Comparison of S-Monovette® cfDNA Exact with two blood collection tubes for stabilization of cfDNA

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Abstract

Genetic material in the form of cell-free DNA (cfDNA) is increasingly used as a biomarker for screening, diagnostics and monitoring of diseases such as various cancers or coronary heart diseases. The use in non-invasive prenatal diagnostics is another growing field of application. However, cfDNA based analytics is complicated by the low abundance of cfDNA and its limited stability in a blood sample.
To address these issues, a variety of blood collection tubes have been introduced throughout the past decade which are dedicated to maintain the level/concentration of non-degraded cfDNA in a blood sample. Nevertheless, these devices show limitations e.g. in their ability to prevent cell lysis.
Hereafter we introduce the S-Monovette® cfDNA Exact as a new blood collection tube for the stabilization of cfDNA after phlebotomy. We compare the stabilization capacity of S-Monovette® cfDNA Exact to two other commercially available cfDNA stabilizing blood collection tubes. Thereby we highlight the unique ability of the S-Monovette® cfDNA Exact to stabilize cfDNA at 37 °C proving cfDNA to be usable in various subsequent analyses and guaranteeing consistent results for up to 14 days.

Introduction

Within the human organism, cells continuously shed DNA into the blood circulation. This so called cell-free DNA (cfDNA) might also be sequestered by both apoptotic or necrotic cells (Wagner 2012). CfDNA paints a representational image of the entirety of the body's cells viability and thus is useful for blood-borne, non-invasive diagnostics, so called liquid biopsies. CfDNA has already been utilized for diagnosis of several cancerous diseases (Mayo-de-Las-Casas et al. 2018; Jahr et al. 2001) or other health conditions (Hummel et al. 2018b) but also for prenatal investigations as it is possible to detect fetal cfDNA in maternal blood (Chiu et al. 2008; Jensen et al. 2013; Lambert-Messerlian et al. 2017; Lo et al. 1997). Further approaches utilize cfDNA not only for diagnosis, but also for disease prognosis and monitoring of therapeutic progress (Oellerich et al. 2017; Christensen et al. 2019).
As a relatively young field within diagnostical methods, cfDNA based analyses bear several hurdles that need to be overcome. A remarkable limitation for the use of cfDNA is its low abundance in blood samples. Highest concentrations of cfDNA are found in certain health conditions such as cancer (Lapin et al. 2018; Volik et al. 2016) or coronary heart diseases (Xie et al. 2018). Elevated cfDNA levels might also be a result of physical exercise (Hummel et al. , 2018a). Thereby, in healthy individuals, the blood plasma cfDNA concentrations range from approx. 1.8 – 44 ng/ml (Breitbach et al. 2014; Fleischhacker und Schmidt 2007). The amount of fetal cfDNA in maternal blood is even lower and comprises approximately only 11 to 13.4 percent of the total cfDNA varying widely among pregnant women (Wang et al. 2013). After phlebotomy, the low amount of cfDNA in the sample is reduced even further due to continuous degradation of DNA e.g. by nucleases (Tamkovich et al. 2006). As a result, cfDNA based analyses are generally accompanied by a working step of DNA amplification.
In addition to the small available amount of genetic material of interest, cfDNA-based analyses are complicated by the release of genomic DNA (gDNA) into the blood sample due to lysis of leukocytes in the pre-analytical phase between phlebotomy and analysis. With increasing pre-analytical time, the released gDNA becomes more and more abundant. As a result, cfDNA is masked by the highly abundant gDNA, causing loss of specificity of cfDNA based analyses.
Thus, the stabilization of both cfDNA (=reduction of degradation) and nucleic acid containing blood cells (=reduction of gDNA contamination) is an important and critical pre-analytical factor.
To avoid contamination of samples with gDNA during transport and storage, one possible strategy is to separate plasma immediately in a two-step centrifugation process after phlebotomy (Sorber et al. 2019). However, this procedure requires laboratory equipment and is often not possible at the site of blood collection.
An alternative approach to maintain sample integrity is to prevent release of gDNA. To this end, there are several pre-evacuated blood collection tubes by different

manufacturers containing additives that stabilize white blood cells immediately after phlebotomy. Furthermore, these additives prevent degradation of cfDNA aiming to maintain cfDNA level and integrity for up to 14 days at ambient temperatures (Fernando et al. 2010; Norton et al. 2013; Grölz et al. 2018). These tubes reduce the need for immediate plasma preparation, allow for convenient sample collection, transport and storage and thus support the efforts to establish standardized pre-analytical workflows.

Herewith, we introduce the new S-Monovette® cfDNA Exact for effective stabilization of cfDNA in blood samples. We compare the stabilization performance of the S-Monovette® cfDNA Exact to widely used blood collection tubes by PAXgene™ (PAXgene™ Blood ccfDNA Tubes) and STRECK (Cell-Free DNA BCT®). We demonstrate that using the S-Monovette® cfDNA Exact, cfDNA in whole blood samples is preserved for up to 14 days at temperatures up to 37 °C.

Materials & Methods

Blood sample collection and storage

Blood from healthy donors was drawn into S-Monovette® cfDNA Exact blood collection tubes (Cat. No. 01.2040.001, SARSTEDT AG & Co. KG, Nümbrecht, Germany), STRECK Cell-Free DNA BCT® (STRECK Inc., Omaha, NE, USA) or PAXgene™ Blood ccfDNA Tube (PreAnalytiX GmbH, Hombrechtikon, Switzerland). Samples were incubated at different temperatures (see Table 1, according to the manufactures guidelines. After incubation, plasma was separated by two step centrifugation (see Table 2) and plasma was stored at -80 °C until cfDNA isolation.

Table 1. Storage times in days and temperatures for the three compared stabilization tubes.

cfDNA stabilization tube	storage time [days]	T [°C]	T [°C]	T [°C]
S-Monovette® cfDNA Exact	0	6	25	37
S-Monovette® cfDNA Exact	1			37
S-Monovette® cfDNA Exact	2			37
S-Monovette® cfDNA Exact	3	6	25	37
S-Monovette® cfDNA Exact	5		25	
S-Monovette® cfDNA Exact	7	6	25	37
S-Monovette® cfDNA Exact	10	6	25	37
S-Monovette® cfDNA Exact	14	6	25	37
STRECK Cell-Free DNA BCT®	0	6	25	37
STRECK Cell-Free DNA BCT®	1			37
STRECK Cell-Free DNA BCT®	2			37
STRECK Cell-Free DNA BCT®	3	6	25	37
STRECK Cell-Free DNA BCT®	5		25	
STRECK Cell-Free DNA BCT®	7	6	25	37
STRECK Cell-Free DNA BCT®	10	6	25	37
STRECK Cell-Free DNA BCT®	14	6	25	37
PAXgene™ Blood ccfDNA Tube	0	6	25	37
PAXgene™ Blood ccfDNA Tube	1			37
PAXgene™ Blood ccfDNA Tube	2			37
PAXgene™ Blood ccfDNA Tube	3	6	25	37
PAXgene™ Blood ccfDNA Tube	5	6	25	
PAXgene™ Blood ccfDNA Tube	7	6	25	
PAXgene™ Blood ccfDNA Tube	10	6	25	

Table 2. Centrifugation conditions for harvesting plasma.

Tube	1st Centrifugation	2nd Centrifugation
EDTA	3,000 x g for 10 min	15,000 x g for 15 min
S-Monovette® cfDNA Exact	3,000 x g for 10 min	15,000 x g for 15 min
PAXgene™ Blood ccfDNA Tube	1,900 x g for 15 min	15,000 x g for 15 min
STRECK Cell-Free DNA BCT®	3,000 x g for 20 min	15,000 x g for 15 min

Hemolysis and cfDNA Isolation

Hemolysis in human plasma was analysed photometrically using a spectral photometer (DU® 640 Spectrophotometer, Beckman Coulter GmbH, Krefeld, Germany). CfDNA from S-Monovette® cfDNA Exact and STRECK Cell-Free DNA BCT® was isolated from plasma using the InviGenius® PLUS and the corresponding InviMag® Free circulation DNA Kit (REF 2439326400, INVITEK Molecular, Berlin, Germany) according to manufacturer's instructions. CfDNA from PAXgene™ Blood ccfDNA Tubes was isolated using the QIAamp Circulating Nucleic Acid Kit Cat. No. 55114, QUIAGEN GmbH, Hilden, Germany) according to manufacturer's instructions. To further assess efficacy of the blood collection tube-kit compatibility of cfDNA isolation and cfDNA fragment length analysis, cfDNA isolation was performed with the NucleoSnap® cfDNA Kit (REF 740300.50, Macherey-Nagel GMBH & Co. KG, Düren, Germany).

cfDNA fragment length analysis

DNA fragment length distribution was assayed by capillary electrophoresis with a Bioanalyzer 2100 (Agilent, Waldbronn, Germany) using the High Sensitivity DNA Kit (Cat. No. 5067, Agilent) according to manufacturer's instructions.

qPCR analysis

All RT-qPCRs were performed with the Maxima SYBR Green/ROX qPCR Master Mix (#K0223, Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to manufacturer's protocol on a Mastercycler ep realplex 4S (Eppendorf, Hamburg, Germany) or Real-time Thermal cycler qTOWER3 (Analytic Jena GmbH, Jena, Germany). Primers (see Table 3) were used in a final concentration of 0.5 µM. After an initial denaturation of 10 minutes at 95 °C, 40 cycles of denaturation (20 sec. at 95 °C), annealing (20 sec. at 60 °C) and elongation (15 sec. at 68 °C) were performed.

Table 3. Primer sequences

Primer	Sequence	Annealing temp.	Fragment length
ERV-3fw (Devonshire et al. 2014)	5'-CATGGGAAGCAAGGGAACTAATG-3'	60°C	135 bp
ERV-3rev	5'-CCCAGCGAGCAATACAGAATTT-3'		
MSTNfw (Breitbach et al. 2014)	5'-TTGGCTCAAACAACCTGAATCC-3'	60°C	88 bp
MSTNrev	5'-TCCTGGGAAGGTTACAGCAAG-3'		

DNA concentration measurement

DNA concentration was measured using the Quantus™ Fluorometer (Promega GmbH, Walldorf, Germany) and the corresponding QuantiFluor® dsDNA System (E2670, Promega GmbH).

Data analysis

Median and 25 % – 75 % interquartile range of CT values were calculated to present data with respect to its biological variations (inter donor variations in white blood cell content). For statistical analysis

and plotting of data, GraphPad Prism® 7.03 was used (GraphPad Software Inc., LaJolla, CA, USA). Statistical calculations were performed as follows. For each dataset a Two-Way ANOVA was performed with a Sidak Post-Hoc test and a family-wise significance and confidence level of 0.05 (95 % confidence interval). Data was always only compared to the corresponding time points and temperatures in the S-Monovette® cfDNA Exact.

Results

DNA is released into blood samples within recommended storage time

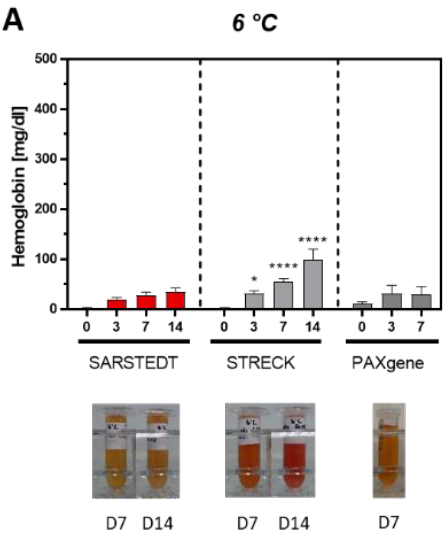
When blood is drawn from the human body and subsequently stored, blood cells start dying shortly after. The lysis of erythrocytes (hemolysis) due to the release of hemoglobin is causing a visually evident, reddish dye of the separated plasma. Furthermore, also leukocytes may undergo lysis and thus release their cellular content, including nucleic acids, into the blood sample.

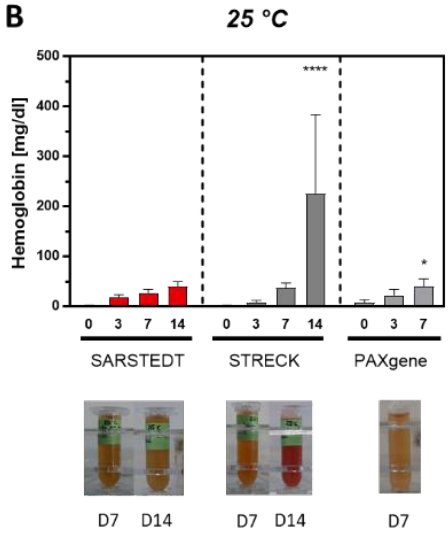
To assess DNA release levels, first blood was drawn into blood collection tubes from PAXgene™ (PAXgene™ Blood ccfDNA Tubes), SARSTEDT (S-Monovette® cfDNA Exact) or STRECK (Cell-Free DNA BCT®) and then stored for up to 14 days at 6 °C, 25 °C or 37 °C (according of the manufacturer's guidelines, see method section). Subsequently, hemolysis levels were measured by determining levels of free hemoglobin.

When blood was stored at 6 °C (fridge temperature), hemolysis was observed in all blood collection tubes. There was comparable hemolysis levels in the S-Monovette® cfDNA Exact and the PAXgene™ tubes, not exceeding 30 mg/dl hemoglobin after day 7 and maximum 34,5 mg/dl at day 14 in the S-Monovette® cfDNA Exact and PAXgeneTM tubes, respectively. In STRECK tubes, hemolysis was most prominent and even visible by naked eye, reaching 31,7 mg/dl at day 3, 54,5 mg/dl at day 7 and going up to 98,5 mg/dl at day 14 (Figure 1A).

Upon storage at 25 °C, continuous increase of hemolysis levels was observed in all blood collection tubes. Moderate hemolysis, not exceeding 40,7 mg/dl, was observed after 14 days of storage in the S-Monovette® cfDNA Exact and 7 days of storage in the PAXgeneTM tubes. For STRECK tubes, there was a highly significant increase of free hemoglobin between day 7 and day 14, reaching more than 226 mg/dl (Figure 1B).

At 37 °C, hemolysis was generally stronger than at 25 °C. While there was comparably low hemolysis in the S-Monovette® cfDNA Exact (reaching approx. 100 mg/dl at day 14), in PAXgene™ tubes the hemolysis level was already significantly increased, reaching approx. 125 mg/dl at day 3. In STRECK tubes, severe hemolysis was observed already at day 7, reaching approx. 172 mg/dl, and at day 14 almost 400 mg/dl - impressively visible by the dark-red to black tone of blood (Figure 1C).





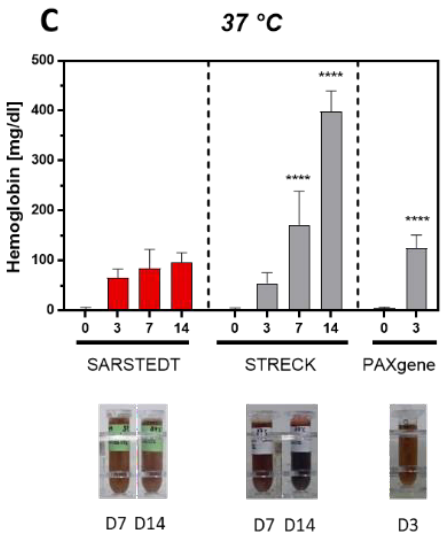


Figure 1: Hemolysis occurs in blood collection tubes. Blood was drawn into SARSTEDT, PAXgene™ and STRECK tubes. (A) Upon storage at 6 °C hemolysis was detected in all blood collection tubes. STRECK tubes showed highest hemolysis levels continuously increasing with storage time and clearly visible with the naked eye. There was less hemolysis in PAXgene™ tubes and in the S-Monovette® cfDNA Exact. (n=7, p≤ 0.05) (B) Upon storage at 25 °C, hemolysis was visible in all blood collection tubes. While the detected hemoglobin levels remained almost constant in PAXgene™ tubes and the S-Monovette® cfDNA Exact, STRECK tubes showed prominent free hemoglobin levels after day 14. (n=7, p≤ 0.05) (C) Upon storage at 37 °C, hemolysis is more prominent in all blood collection tubes. Similar to storage at 25 °C, there is strong hemolysis after 7 days in STRECK tubes compared to less hemolysis in the S-Monovette® cfDNA Exact. In PAXgene™ tubes significant hemolysis could also be detected after day 3. (n=7, p≤ 0.05).

To investigate whether the observed hemolysis is accompanied by leukocyte lysis and thus the release of DNA into the blood samples, we determined the DNA concentration in the samples by quantitative PCR and by measuring the DNA concentrations.
Thereby, we could show that there is release of DNA into the blood samples at all storage temperatures tested (6 °C, 25 °C and 37 °C) and further, demonstrate that DNA release is strongly dependent on the blood collection tube as well as the storage time and temperature.

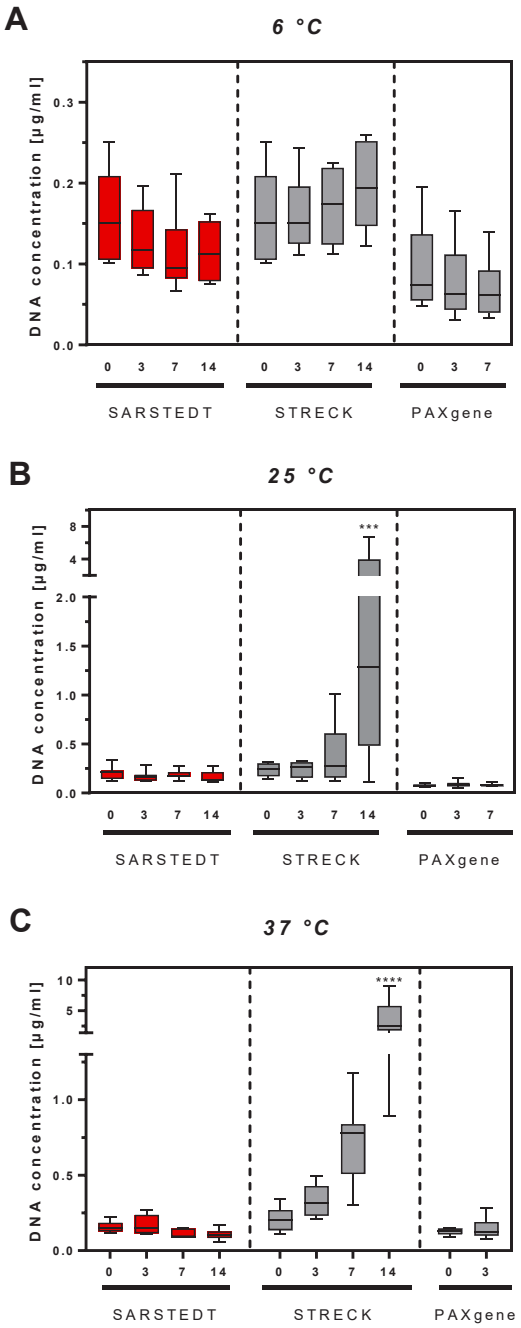


Figure 2: Performance comparison of three different cfDNA stabilizing blood collection tubes. Blood was drawn into PAXgene™ tubes, the S-Monovette® cfDNA Exact or STRECK tubes and stored at 6 °C (A), 25 °C (B) or 37 °C (C). (A) Upon storage at 6 °C, DNA levels were stable in samples stored in the S-Monovette® cfDNA Exact and PAXgene™ tubes as well as in STRECK tubes. (n≥7). (B) Upon storage at 25 °C, DNA levels were stable in samples stored in the S-Monovette® cfDNA Exact and PAXgene™ tubes. In STRECK tubes, there is a strong release of DNA into the samples increasing with storage time. (n>=7). (C) Upon storage at 37 °C, DNA levels were stable in samples stored in the S-Monovette® cfDNA Exact and PAXgene™ tubes. In contrast, in STRECK tubes, there was release of DNA into the samples after three days of storage. (n>=7).

When blood was stored in PAXgene™ tubes or the S-Monovette® cfDNA Exact at 6 °C and cfDNA was isolated with the Qiagen or the InviMag kit, elevated levels of DNA in the S-Monovette® cfDNA Exact (approx. 0.16 µg/ml compared to 0.08 µg/ml) were detectable in comparison to PAXgene™ tubes. The DNA levels remained constant in both the S-Monovette® cfDNA Exact an PAXgene™ tubes for the maximum indicated storage time of PAXgene™ tubes which is 10 days (shown only until day 7). When STRECK tubes where compared for cfDNA-stabilization performance at 6 °C, cfDNA was isolated with the InviMag kit. The DNA levels at day 0 were nearly equal in all investigated tubes (between 0.1 – 0.2 µg/ml). Upon storage, there was no measurable release of DNA into samples stored in the S-Monovette® cfDNA Exact, PAXgene™ or STRECK tubes (Figure 2A).

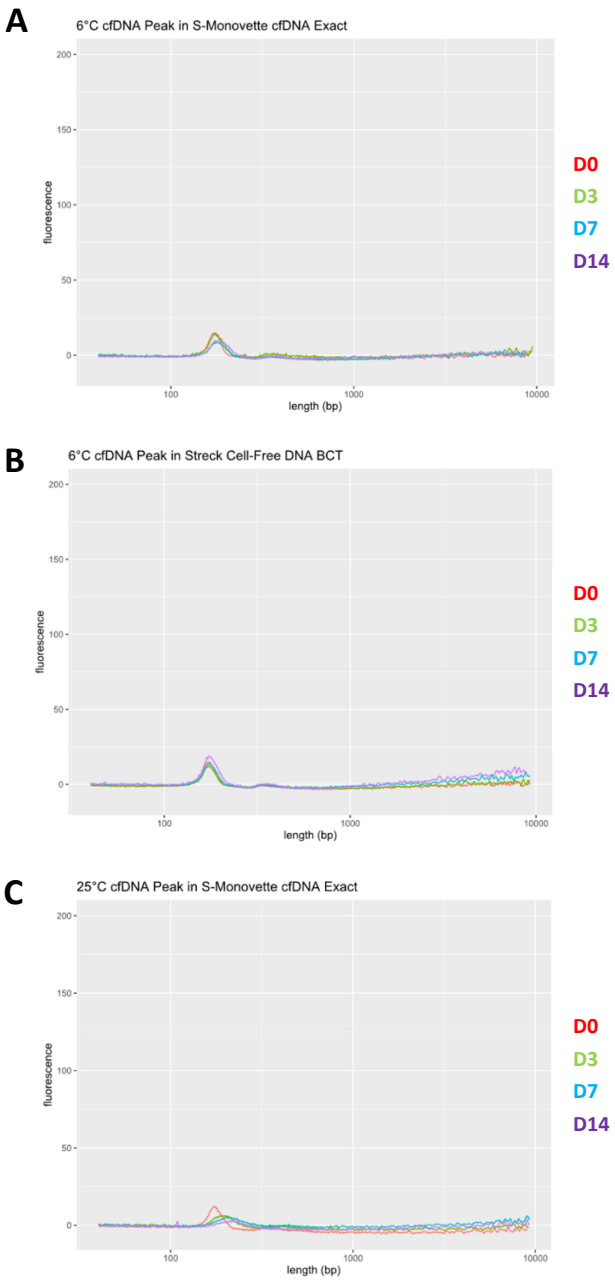
When blood was stored at 25 °C in PAXgene™ tubes, STRECK tubes or the S-Monovette® cfDNA Exact and cfDNA was isolated, the DNA levels on day 0 were nearly the same in between STRECK tubes and S-Monovette® cfDNA Exact (approx. 0.2 µg/ml). In the PAXgene™ tubes the DNA levels were reduced (0.08 – 0.09 µg/ml). When comparing for DNA stabilization performance, DNA levels remained stable for 10 days in the S-Monovette® cfDNA Exact and also in PAXgene™ tubes. STRECK tubes where compared for cfDNA-stabilization performance at 25 °C, again, the DNA levels at day 0 were nearly equal to the S-Monovette® cfDNA Exact (0.23 µg/ml). Notably, in the S-Monovette® cfDNA Exact, there was a slight decrease in DNA levels even after 14 days of storage meaning no DNA was released. In contrast, high amounts of DNA were released into blood stored in STRECK tubes after day 7 and the DNA concentration massively increased to 2,17 µg/ml at day 14 (Figure 2B).

When blood was stored at 37 °C in PAXgene™ tubes, STRECK tubes or the S-Monovette® cfDNA Exact and cfDNA was isolated the DNA levels on day 0 were 0.16 µg/ml in S-Monovette® cfDNA Exact, slightly more in STRECK tubes (0.2 µg/ml) and comparable in PAXgene™ tubes (0.13 µg/ml). The DNA levels remained constant in both, the S-Monovette® cfDNA Exact an PAXgene™ tubes for the maximum indicated storage time of PAXgene™ tubes which is 3 days. After day 3 the DNA level in the S-Monovette® cfDNA Exact showed a continuous slight decrease over until 14 days of storage as the latest time point of analysis. In STRECK tubes, there was release of DNA into the samples early-on culminating in a drastic increase to a maximum of approx. 3.56 µg/ml at day 14 of storage (Figure 2C).

cfDNA is stabilized in blood collection tubes

The previous findings show that, depending on the storage system, there is release of DNA into stored blood samples, the storage temperature and time. Next, we aimed to determine the fragment length of the DNA in the blood samples. To this end, isolated cfDNA was analyzed by capillary electrophoresis with an Agilent Bioanalyzer 2100.

We compared cfDNA from aforementioned samples from S-Monovette® cfDNA Exact and STRECK tubes (stored for up to 14 days at 6 °C, 25 °C or 37 °C). Samples stored in PAXgene™ tubes were not included in the latter analysis, was the capillary electrophoresis presented was not evaluable.



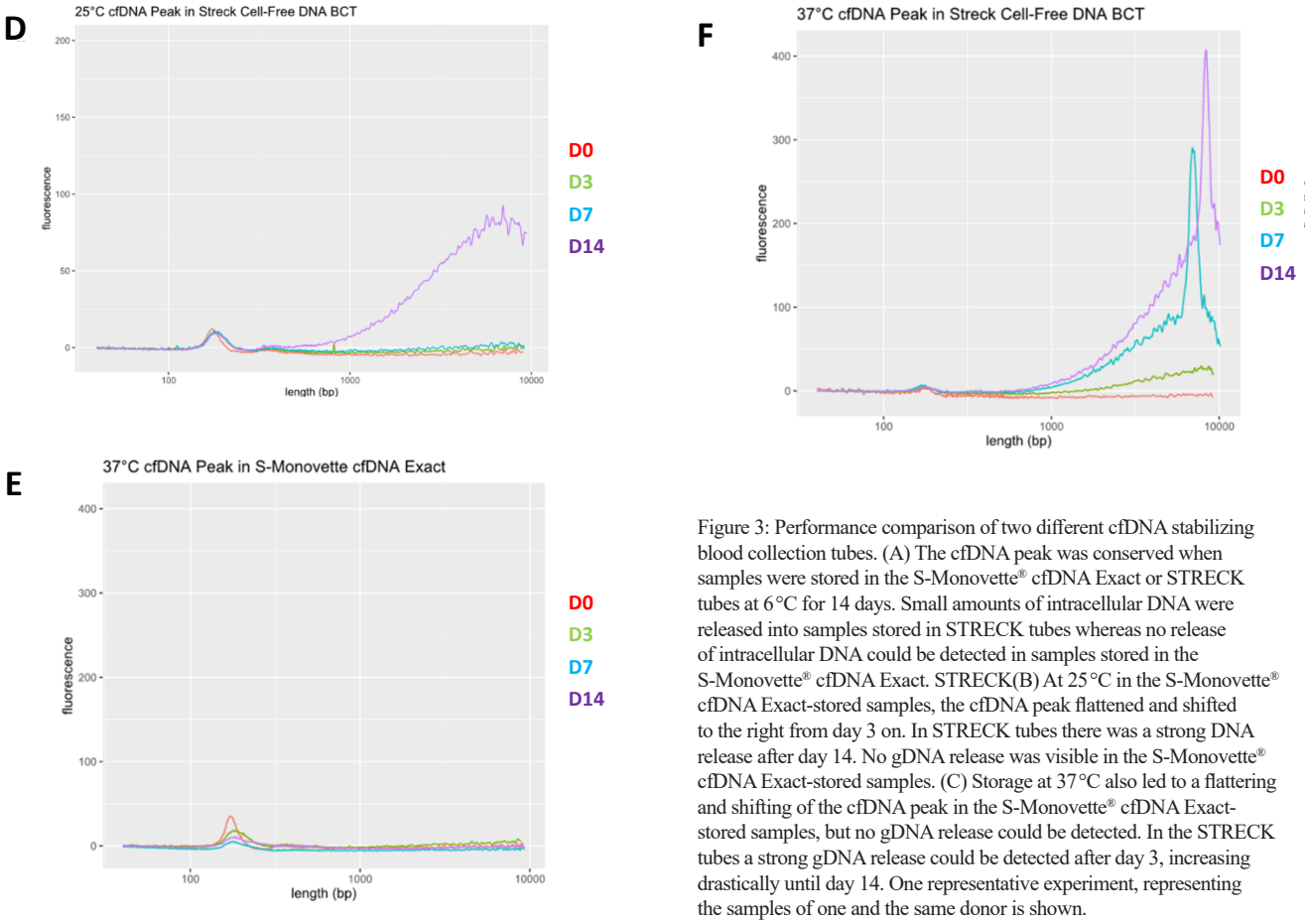


Figure 3: Performance comparison of two different cfDNA stabilizing blood collection tubes. (A) The cfDNA peak was conserved when samples were stored in the S-Monovette® cfDNA Exact or STRECK tubes at 6 °C for 14 days. Small amounts of intracellular DNA were released into samples stored in STRECK tubes whereas no release of intracellular DNA could be detected in samples stored in the S-Monovette® cfDNA Exact. STRECK(B) At 25 °C in the S-Monovette® cfDNA Exact-stored samples, the cfDNA peak flattened and shifted to the right from day 3 on. In STRECK tubes there was a strong DNA release after day 14. No gDNA release was visible in the S-Monovette® cfDNA Exact-stored samples. (C) Storage at 37 °C also led to a flattering and shifting of the cfDNA peak in the S-Monovette® cfDNA Exact-stored samples, but no gDNA release could be detected. In the STRECK tubes a strong gDNA release could be detected after day 3, increasing drastically until day 14. One representative experiment, representing the samples of one and the same donor is shown.

Samples isolated from the S-Monovette® cfDNA Exact showed a highly similar and stable graph pattern for all storage temperatures. On day 0, a prominent peak was visible at approx. 160 bp which represents the cfDNA. On day 7, the aforementioned peak at 160 bp is still present, however, smaller in size and has faded to the right when stored at 25 °C or 37 °C (Figure 3C,E). No additional peaks and therefore no release of intracellular DNA was visible in samples isolated from the S-Monovette® cfDNA Exact. Day 14 peaks did not differ from peaks measured from samples on day 7 displaying a cfDNA peak at approx. 160bp and no additional, longer DNA fragments (Figure 3A,C,E).

In contrast to the stable DNA peaks of the samples stored from the S-Monovette® cfDNA Exact, the samples stored in STRECK tubes at 6 °C showed vastly different result. On day 0, a prominent peak at 160 bp (cfDNA) could also be detected and remained stable even after the storage time of 14 days. But, from day 7 to day 14, high molecular weight DNA molecules (>1000 bp) became more abundant (Figure 3B). When stored at 25 °C, the aforementioned peak for high molecular weight DNA becomes more pronounced and reaches its maximum after a storage time of 14 days (Figure 3D). Sample storage at 37 °C allowed for a visible cfDNA peak maintained over the complete storage period, but also led to detection of high molecular weight DNA after only three days of storage with the amount tremendously increasing with prolonged storage time (Figure 3F).

Since the cfDNA peak faded over time in the samples stabilized in the S-Monovette® cfDNA Exact but there was no indication for loss of DNA (see Figure 2), we asked whether the cfDNA is still present in the sample but not detectable by capillary electrophoresis. To this end, blood was drawn into the S-Monovette® cfDNA Exact and, as this effect was most pronounced upon sample storage at 37 °C, stored at said 37 °C for up to 7 days. cfDNA was isolated (with the NucleoSnap® cfDNA Kit ,Macherey-Nagel) and analyzed by capillary electrophoresis. On day 0, a clear single peak of approx. 160 bp indicated the presence of cfDNA. However,

when samples were stored for 7 days at 37 °C and cfDNA was isolated according to the manufacturer's instructions ("standard"), the cfDNA peak vanished. When the digestion step with Proteinase K – which is part of the cfDNA isolation procedure – was extended to an overnight incubation at 56 °C (temperature in standard protocol), the cfDNA peak was partly restored (see Figure 4).

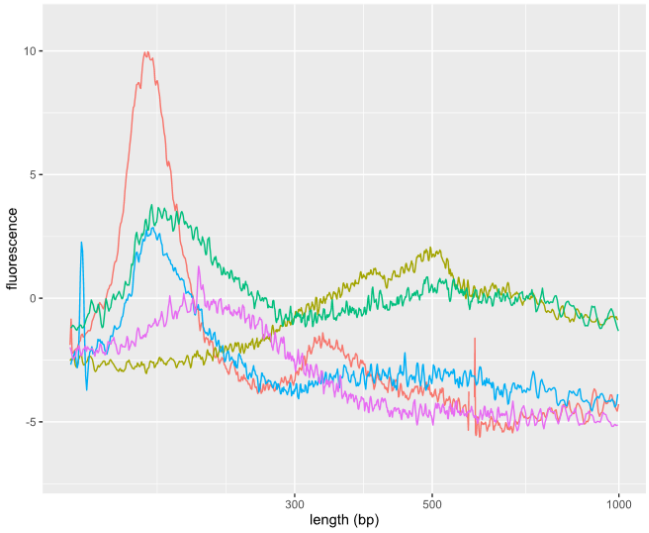


Figure 4: Optimization of cfDNA isolation using the Macherey-Nagel cfDNA isolation kit. As control, EDTA blood was collected and cfDNA was isolated rightafter. One cfDNA Exact sample was stored at 37 °C for 7 days and then analysed with either the standard protocol, overnight digestion of proteinase K at 56 °C, over-night lysis at 56 °C or proteinase K digestion for 3h at 56 °C.

Stabilized cfDNA is amplifiable by PCR and amplified cfDNA levels are maintained over time

CfDNA has been described as potent source for blood-borne diagnostics of diseases such as breast cancer (Fernandez-Garcia et al. 2019). Such diagnostics most often utilize PCR techniques. Thus, we investigated whether stabilized cfDNA is usable for PCR based diagnostics. Furthermore, after qualitatively demonstrating cfDNA stabilization, we addressed the question to which extent blood collection tubes maintain the level of cfDNA over time at different temperatures. To this end, blood was drawn into PAXgene™ tubes, the S-Monovette® cfDNA Exact or STRECK tubes and stored at different temperatures (6 °C, 25 °C or 37 °C) for up to 14 days. After, cfDNA was isolated using the QIAamp Circulating Nucleic Acid Kit or the InviMag® Free circulation DNA Kit and levels of *ERV-3* and *MSTN* were determined by qPCR.

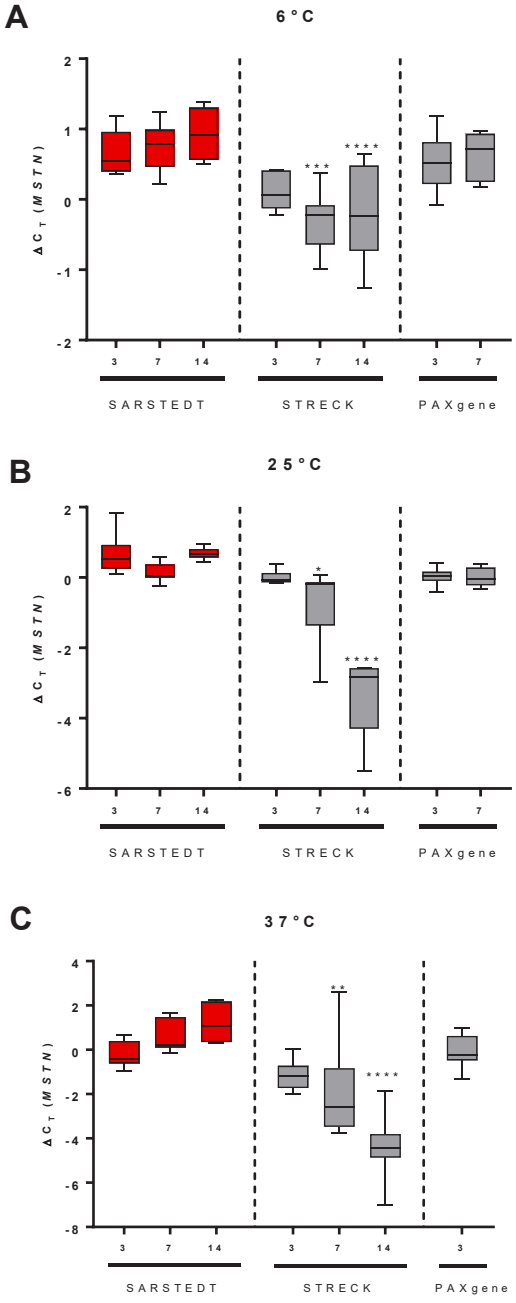


Figure 5: Abundance of cfDNA stabilization marker gene *MSTN* depends on blood collection tube, storage time and storage temperature. Evaluation of cfDNA stability by qPCR according to the gene *MSTN*. (A) Upon storage at 6 °C, *MSTN* abundance was stable in the samples drawn by S-Monovette® cfDNA Exact and PAXgene™ tubes for up to 14 days. However, in STRECK tubes, abundance of *MSTN* continuously increased throughout 14 days of storage (n=7). (B) Upon storage at 25 °C, the levels of *MSTN* were stable in the S-Monovette® cfDNA Exact and also in PAXgene™ tubes for 14 days. However, in STRECK tubes, gene levels of *MSTN* strongly increased after 7 days of storage. (n=7). (C) Upon storage at 37 °C, stability of *MSTN* was generally maintained in the S-Monovette® cfDNA Exact and also in PAXgene™ tubes for 3 days (n=7). Upon storage at 37 °C, stability of *MSTN* was generally maintained in the S-Monovette® cfDNA Exact for 14 days, while stabilization was not efficient in STRECK tubes after day 3 (n=7).

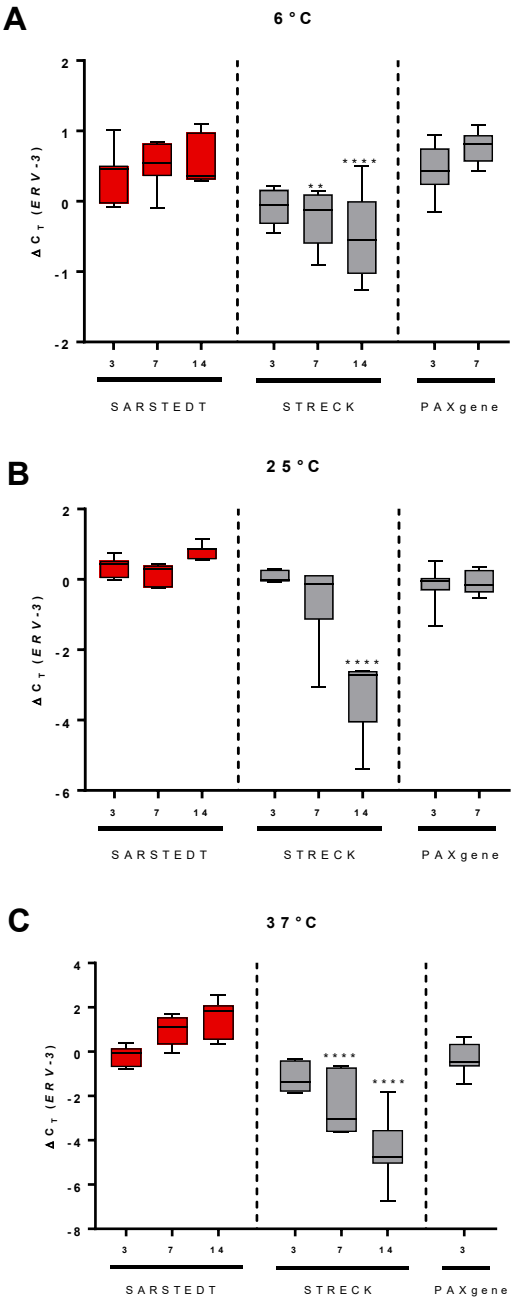


Figure 6: Abundance of cfDNA stabilization marker gene *ERV-3* depends on blood collection tube, storage time and storage temperature. Evaluation of cfDNA-stability by qPCR according to the gene *ERV-3*. (A) Upon storage at 6 °C, the levels of *ERV-3* were stable in the S-Monovette® cfDNA Exact and also in PAXgene™ tubes for 10 days. However, in STRECK tubes, gene levels of *ERV-3* continuously increased throughout 14 days of storage (n=7). (B) Upon storage at 25 °C, the levels of *ERV-3* were stable in the S-Monovette® cfDNA Exact and also in PAXgene™ tubes for 10 days. Furthermore, the levels of *ERV-3* were maintained for up to 14 days in the S-Monovette® cfDNA Exact. However, in STRECK tubes, gene levels of *ERV-3* strongly increased after 7 days of storage (n=7). (C) Upon storage at 37 °C, stability of *ERV-3* was generally maintained in the S-Monovette® cfDNA Exact and also in PAXgene™ tubes for 3 days. Furthermore, the levels of *ERV-3* were generally maintained in the S-Monovette® cfDNA Exact for 14 days, while stabilization was not efficient in STRECK tubes after day 3 (n=7).

When blood was stored in the S-Monovette® cfDNA Exact or PAXgene™ tubes at 6 °C for up to 14 days and cfDNA was isolated, the levels of both, *ERV-3* and *MSTN* laid within 1 CT for all time points. In contrast, when blood was stored in the STRECK tubes at 6 °C for up to 14 days, expression levels of both, *ERV-3* and *MSTN* showed a continuous, significant decrease over time, indicating an increase of genetic material available (potentially due to release of gDNA into the samples) (Figure 5A, Figure 6A).

When blood was stored in the S-Monovette® cfDNA Exact, PAXgene™ tubes or STRECK tubes at 25 °C for up to 14 days and cfDNA was isolated, levels of both, *ERV-3* and *MSTN*, laid within 1 CT for all time points. In contrast, STRECK tubes showed a significant drop of CT-values after day 7, indicating an increase of available genetic material (see above) (Figure 5B and Figure 6B).

When blood was stored in the S-Monovette® cfDNA Exact, PAXgene™ tubes or STRECK tubes at 37 °C for up to 14 days the levels of both, *ERV-3* and *MSTN* increased in the S-Monovette® cfDNA Exact over time by a maximum +2 CT, indicating a slight immobilization of DNA due to increasing stabilization of the components. Levels of DNA after day 3 in the PAXgene™ tubes were comparable to S-Monovette® cfDNA Exact, no longer storage is indicated for PAXgene™ tubes. For samples stored in STRECK tubes, there was a strong drop of CT-values after day 3, indicating a significant increase of available genetic material (see above) (Figure 5C and Figure 6C).

Discussion

gDNA release is effectively prevented for up to 14 days in the S-Monovette® cfDNA Exact

Taken together, samples stored at 6, 25 or 37 °C in the S-Monovette® cfDNA Exact exhibited consistently low levels of hemolysis and no high molecular weight DNA contamination when compared to commercially available PAXgene™ or STRECK tubes.

In brief, at 6 °C almost no hemolysis and release of DNA into the samples was measured in samples stored in the S-Monovette® cfDNA Exact and also PAXgene™ tubes. However, there was a clear increase in hemolysis as well as in the DNA content of samples with increasing storage time in samples stored in STRECK tubes. At 25 °C, PAXgene™ tubes caused equally low hemolysis as the S-Monovette® cfDNA Exact but clearly more DNA was released into STRECK tubes. Similar results were obtained at 37 °C when samples from the S-Monovette® cfDNA Exact and PAXgene™ tubes showed almost equally low levels of hemolysis. However, PAXgene™ tubes are indicated only for storage times of up to 3 days at 37 °C while the S-Monovette® cfDNA Exact shows stable hemolysis levels for up to 14 days at 37 °C. STRECK tubes led to strongest hemolysis and DNA release among all tested blood collection tubes also at 25 °C and 37 °C. Hemolysis is a measure for cellular stress that leads to rupture and thus to lysis of red blood cells and also other blood cells such as leukocytes. The red colour indicative of free hemoglobin makes lysis of erythrocytes evident by optical investigation and thus also gives hint about lysis of DNA containing leukocytes. Release of genomic DNA from leukocytes will increase the total DNA amount in the tested samples and might interfere with subsequent cfDNA based analysis. The results clearly show that the observed hemolysis corresponds to the DNA content in the respective sample.

Thus it becomes obvious that among the tested blood collection tubes, the S-Monovette® cfDNA Exact most efficiently inhibits lysis of red and white blood cells and thus also prevents release of DNA into the samples at all tested storage temperatures (6 °C, 25 °C and 37 °C).

cfDNA is stabilized in blood collection tubes

Within all investigated blood collection tubes, cfDNA is stabilized. However, only in the S-Monovette® cfDNA Exact, release of gDNA is effectively prevented for up to 14 days at all tested temperatures (6 °C, 25 °C and 37 °C). Tubes from PAXgene™ show efficient stabilization of cfDNA levels, indicating no release of gDNA from dying cells into the sample at all investigated temperatures for the short storage time recommended. However, the indicated stabilization in PAXgene™ tubes does not exceed 10 days at 6 °C, 10 days at 25 °C and 3 days at 37 °C. Upon sample storage at 37 °C, release of gDNA was evident into samples stored in STRECK tubes from day 3 on. There was no release of gDNA into samples stored in the S-Monovette® cfDNA Exact for up to 14 days. In all tested blood collection tubes, a clear cfDNA peak was visible on day 0. In STRECK tubes, the cfDNA peak was present for up to 14 days, however, size reduced over time. The cfDNA peak faded in the samples stabilized in the S-Monovette® cfDNA Exact but there was no indication for loss of DNA (Figure 2). Thus, we asked whether the cfDNA is still present in the sample but not displayable in the Bioanalyzer graph. It is known that upon fixation there is crosslinking of nucleic acids and proteins. We hypothesized that in the S-Monovette® samples, cfDNA was covalently linked to proteins (such as histones) and thus cannot run properly in the capillary electrophoresis based Bioanalyzer Chip. If so, it might be possible to degrade the covalently linked proteins by proteolytic digestion and thus liberate DNA. This treatment should allow the cfDNA to run properly in the Bioanalyzer Chip and thus restore the cfDNA peak at approx. 160 bp. Indeed, when samples were stored at 37 °C for 7 days and cfDNA was then isolated according to the manufacturer's instructions ("standard"), the cfDNA peak had vanished. When digestion with Proteinase K – which is part of the cfDNA isolation procedure – was extended to overnight incubation at 56 °C, the cfDNA peak was partly restored (Figure 4). The restoration of the cfDNA peak is in accordance with previous findings, where the authors report that elongated digestion with proteinase K leads to reversal of an observed drop in cfDNA concentration due to stabilization (Warton et al. 2017). Taken together, these findings confirm that within the S-Monovette® cfDNA Exact, cfDNA is effectively stabilized but to some extent its binding to proteins increases upon prolonged storage time. Nevertheless, stabilized cfDNA was fully accessible for qPCR and not impaired in quality or quantity even when the standard cfDNA isolation procedure was used.

To sum up, the S-Monovette® cfDNA Exact prevents the release of nucleic acids into the blood sample and furthermore is the only tested device to (1) effectively stabilizing cfDNA from in whole blood samples and (2) fully protecting blood samples from contamination with gDNA at 37 °C storage temperature.

Stabilized cfDNA is amplifiable by PCR and cfDNA levels are maintained over time

The S-Monovette® cfDNA Exact effectively stabilizes the level of cfDNA at 6 °C, 25 °C and 37 °C. The abundance of both *ERV-3* and *MSTN* are nearly constant in samples stored in the S-Monovette® cfDNA Exact at all investigated temperatures. Comparable results were

obtained in samples stored in PAXgene™ tubes, however only for the shorter storage periods as mentioned before. Another picture is obtained with STRECK tubes, which fail to stabilize both *ERV-3* and *MSTN* at 6 °C, 25 °C and also at 37 °C according of the manufacturer's stated guidelines lines and storage times. The gene levels of *ERV-3* and *MSTN* are measures for the concentration of DNA in the tested samples. The primer pairs for amplification of *ERV-3* and *MSTN* have been chosen to yield products of distinct length that are specific for cfDNA sequences (Breitbach et al. 2014; Devonshire et al. 2014). However, these target sequences are also present in long DNA fragments of necrotic origin. As a result, the presence of gDNA in a DNA sample will lead to lower CT-values ($\Delta CT < 0$). It has been shown that short DNA fragments are derived from apoptotic cells in which DNA was degraded in a controlled manner while long fragments are originated from uncontrolled DNA decay in necrotic cells (Giacona, 1998). To this end, the lower CT-values of both, *ERV-3* and *MSTN*, in PAXgene™ and STRECK tubes indicate the presence of gDNA in these samples which is highly likely to be a result of cell lysis. These findings confirm the aforementioned observations of gDNA being released into blood samples stored in STRECK tubes (Figure 2, Figure 5 and Figure 6).

Taken together, the S-Monovette® cfDNA Exact is the only tested device that effectively prevents the release of additional DNA into the blood samples and thus maintains the level of cfDNA. The fading of the cfDNA peak in capillary electrophoresis analysis of cfDNA isolated from blood stored in blood collection tubes, an effect most prominent in the S-Monovette® cfDNA Exact, does not influence the cfDNA availability in subsequent analyses (see above). Fading and shifting of cfDNA peaks to higher molecular weight is caused by the stabilization mechanism of chemical fixation with other sample components. It was already shown that crosslinking effects, which influence capillary electrophoretic assays do not affect subsequent analysis. STRECK tubes have been proved for mutation detection by qPCR in cancer-related applications (Denis et al. 2015), used for droplet digital PCR (Sacher et al. 2016), and with BEAMing and Safe-Sequencing (Medina Diaz et al. 2016).

The tested liquid biopsy devices are designated for maintaining sample integrity under challenging conditions such as limited infrastructure. This might include no opportunity for instant plasma separation or incapability of cooling the samples. Under such conditions, the S-Monovette® cfDNA Exact represent a novel and superior device for stabilization of cfDNA and subsequent diagnostic applications.

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