma samples from the same donor were compared using the Agilent BioAnalyzer. Representative profiles are shown in **Figure 1**. A close look at the main ccfDNA peak at ~170 bp (**Figure 1B**) reveals a difference between PAXgene tubes and Streck tubes. The peak for ccfDNA isolated from PAXgene tubes on day 7 overlaps well with that of PAXgene tubes on day 0. The size of the main peak in PAXgene (170 and 172 bp) is very close to that of EDTA on day 0 (173 bp), indicating ccfDNA preservation and absence of modification to the ccfDNA in PAXgene tubes. In comparison, the main ccfDNA peak from Streck tubes at day 0 and 7 exhibits a shift towards higher molecular weight, 181 and 196 respectively, which could be potentially due to ccfDNA modification by crosslinking reagents.



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

Figure 1. Agilent Bioanalyzer ccfDNA profiles. **(A)** A representative total profile. **(B)** ccfDNA peak region.

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To determine if the additives of the different collection tubes include formaldehyde or formaldehyde-releasing substances (e.g., diazolidinyl urea) that can lead to crosslinking, the additives alone, as well as plasma generated from blood drawn into the tubes, were subjected to the MQuant Formaldehyde Test **(Figure 2)**.

In the MQuant method, formaldehyde content is represented on a color scale ranging from 0 to 100, where 0 is absence of formaldehyde and 100 is the test maximum (i.e., 100 mg/l HCHO, or 0.1% w/v). As expected, water or plasma generated from blood drawn into EDTA tubes (first row, **Figure 2**) exhibited no formaldehyde content. Also, like EDTA tubes, the PAXgene additive alone and plasma generated from blood stored in PAXgene tubes exhibited a reaction color corresponding to zero, indicating an absence of formaldehyde or a formaldehyde-releasing substance. In contrast, positive controls with diluted NBF solution or plasma generated from EDTA tubes mixed with NBF, as well as Streck additive alone and plasma generated from Streck tubes, showed reaction colors corresponding to upper levels on the scale.

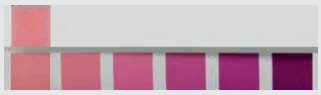
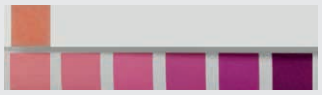
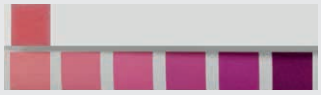
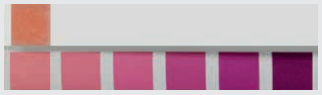
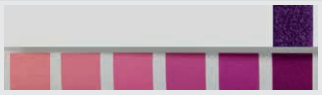
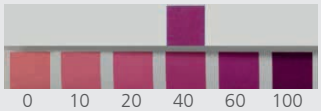
Tube type	Additive	Plasma
Negative control EDTA tube	 **Water** **0 = formaldehyde-free**	 **Undiluted EDTA plasma** **0 = formaldehyde-free**
PAXgene Blood ccfDNA Tube	 **Undiluted tube additive** **0 = formaldehyde-free**	 **Undiluted plasma** **0 = formaldehyde-free**
Streck Cell-Free DNA BCT	 **1:200 diluted tube additive** **60 mg/l = 0.006% formaldehyde (1.2% before dilution)**	 **Undiluted plasma** **100 mg/l = 0.01% formaldehyde**
Positive control	 **1:1000 diluted NBF** **40 mg/l = 0.004% formaldehyde (4% before dilution)**	 **1:10 diluted EDTA plasma + NBF** **60 mg/l = 0.006% formaldehyde (0.06% before dilution)**

Figure 2. MQuant Formaldehyde Test results. The results for formaldehyde concentrations are approximate based on the best color match with the scale by visual assessment.

In situ ccfDNA stability

The stability of ccfDNA was compared between PAXgene tubes and Streck tubes stored for up to 14 days at a temperature range of 2–8°C to 35°C. The relative copy number of a 66 bp fragment of 18S rDNA was measured as a marker for DNA release from apoptotic cells, which dilutes the ccfDNA fraction. Relative copy number value was calculated as the ratio of C_T measured after a storage period of 7 or 14 days to C_T measured on day 0. A value of 1 was considered ideal, indicating minimal DNA release. As shown in **Figure 3**, the greatest gDNA release (approximately 35-fold increase) was observed in samples stored in EDTA tubes for 7 days at 25°C. PAXgene tubes and Streck tubes exhibited comparable performance for a storage duration of 7 days at temperatures of 2–8°C to 25°C. After 14 days at 25°C, total DNA from Streck tubes increased

on average by 3.7-fold, whereas PAXgene tubes still performed close to ideal. At higher temperatures of 30°C and 35°C on day 14, cellular DNA release increased in both tube types; up to 28-fold in Streck tubes and 6-fold in PAXgene tubes.

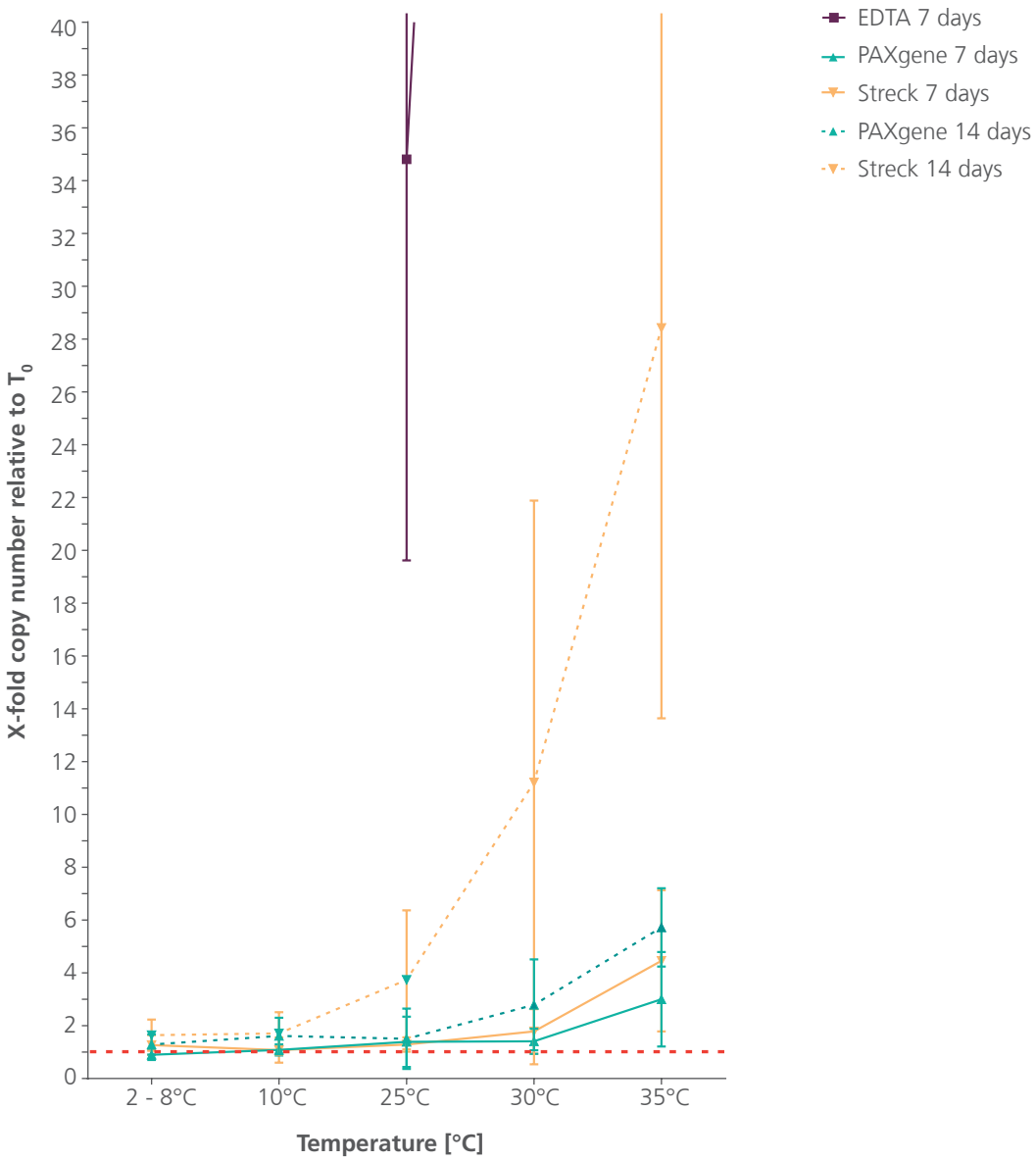


Figure 3. ccfDNA stability in PAXgene and Streck tubes. Copy numbers of 18S rDNA 66 bp fragment measured by qPCR in plasma from EDTA, PAXgene and Streck tubes stored at different temperatures for 7 and 14 days relative to measurements directly after blood draw (T_0). The dashed red line indicates no change relative to T_0. The EDTA storage for 7 days was performed only at 25°C.

Hemolysis

Hemolysis results for 4 tube types are presented in **Figure 4**. Hemolysis is characterized by the release of hemoglobin into plasma as a result of red blood cell damage during blood collection (typically not expected with healthy donors) or during storage. The degree of hemolysis correlates with plasma color from faint pink (trace hemolysis, pfHb <50 mg/dL) to bright red (gross hemolysis, pfHb >300 mg/dL) and can be assessed visually or by measuring plasma free hemoglobin directly. Representative samples stored for 7 days in the four tube types are shown in **Figure 4A**. EDTA, Roche and Streck tubes exhibited visible hemolysis after sample storage in comparison to the PAXgene tube, which showed no or minimal hemolysis.

Figure 4B displays average change in measured pfHb for each tube type during 7 days of storage. On day 3, Roche tubes showed the highest hemolysis compared to the other tubes. On day 7, all tubes except for PAXgene were moderately hemolyzed on average (pfHb was above trace levels of 50 mg/dL).

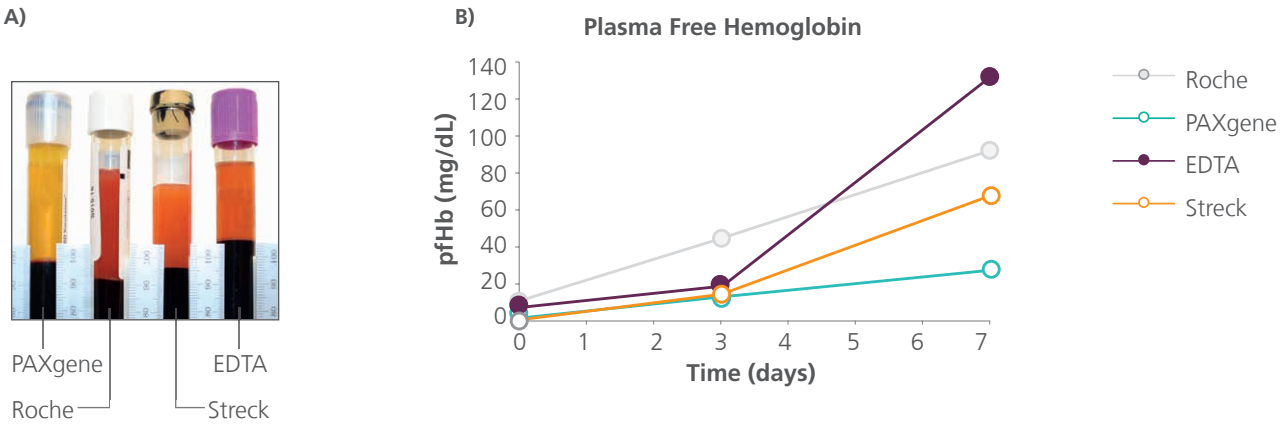


Figure 4. Comparison of 4 tube types for hemolysis. (A) Representative samples after 7 days of sample storage. **(B)** Average plasma free hemoglobin (pfHb) concentration after 0, 3 and 7 days of sample storage (n = 20).

Performance in ccfDNA applications

Results from the *therascreen* EGFR Plasma RGQ PCR Kit are presented in **Figure 5A**. EGFR T790M ΔC_T values from the test are compared for EDTA, PAXgene and Streck tubes. Higher ΔC_T values indicate lower assay sensitivity which may lead to a higher likelihood of false negative results. qPCR assay sensitivity was better maintained with samples stored in PAXgene tubes compared to EDTA and Streck tubes for both storage durations (7 and 14 days), as indicated by a lower median ΔC_T value.

Results from NGS testing of samples from PAXgene and Streck tubes are presented in **Figure 5B**. Variant frequency from QIAGEN QCI-A™ analysis was compared between PAXgene and Streck tubes after 7 days of storage. Variant frequency is interpreted as the number of mutated DNA fragments (L858R or T790M) in a background of wildtype DNA in a sample. For both L858R and T790M, the rate of variant detection was higher from samples stored in PAXgene tubes compared to Streck tubes.

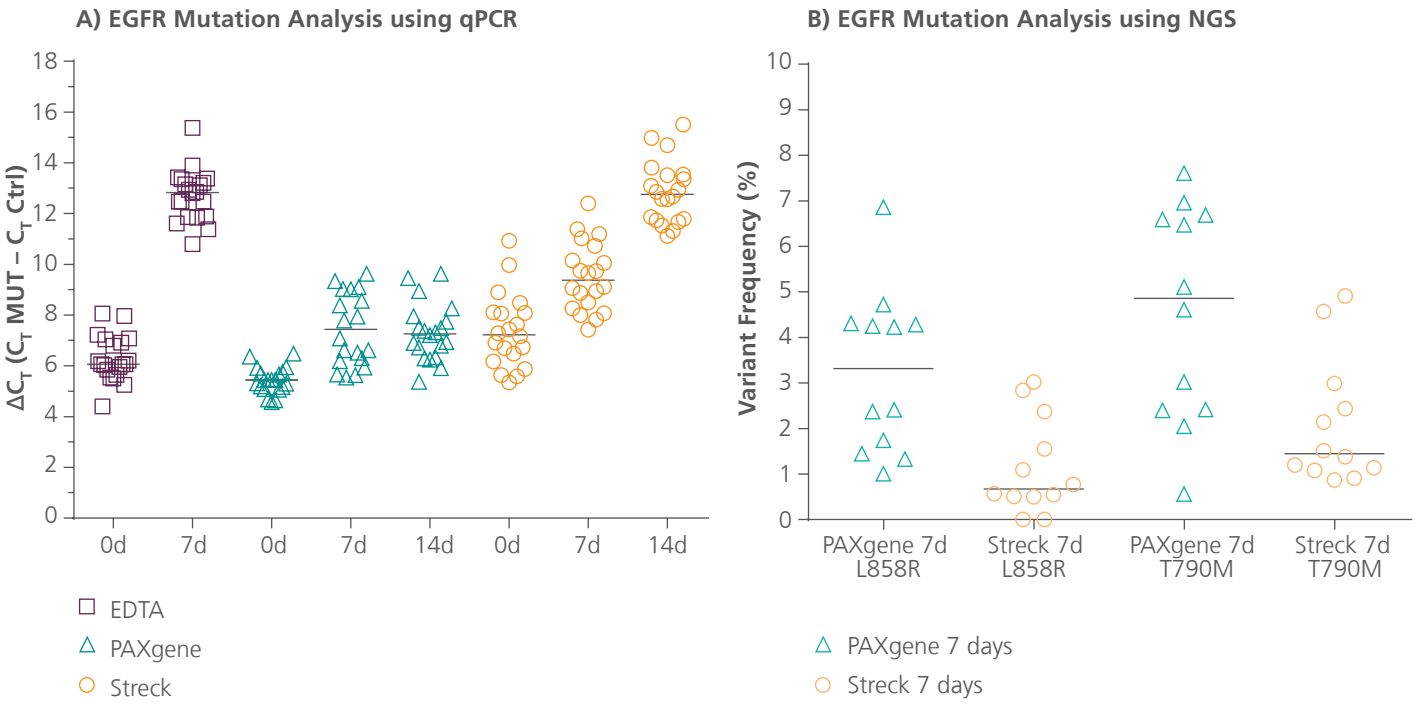


Figure 5. Results from mutation detection assays. (A) Results from analysis of the EGFR T790M variant with the *therascreen* EGFR Plasma RGQ PCR Kit reported as change of ΔC_T from EDTA, PAXgene and Streck tubes stored for 0, 7 and 14 days. **(B)** Variant frequencies for EGFR mutations L858R and T790M detected with the GeneReader NGS AIT Panel after storage in PAXgene and Streck tubes for 7 days. The solid line indicates the median.

Discussion

Blood collection tubes with additives to stabilize ccfDNA allow for extended shipping and storage at a wider range of temperatures prior to plasma separation. The choice of stabilization chemistry is critically important because it can negatively impact the sensitivity of downstream assays. PAXgene tubes are free of crosslinking chemicals, such as formaldehyde, and exhibited no evidence of ccfDNA modification (**Figures 1 and 2**). Crosslinking reagents chemically modify DNA and protein, and create protein-DNA, formaldehyde-DNA, protein-protein complexes and intrastrand DNA crosslinks which, if not completely reversed, may limit the suitability of DNA for downstream enzymatic or amplification reactions (6). Even extensive digestion with Proteinase K as part of ccfDNA purification protocols may not completely resolve these complexes (7, 8), and the negative impact from crosslinking chemistry may become more evident when the input concentration of ccfDNA is close to an assay's limit of detection (LoD). A study aimed at evaluating the impact of blood collection tube types on the LoD of a PCR-based EGFR mutation detection kit, found better sensitivity with PAXgene tubes than with Streck tubes at lower ranges of DNA input (9). This study, as well as our own data, suggest that the stabilization chemistry in PAXgene tubes may help to optimize assay detection sensitivity.

Among important applications of liquid biopsies is the analysis of DNA methylation as a biomarker for cancer. A commonly used method is to treat DNA with bisulfite to convert unmethylated cytosine to uracil. Methylated cytosine is protected from conversion by bisulfite and hence, can be specifically detected in subsequent PCR. During crosslinking, formaldehyde forms a methyl bridge with cytosine or guanine bases through a covalent bond (10), potentially interfering with downstream bisulfite modification. Reports indicate that the choice of blood collection tube may impact the success and sensitivity of methylation

detection assays. Schmidt et al. (11) reported no effects from the stabilization solution of PAXgene tubes, while experiments to quantify methylated SHOX2 DNA (mSHOX2) as a marker for advanced stage non-small cell (NSCLC) and small-cell lung cancer (SCLC) were unsuccessful with Streck tubes. Distler et al. (12) used spiked samples to assess methylated SEPT9, a screening marker for colorectal cancer, and found that sensitivity and specificity from samples stored in Streck tubes for 7 days at 25–30°C were low. They indicated that increased gDNA from lysed lymphocytes and reduced stability of ccfDNA were the possible causes for the negative impact of Streck tubes on assay specificity and sensitivity, respectively. Finally, Alsobrook et al. (13) successfully validated the PAXgene Blood ccfDNA Tubes and QIAsymphony PAXgene Blood ccfDNA Kit and protocols for use with the Colvera™ Colorectal Cancer (CRC) Recurrence Assay (Clinical Genomics Technologies Pty Ltd, Australia), which targets methylated BCAT1 and IKZF1 genes. They also demonstrated that PAXgene tubes achieved lower LoD than EDTA tubes.

The in situ ccfDNA stability results in **Figures 3 and 5A** include data for PAXgene tubes after 14 days of sample storage, which is beyond the 7 days indicated in the product performance claims of the PAXgene ccfDNA Tube. These data are included for information purposes only. Results from the qPCR-based 18S rDNA assay demonstrated comparable performance between PAXgene and Streck tubes for samples stored at room temperature for up to 7 days. PAXgene tubes, however, showed better ccfDNA stability at the longer storage duration of 14 days at temperatures of 25 to 35°C. Comparable performance between PAXgene and Streck tubes in the preservation of DNA concentration after 4 days of storage has been reported independently (14). The authors of the study also noted that 4 days of room temperature storage slightly increased high-molecular weight bands in the Streck tubes compared to samples processed 1 h after collection. Nikolaev et al. (15) compared EDTA, PAXgene and Roche tubes stored at different temperatures and times, and found that PAXgene and Streck tubes performed better than Roche tubes at elevated temperatures, with no detectable gDNA contamination for up to a week when stored either at room temperature (22°C) or summer-like temperatures (30°C daytime and 22°C nighttime temperatures).

qPCR and NGS are among the most widely used mutation detection methods in ccfDNA applications. Higher sensitivity for qPCR (*therascreen* EGFR Plasma RGQ PCR Kit) and improved variant detection rates in NGS assays were obtained with samples stored for 7 to 14 days in PAXgene tubes compared to Streck tubes (Figures 5A and 5B). Reports from various groups have demonstrated equivalent or superior performance of PAXgene tubes for such applications compared to blood collection tubes from other suppliers. Alidousty et al. (9) showed improved LoD with samples from PAXgene tubes compared to Streck tubes using amplification-refractory mutation system PCR. Using samples stored in PAXgene tubes, Taniere et al. (16) were able to detect all mutations in the cobas® EGFR Mutation Test v2 (Roche Diagnostics GmbH, Germany), which covers 29 deletions in exon 19, T790M, L858R, G719X, S768I, L861Q and 5 insertions in exon 20.

Reports about potential interference of hemolysis on downstream ccfDNA applications is limited. Kim et al. (17) highlights that avoiding hemolysis is an essential step in isolating ccfDNA from plasma. Hemoglobin at a concentration of 2.0 g/L in plasma is also been noted to interfere with the Roche cobas EGFR Mutation Test on the assay package insert. Even in the absence of direct interference from hemoglobin, hemolysis is perceived as an indication of poor sample quality. Thus, visual or automated evaluation of clinical samples for hemolysis is common practice. In addition to red blood cell damage, hemolysis may indicate compromised integrity of other cell types, such as leukocytes, that can release gDNA. Furthermore, minimizing hemolysis ensures a clearly visible plasma layer, which helps avoid aspirating cells from the buffy coat during plasma transfer and thus, maximizes plasma recovery and reduces the risk of increased gDNA background.

Conclusion

The PAXgene Blood ccfDNA Tube and Streck Cell-Free DNA BCT showed comparable performance in preventing gDNA release for up to 7 days at room temperature, based on results from the 18S rDNA 66 bp amplicon qPCR assay. However, PAXgene tubes showed better performance in this regard when blood samples were stored for 14 days. In contrast to the Streck tube, the PAXgene tube was also free of any potential crosslinking formaldehyde substances and, based on the analysis of ccfDNA profiles, induced no chemical modifications to ccfDNA. Most importantly, results of our studies and evidence found in the literature indicate that ccfDNA purified from PAXgene tubes helps to optimize sensitivity of mutation detection assays, as well as performance of assays for the quantitation or detection of methylated ccfDNA markers over other collection methods. This improvement could be due to the non-crosslinking stabilization additive used in PAXgene Blood ccfDNA tubes that results in more accurate preservation of isolated ccfDNA.

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