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Comparison of Roche Cell-Free DNA collection Tubes® to Streck Cell-Free DNA BCT®s for sample stability using healthy volunteers

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ABSTRACT

Objectives: To compare the Roche Cell-Free DNA Collection Tubes® against the Streck Cell-Free DNA BCT®s for sample stability using Cell Free DNA (cfDNA) from healthy volunteers (n = 20).
Design & methods: Whole blood was drawn into five Roche and five Streck tubes per volunteer, stored at room temperature and processed at five different time points (Days 0, 4, 7, 10 and 14). One volunteer had blood drawn into ×10 K_3EDTA tubes to observe the effect of no preservation buffer on White Blood Cell (WBC) lysis. DNA was extracted from the plasma and the concentration (ng/μL) measured using the Qubit Fluorometer® at each time point. The eluted DNA was further analysed by capillary electrophoresis to determine the proportion of cfDNA and gDNA contamination in the samples over the 14 days.
Results: There was no difference in individual ($p = 0.097$) and median paired ($p = 0.26$) DNA concentration across the five time points between the two tubes. However, a difference was observed for samples in the Roche tubes for pair days 0–7 ($p = 0.01$), 0 to 10 ($p = 0.046$) and 0 to 14 ($p = 0.0016$) in contrast to the Streck tubes after adjustment for multiple testing.
Conclusion: The findings of this study indicate that the Roche Cell-Free DNA Collection Tubes® are a suitable alternative for sample collection and storage at room temperature, albeit for a duration of less than 7 days.

1. Introduction

Current clinical practice for cancer surveillance typically involves radiological imaging. This procedure has significant disadvantages such as high operating costs, patient inconvenience and radiation exposure [1,2]. The presence of nucleic acids within the blood circulation are currently being developed as clinical tool for cancer surveillance. Cell Free DNA (cfDNA) constitutes short DNA fragments (130bp-160bp) which are released into the blood stream via apoptosis, necrosis and active secretion [3]. Circulating tumour DNA (ctDNA) is a tumour derived subset of cfDNA and is currently under development as a clinical tool for cancer diagnosis and disease surveillance [4,5]. Acquisition of ctDNA occurs through a routine blood draw and analysis involves mutation detection and quantification using techniques such as targeted Next Generation Sequencing (NGS) and digital PCR methods (Droplet Digital PCR (ddPCR) and BEAMing) [3]. Results of such analysis provide a real-time molecular view of the tumour dynamics in terms of disease progression and the tumour's response to treatment [6].While many studies have demonstrated the validity of utilising ctDNA within a clinical diagnostic scenario, its application is hindered by a lack of standardised operating protocols [7]. Specifically, pre-analytical variables such as

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sample collection, storage and transport to distant laboratory sites are of particular concern for protecting the integrity of ctDNA for further downstream analysis. Regular analysis of ctDNA requires serial blood samples to be drawn from patients via venipuncture into blood collection tubes consisting of an anti-coagulant buffer such as potassium ethylenediamine tetra-acetic acid (K_3EDTA). Early validation studies have identified the short stability period of cfDNA stored in K_3EDTA tubes as a significant barrier, as samples drawn must be processed within 24 h of collection to prevent significant genomic DNA (gDNA) contamination [8]. While the storage period can be extended by refrigerating samples (4-6 °C) this increases the cost for shipment [9]. Furthermore, shipment of samples to distant processing laboratories experience variable conditions such as constant agitation and fluctuating temperatures, which are known to increase white blood cell (WBC) lysis significantly [10]. In cancer patients, a further implication of high gDNA contamination is the biological phenomenon known as clonal haematopoiesis, in which gDNA contain background mutations [11]. Several recent ctDNA studies have shown that high levels of such mutations can confound the discovery of disease associated variants. Therefore, technical adaptations such as the use of cfDNA collection tubes during sample collection are essential for accurate ctDNA variant detection and quantification in future clinical studies [11,12].

Streck Cell-Free DNA BCT® (Streck, USA) are blood collection tubes with a proprietary stabilisation buffer, intended for the collection, shipping and storage of whole blood samples for clinical cfDNA analysis. Samples drawn into these tubes are stable for up to 14 days post collection within 6ºC-37 °C. Extensive validation studies against standard K_3EDTA tubes have shown significant reductions in gDNA release from WBCs under various sample shipment conditions [10,13,14]. A similar product, Roche Cell-Free DNA Collection Tubes® (Roche, Switzerland) has since become commercially available. The Roche tubes also contain a stabilisation buffer and claim to prevent gDNA release for up to 7 days post collection under various shipment conditions [15]. Nevertheless, the Roche tubes have yet to be recommended as an alternative cfDNA collection tube (at the time of this study; 2016) in comparison with Streck Cell Free DNA BCT®s.

The purpose of this study was to compare the Roche Cell-Free DNA Collection Tubes® against Streck Cell-Free DNA BCTs® to determine any significant differences in sample stability with the two tube types. Comparisons were made between the tubes for the concentration of cfDNA and the level of gDNA contamination at room temperature (22 °C). A 14-day post collection period was used in this study as this was the maximum time frame indicated by the manufacturer of the Streck tubes to maintain sample stability.

2. Materials and methods

2.1. Ethics and volunteer recruitment

Ethical approval was obtained from the New Zealand Ministry of Health; Health and Disability Ethics Committees (reference number LRS/09/05/018/AM04) for using samples from healthy volunteers for the development of blood and urine markers for cancer. Twenty healthy volunteers were recruited; those who were pregnant, self-reported as unwell (common flu or cold) or presently taking prescription medication were excluded. Written informed consent was obtained from all volunteers prior to their participation in the study.

2.2. Sample collection and storage

Blood was drawn from the healthy volunteers by venipuncture into ×5 Streck Cell-Free DNA BCT® and ×5 Roche Cell-Free DNA Blood Collection Tubes® by phlebotomists at Southern Community Laboratories (Dunedin, New Zealand). Blood was drawn to fill the tubes (~8.5–10 mL) as per the manufacturer's recommendations and brought back to the processing laboratory (Cancer Genetics Laboratory, Department of Biochemistry, University of Otago) for storage and processing. One volunteer also had blood drawn into ×10 2 mL K_3EDTA BD Vacutainer® tubes. All samples were stored upright in a test tube rack in a styrofoam container placed in a temperature (22 °C) regulated room. Subjects 4, 11, 13, 16 and 17 were unable to provide blood samples for all time points.

2.3. Sample processing

Samples were processed on days 0, 4, 7, 10 and 14 post collection with day 0 samples processed within 2 h of blood draw. The plasma was separated from the red blood cells (RBC) by centrifugation in an Eppendorf Centrifuge 5810R. The samples were first spun down at a speed of 200 rcf then at 1600 rcf for 10-min intervals with both break and acceleration set at zero. The plasma was transferred into 15 mL centrifuge tubes and spun again at 1600 rcf to remove intact WBCs [16]. DNA was extracted from the healthy volunteer plasma using the QIAmp Circulating Nucleic Acid Kit as per the manufacturer's protocol for 4 mL of plasma. Modifications to the protocol involved using an Eppendorf 5417C centrifuge set to 18,000 rcf and 20,000 rcf to draw Buffer ACW1, ACW2 and Absolute Ethanol (100%) through the QIAamp Mini Column rather than the vacuum pump. The final DNA was eluted in 35 μL of UltraPure DNase/RNase Free distilled water, rather than the provided Buffer AVE.

Concentration of plasma DNA was measured using the Qubit 2.0 Fluorometer® with the dsDNA High Sensitivity Kit as per the manufacturer's protocol. Two microlitres of the DNA sample was diluted in 198 μL of dsDNA HS buffer and reagent and then measured against the provided DNA standards in ng/μL. The final concentration of DNA (in ng/mL of plasma) was calculated using the following formula.

$$\frac{Qubit\ concentration\ (ng.\ \mu L)\ x\ 100}{1000}\ x\ \frac{Volume\ of\ Eluted\ DNA\ (35\ \mu L)}{Volume\ of\ plasma\ (4mL)}$$

Fragment size profiling of the eluted DNA was carried out by capillary electrophoresis, using the Agilent 2100 Bioanalyzer with the High Sensitivity DNA Analysis Kit. Samples were diluted to 1 ng/μL and loaded onto the HS dsDNA chip with the appropriate reagents and run on the system as per the manufacturer's protocol. The Agilent 2100 Expert Software was used to generate an electropherogram which quantified DNA fragments as fluorescent peaks (mass/molarity) at various base pairs (bp) in length between two markers (lower = 35bp, upper = 10, 380bp). This provided a visual representation of cfDNA (~165bp in length) in relation to genomic DNA (gDNA) contamination.

2.4. Statistical analysis

Statistical analysis was carried out using R Studio (Version 1.0.143, 2009–2016). For each subject, differences in cfDNA concentrations were calculated (Streck *minus* Roche) and one-way Analysis of Variance (ANOVA) was performed on the difference data, with "Day" included as a main effect. The normality of the model residuals was tested using the Shapiro-Wilk and Kolmogorov-Smirnov tests. Follow up analysis was performed using the non-parametric Kruskal-Wallis test to investigate differences in cfDNA concentration across the five time points for both tube types. The Wilcoxon rank-sum test was used to investigate for differences in cfDNA concentration compared to day zero, (assuming no day effect) for the two tubes. Any differences in the performance of the tubes with regard to sample stability were considered to be statistically significant if the associated p values were less than or equal to 0.05.

The Wilcoxon signed-rank test (a non-parametric paired student t-test) was used to investigate paired differences of change in cfDNA concentrations for days 0–4; 0 to 7; 0 to 10 and 0 to 14 for the two tubes individually as well as between the two tube types for the respective paired days. The Bonferroni correction method was applied to adjust for multiple hypothesis testing. Paired sample analysis was considered statistically significant if the adjusted p value was less than or equal to 0.05.

3. Results

3.1. cfDNA concentration

The concentration of eluted DNA from each volunteer for both Streck and Roche tubes are presented in Fig. 1. Concentration values varied between individuals and tube type with a range of 2–15 ng/mL across all volunteers. Boxplots depicting the distribution of the concentrations for each day and tube type are shown in Fig. 2.

3.2. Statistical analysis

Analysis of variance using the difference data (Streck *minus* Roche for each subject at each timepoint) showed that distributed residuals were not non-normally distributed (Shapiro-Wilk: $W = 0.96$, $p = 4.4 \times 10^{-3}$; Kolmogorov-Smirnov: $D = 0.18$, $p = 6.0 \times 10^{-3}$). The Kruskal-Wallis test, used to assess changes in the difference of DNA concentration across days, showed no significant association with tube type ($p = 0.097$). Subsequent analysis with the Wilcoxon rank-sum test found no evidence that the distribution of differences in the data for the time points was not centred around zero ($p = 0.26$).

The Wilcoxon signed-rank test was used to investigate the paired differences between the change in the DNA concentrations for days 0–4; 0 to 7; 0 to 10 and 0 to 14, for the individual tubes as well as between the two tube types. After adjusting for multiple comparisons, no statistical significance was observed for the change in total DNA concentrations between day 0–4, 0 to 7, 0 to 10 or 0 to 14 for the Streck samples ($p > 0.05$, Table 1). For the Roche samples a statistically significant difference was observed for the change in total DNA concentration for comparison between days 0–7, 0 to 10 and 0 to 14. ($p < 0.05$, Table 1). The paired differences of change in DNA concentrations between the tube types was not statistically significant ($p > 0.05$, Table 1).

3.3. Capillary electrophoresis analysis

Capillary electrophoresis was carried out to distinguish between cfDNA and gDNA contamination over the various time points. Electropherograms were generated for each sample and the fragment size of the DNA measured against fluorescence intensity (FU) [17]. Previous studies have identified the majority of cfDNA fragments are approximately 165 bp in length, whilst gDNA consist of longer and higher molecular weight fragments. We attributed gDNA as high molecular weight fragments clustered close to the upper marker (10, 380bp) Therefore, this study looked to identify samples with increasing gDNA contamination over the five time points.

Electropherograms for samples stored in Streck, Roche and K_3EDTA tubes from three different volunteers for their respective "Day 0" and "Day 14" samples are shown in Fig. 3 to provide a visual depiction of the cfDNA peak and onset of gDNA contamination. Subjec 20's electropherogram for their Streck Day 0 sample is shown in Fig. 3A and depicts a cfDNA peak of 182bp at 40 FU (Fig. 3A). This sample also consisted of two further peaks at 300bp and 500bp as well as gDNA fragments of 4000 to 6000bp at 10 FU (Fig. 3B). In the volunteer's day 14 sample, the fluorescence level of cfDNA peak had decreased to 20 FU and there was an obvious increase in the number of DNA fragments within this sample, however their fluorescence level appeared relatively consisting to their corresponding Day 0 sample. Subject 2's Roche day 0 sample had a cfDNA peak of 174bp at 40 FU (Fig. 3C). The corresponding Day 14 sample this cfDNA peak at 180bp and with a measured fluorescence just over FU (Fig. 3D). No further DNA fragments were detected in this sample however the jagged increase of the baseline (in comparison to Day 0) may be a result of residual ethanol during the extraction process (Fig. S2). Visual comparison were made against a third subjects sample stored in K3EDTA tubes to identify gDNA contamination from WBC lysis in samples lacking a preservation cocktail. In the Day 0 sample, the cfDNA peak of 181bp had a fluorescence intensity of 18 FU and a few

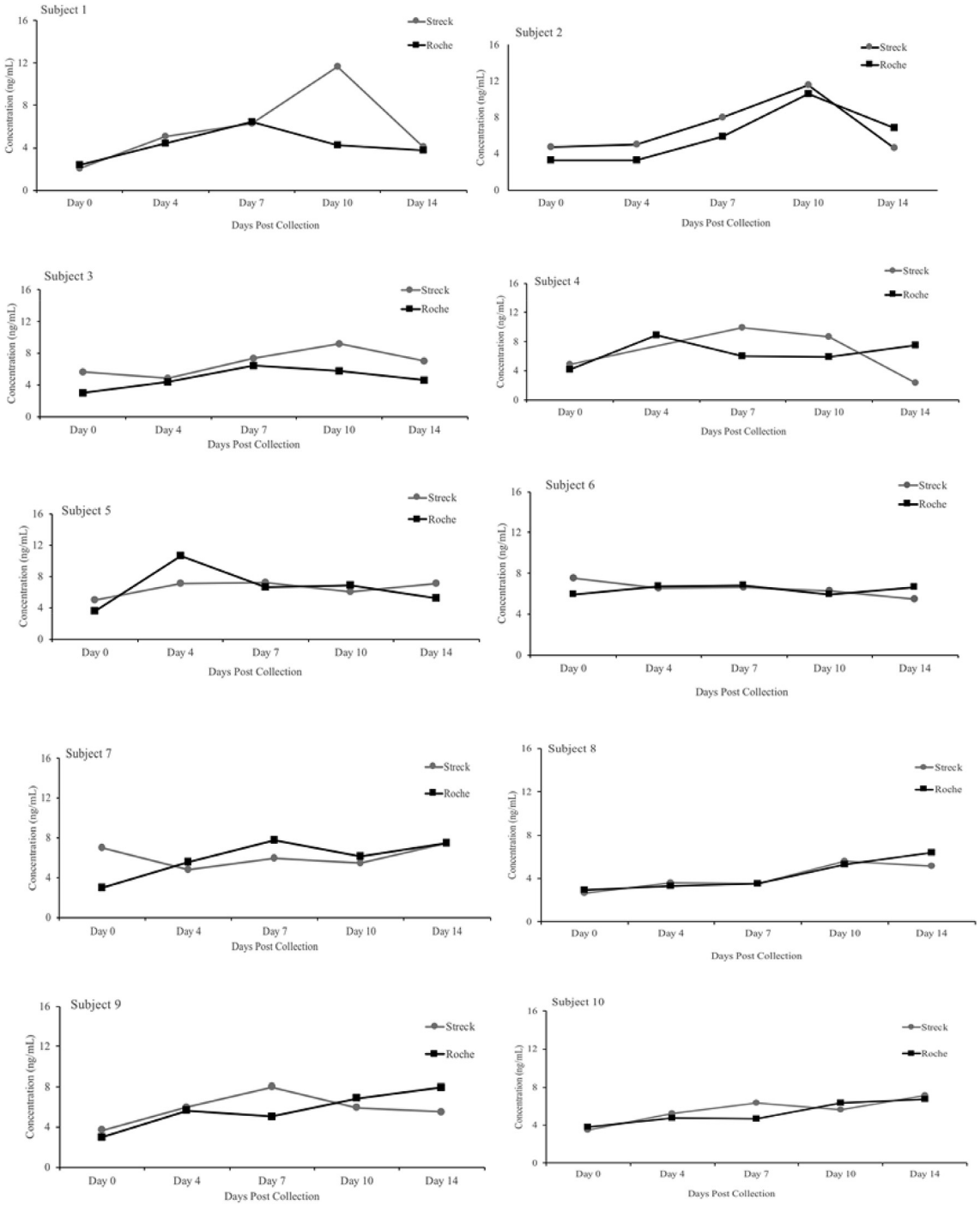

Fig. 1. Concentration of eluted DNA of samples drawn from 20 healthy volunteers and stored in either Streck or Roche cfDNA blood collection tubes. Samples were drawn as per the manufacturer's recommendations and stored at 22 °C until processing at five time points (Day 0, 4, 7, 10 and 14). Concentrations are reported as ng/mL of plasma.

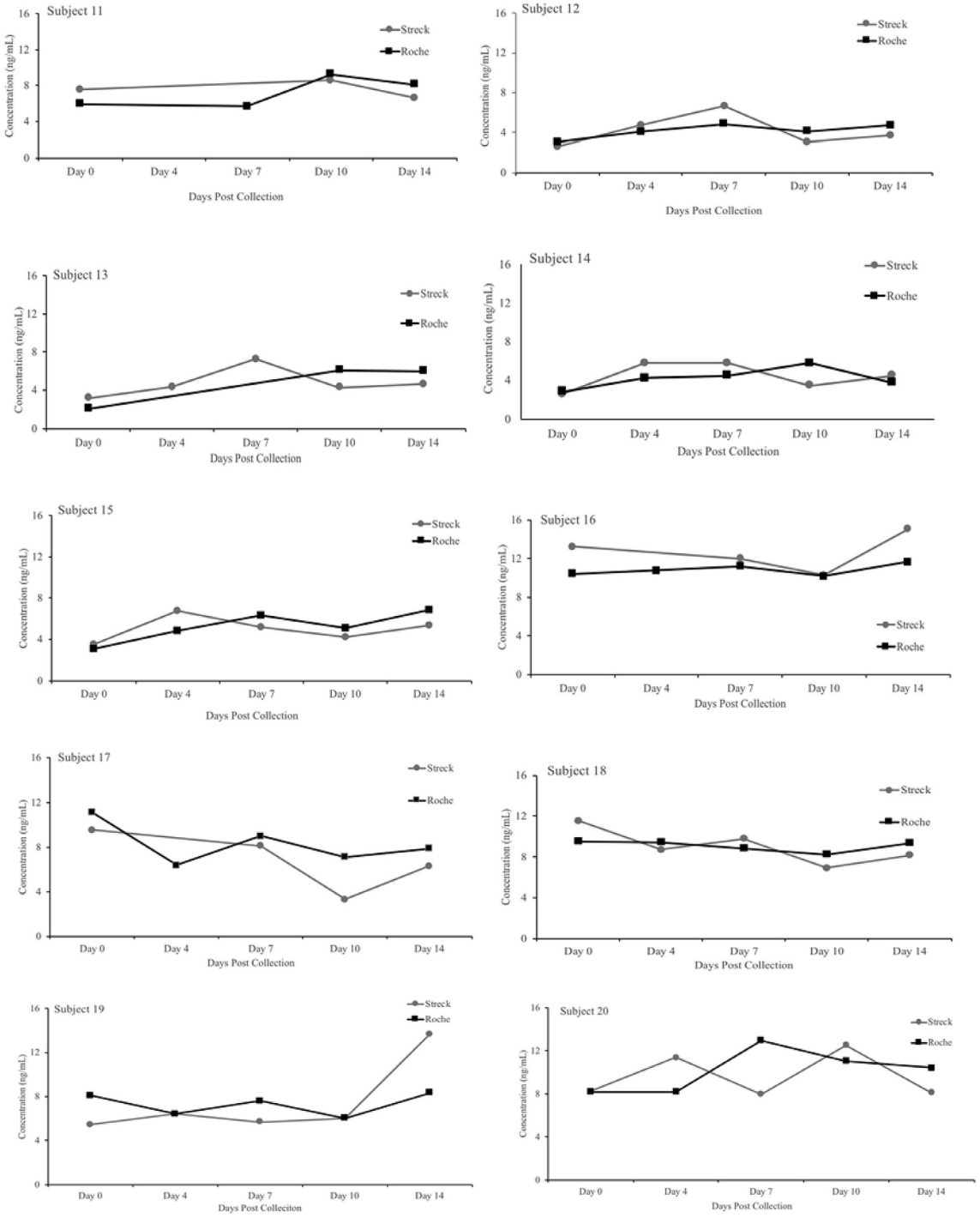

Fig. 1. . (*continued*).

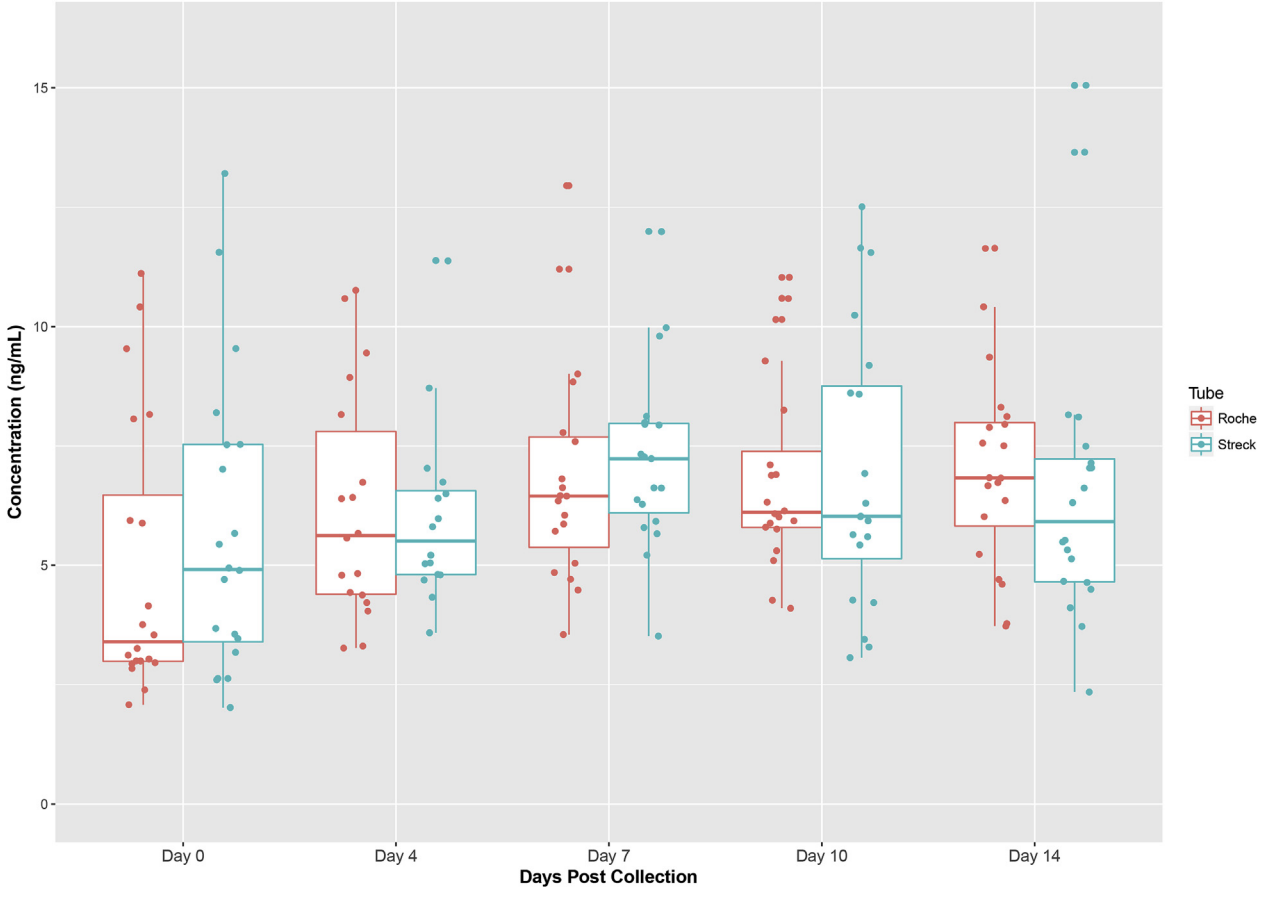

Fig. 2. Concentration of plasma DNA in Roche and Streck blood collection tubes over 14 days post collection. Whole blood was drawn from healthy volunteers (n = 20) into both Roche and Streck tubes, stored and processed at five time points (Day 0, 4, 7, 10 and 14).

Table 1
Wilcoxon signed rank test results for paired sample analysis within and between streck cell-free DNA BCT®s and roche cell free DNA collection Tubes®.

Streck Sample Pair	V	Adjusted p-value
Day 0 and 4	29	0.36
Day 0 and 7	34	0.096
Day 0 and 10	65	1.12
Day 0 and 14	70	1.6
Roche Sample Pair		
Day 0 and 4	27	0.163
Day 0 and 7	19	0.01
Day 0 and 10	33	0.046
Day 0 and 14	14	0.0016
Streck and Roche Pair		
Day 0 and 4	62	7.44
Day 0 and 7	91	6.64
Day 0 and 10	149	0.88
Day 0 and 14	160	0.32

gDNA fragments ranging between 3000 and 8000bp which measured <10 FU (Fig. 3E). The Day 14 sample showed a considerable increase fragments and their fluorescence intensity of with the largest at 9000bp peaked at 2000 FU (Fig. S3). The fluorescence of these longer fragments also appears to partially obscure the cfDNA peak (Fig. 3F).

From these results it was observed that the gDNA contamination from WBC lysis was substantially less in the Streck and Roche tubes over the 14 days in comparison with the K_3DTA tubes.

4. Discussion and conclusion

Pre-analytical variables such as sample collection, storage and shipment have been previously shown as crucial factors for consideration for ctDNA clinical studies [18,19]. The availability of commercial cfDNA collection tubes integrates collection, shipment and long-term storage. CfDNA collection tubes consist of a preservation buffer which aims to stabilise the sample and prevent WBC lysis under fluctuating temperatures and agitation over an extended period (4–14 days). Previous studies have extensively compared the use of K_3EDTA tubes against the now widely used Streck Cell-Free DNA BTC®s. These studies have shown K_3EDTA are substantially inadequate for long term storage of samples (>24 h) at room temperature [10].

In this study, we compared the stability of samples drawn in Streck Cell-Free DNA BCT® and Roche Cell-Free DNA Collection Tubes® over a 14-day period at room temperature.

Our findings showed that mean cfDNA concentrations differed between day 0–7; 0 to 10 and 0 to 14 for samples in the Roche tubes ($p = 0.01, p = 0.045, p = 0.002$; Table 1), suggesting an increased concentration of gDNA from WBC lysis over time from day 7 onwards. Capillary electrophoresis was used to determine the presence of various DNA fragments in each day sample for all 20 volunteers. It was observed that the Streck and Roche samples had considerably less high molecular weight gDNA fragments over the 14-day period upon comparison with samples stored in K_3EDTA.

Two recent publications have also compared Roche Cell-Free DNA Collection Tubes® against the Streck Cell-Free DNA BCT®s and standard K_3EDTA tubes [20,21]. Both these studies demonstrated the stabilisation of whole blood from healthy volunteers. Nikolaev et al. (2018) used a PCR quality control test to determine levels of gDNA contamination over a 7 day period. They reported no detectable gDNA contamination of samples within one week of collection for either the Roche or Streck tubes stored at room temperature. However they did identify a significant increase ($p < 0.01$) in the 305/41bp ratio, used to measure gDNA amplicons one week post collection. Further findings showed that samples drawn into Roche tubes and stored under fluctuating temperatures (22ºC-30 °C) had detectable gDNA levels from day 5 onwards, whereas no gDNA was detected in the Streck tubes under the same regime [20]. Alidousty et al. (2017) carried out a spike in experiment of *EGFR* T790M into whole blood samples of one healthy volunteer to assess whether the length of storage at room temperature affects the detection capability of ctDNA. No statistical significant difference was observed between the Roche and Streck tubes for the detection of *EGFR* T790M over 7 days [21].

It was interesting to note the variance in DNA fragment sizes observed within healthy volunteer samples. This study only looked at the fluorescence intensity of the previously established cfDNA peak at 150-200bp [5]. However several recent publication have eluded that cfDNA may also consist of much longer fragments. Of particular note is a 2000bp fragment observed by Bronkhorst et al. (2016), thought to also be part of the "cfDNA pool" [22]. However the clinical utility of such fragments have yet to be determined as the majority of research has focussed on the clinical application of ctDNA which is even shorter than cfDNA [4].

The current study has several limitations one being that the sample stability was only measured at room temperature. Further validation of the manufacturer's claim of sample stability at temperatures ranging from 4 to 37 °C under conditions such as constant agitation would help confirm the use of the Roche Cell-Free DNA Collection Tubes® for shipment of patient samples in clinical studies, similar to studies carried out for the Streck tubes. Secondly, blood samples were not drawn by the same phlebotomist for all volunteers. It is possible that differences in experience and technique could have impacted on the initial gDNA concentration in the samples. Finally, the small sample used in the current study should also be taken into consideration while interpreting the findings of this study.

Overall, our findings indicate that the Streck tubes maintained the stability of the sample for up to 14 days post collection in contrast

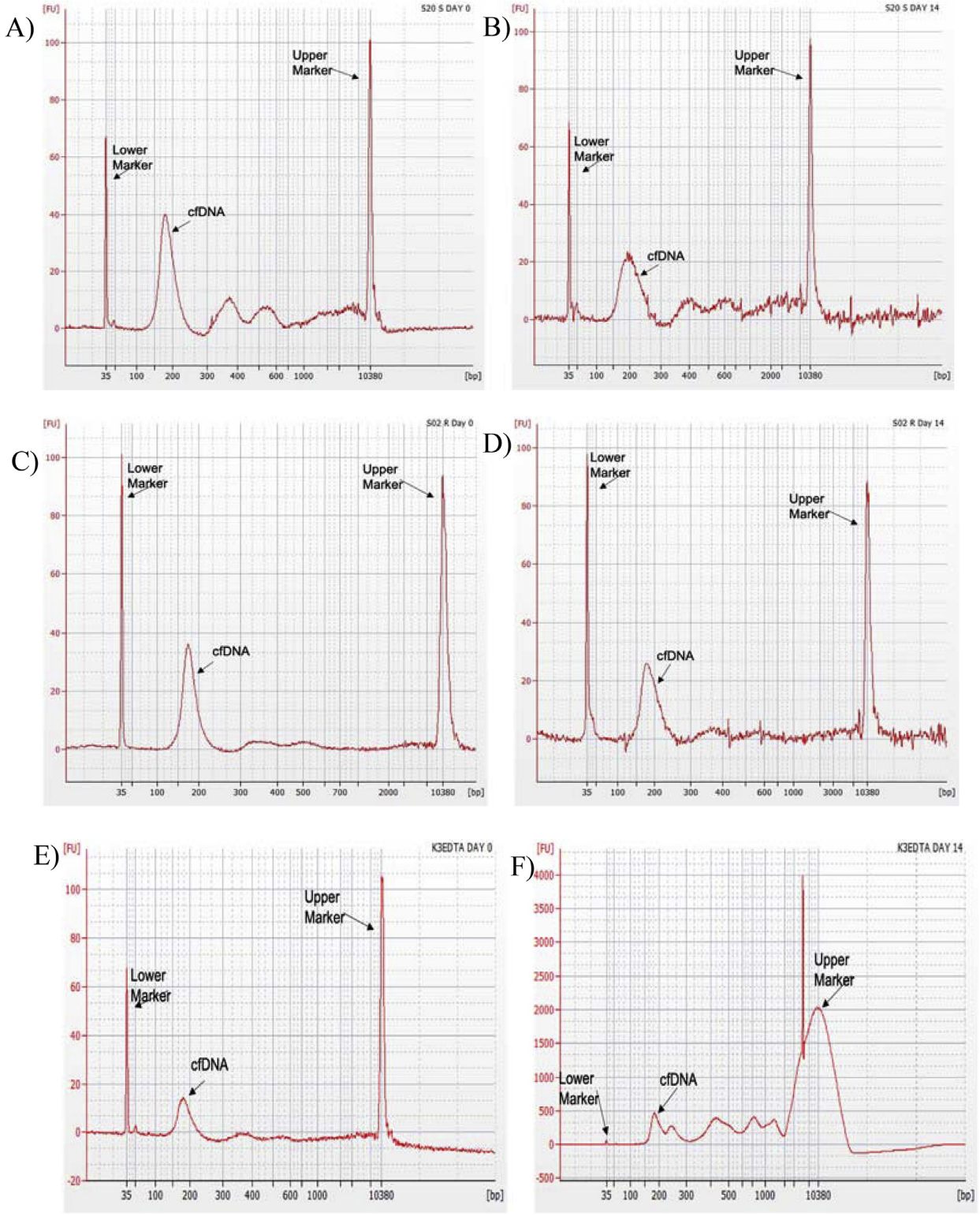

Fig. 3. Electropherograms from healthy volunteers' samples stored in Streck, Roche and K_3EDTA blood collection tubes. DNA peaks are quantified between two markers, lower (35 bp) and upper (10, 380 bp). cfDNA peak for each volunteer was identified between 150 and 200 bp. Genomic DNA peaks clustered around the upper marker. Graphs are shown for three volunteers **A**. Streck Day 0 sample, volunteer had a significant cfDNA, two smaller distinguishable peaks at 375bp and 500bp and further peaks near the upper marker characterized as gDNA. **B.** Streck Day 14 sample, cfDNA

size peak had decreased in fluorescence, however the two previously observed peaks were still apparent. gDNA near the upper marker was at the same fluorescence intensity as the day 0 sample. **C.** Roche Day 0 sample, a significant cfDNA peak was apparent. No gDNA was observed near the upper marker. **D.** Roche Day 14 sample, fluorescence of cfDNA has diminished, increase in "jaggedness" of baseline however no gDNA were detected. **E.** K_3EDTA Day 0 sample, an obvious cfDNA peak was present between 150bp and 200bp. **F.** K_3EDTA Day 14 sample, significant gDNA contamination with very high fluorescence intensity partially obscuring the cfDNA in the sample.

to the Roche tubes which maintained sample stability for seven days. This finding is consistent with the Roche tube manufacturer's recommendations and previous studies [15,20,21].

In conclusion, we have shown that Roche Cell-Free DNA Collection Tubes® are a suitable alternative for sample collection for clinical ctDNA studies for up to seven days as claimed by the manufacturers. However, it is imperative to note that the collection, storage, shipment and processing of such samples should occur within the stipulated time period to limit false negative and positive results due to high gDNA background levels. For storage of samples with potential longer delays (>7 days) between venipuncture and plasma separation, Streck Cell-Free DNA BCT®s may provide a better option.

Author contributions

SP, DZ, RD and PG designed the study. SP carried out the experiment and analysed the data. MB and SP carried out the statistical analysis. SP led the writing of the manuscript with input from MB, RD, DZ, and PG.

Disclosures/conflict of interest

The authors report no conflict of interest. The Roche Cell-Free DNA Collection Tubes® used in this research were supplied as free samples.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plabm.2019.e00125.

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Glossary

BEAMing: Beads, Emulsion, Amplification and Magnets
cfDNA: Cell Free DNA
ctDNA: Circulating Tumour DNA
ddPCR: Digital Droplet PCR
DNA: Deoxyribose Nucleic Acid
EDTA: Ethylenediaminetetra acetic acid
FU: Fluorescence Units
gDNA: Genomic DNA
PCR: Polymerase Chain Reaction
RBC: Red Blood Cells
WBC: While Blood Cells
NGS: Next Generation Sequencing