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A stabilizing reagent prevents cell-free DNA contamination by cellular DNA in plasma during blood sample storage and shipping as determined by digital PCR

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ABSTRACT

Objectives: To study the ability of a stabilizing reagent to prevent cellular DNA contamination of cell-free DNA (cfDNA) in plasma during whole blood sample storage and shipping.

Design and methods: Samples were drawn from healthy donors into K_3EDTA and Cell-Free DNA BCTs (BCT) and stored at room temperature (RT). Aliquots were removed at specified time points and cfDNA was purified from the plasma. A Droplet Digital PCR (ddPCR) assay that amplifies a short β-actin gene fragment (136 bp) was used to measure the total plasma cfDNA (pDNA) concentration while a longer β-actin fragment (420 bp) was used to quantify genomic DNA (gDNA). In a follow-up experiment, blood samples drawn into the same types of tubes were shipped round trip by overnight air before cfDNA was isolated and analyzed.

Results: Blood stored in K_3EDTA tubes at RT showed increases in pDNA and gDNA concentrations over time. However, both pDNA and gDNA levels remained stable in BCT for at least seven days. On day 14, there was a 4.5-fold increase in pDNA in BCT as compared to >200-fold increase in K_3EDTA tubes. Likewise, gDNA increased <2-fold on day 14 in BCT as opposed to a 456-fold increase in K_3EDTA tubes. Similar results were observed after samples were shipped.

Conclusions: Cell-Free DNA BCTs prevent gDNA contamination that may occur due to nucleated cell disruption during sample storage and shipping. This novel blood collection tube provides a method for obtaining stable cfDNA samples for rare target detection and accurate analysis while mitigating the threat of gDNA contamination.

Introduction

The presence of cell-free DNA (cfDNA) in blood has been known for over 50 years [1]. However, the importance of cfDNA in clinical medicine was not recognized until 1977, when Leon and colleagues demonstrated elevated levels of cfDNA in cancer patients [2]. Currently, a number of laboratories worldwide are investigating cfDNA for non-invasive diagnosis and prognosis. Fetal cfDNA present in maternal blood is now used for non-invasive prenatal diagnosis and clinical studies are underway using tumor-derived cfDNA as surrogate markers in cancer patients [3]. Yet, in both pregnant women and cancer patients, the cfDNA of interest (fetal or tumoral in origin) constitutes <10% of total cfDNA [4]. Accurate quantification of low occurrence targets means that any release of genomic DNA (gDNA) from white blood cells (WBCs) following blood draw should be minimized during sample storage and shipping so that the proportion of specific cfDNA targets is preserved. Obscuring cfDNA with gDNA could hamper detection in downstream applications [5].

One way to prevent gDNA release into the plasma fraction is to immediately process blood after phlebotomy [6]. This may limit the diagnostic scope of using cfDNA, particularly in locations that lack facilities for plasma separation from blood and cryopreservation prior to shipment. Alternatively, gDNA release into blood can be prevented by stabilizing WBCs. A previous study had shown that chemicals present in a blood collection tube, Cell-Free DNA™ BCT (BCT), stabilize WBCs in maternal blood samples thereby preserving the original proportion of fetal cfDNA for up to 14 days at room temperature (RT) [7].

Further studies have shown that true cfDNA fragments are generally <200 bp and are likely due to cellular apoptosis [9]. Increases in the concentration of fragments >300 bp may be an indication of a compromised blood sample in which nucleated cells have released gDNA, as Chan and colleagues have shown that the majority of circulating DNA is <313 bp [10]. Therefore, having the ability to assess the degree of gDNA contamination in plasma may be useful in determining sample quality and integrity. However, most manufacturers of real-time quantitative PCR (qPCR) instruments and reagents recommend amplicon lengths of 80–150 bp, since longer products show decreased amplification efficiencies, which can increase variation and decrease the reliability of results [11].

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Digital PCR is a third generation PCR technology in which the exponential analog nature of the qPCR has been transformed into a linear digital signal. Target DNA is diluted or partitioned to the point of having a single target DNA molecule in each reaction. End-point PCR then individually amplifies and analyzes for the presence or absence of target DNA using fluorescence probes [12]. Since digital PCR relies on end-point threshold to assign each replicate reaction as either positive or negative, it can tolerate wide variations in amplification efficiencies without affecting DNA copy number quantification [13]. Therefore, we have utilized digital PCR technology to quantify contaminating gDNA by amplifying a 420 bp DNA fragment from the β-actin gene. We have also developed a second digital PCR assay to quantify pDNA by amplifying a 136 bp shorter β-actin amplicon. Using these assays we determined the quality of a plasma cfDNA sample to evaluate the degree of gDNA contamination. We used this new methodology to analyze pDNA and gDNA changes in whole blood stored over 14 days at RT in either a K_3EDTA collection device or BCT, a cfDNA stabilization tube.

Materials and methods

Recruitment of blood donors

Volunteer donors were recruited from Streck Inc. in Omaha, NE with written informed consent. Donors were from both sexes and presumed to be healthy. The study was approved by the IRB of Methodist Hospital, Omaha, Nebraska USA.

Blood collection

For each experiment, blood samples from all donors were drawn into two different blood collection tubes. Control samples were drawn into K_3EDTA tubes (BD Vacutainer®, Becton Dickinson, Franklin Lakes, NJ) and compared to samples drawn into Cell-Free DNA™ BCT (BCT) (Streck Inc., Omaha, NE). Blood was mixed immediately after the draw by inverting tubes 10 times.

Sample processing

After phlebotomy blood samples were stored at RT (20–24 °C), except where otherwise noted, and plasma was separated at specified time points. Prior to the separation of plasma, stored blood samples were mixed by inverting tubes 10 times and centrifuged at 1600 ×*g* for 20 min at RT. The plasma layer was carefully removed, without disturbing the buffy coat, transferred to a new vial before being centrifuged at RT at 16000 ×*g* for 10 min to remove residual cells.

Total pDNA isolation from plasma

The QIAamp® Circulating Nucleic Acid Kit (Qiagen, Santa Clarita, CA) was used for the extraction and purification of DNA from 0.5 mL plasma at each time point. The manufacturer's recommended protocol was modified slightly by increasing the duration of the Proteinase K treatment from 30 min to 1 h at 60 °C to reverse the effects of chemical fixation. Samples were eluted in 30 μL sterile nuclease-free water and stored at −80 °C until analysis by ddPCR.

Droplet Digital PCR (ddPCR)

For pDNA quantification, primers and probe measuring a short DNA fragment (136 bp) from the human β-actin gene [14] were: forward primer 5′-GCG CCG TTC CGA AAG TT-3′; reverse primer 5′-CGG CGG ATC GGC AAA-3′; probe 6FAM-ACC GCC GAG ACC GCG TC-MGBNFQ. All primers were purchased from Integrated DNA Technologies (IDT) (Coralville, IA). For the quantification of a longer β-actin gene sequence (420 bp), the following primers and probe were used: forward primer 5′-CCG CTA CCT CTT CTG GTG-3′; reverse primer 5′-GAT GCA CCA TGT CAC ACT G-3′; probe 6FAM-CCT CCC TCC TTC CTG GCC TC-BHQ. Primers and probes for the long β-actin fragment were designed using the online primer design tool, PrimerQuest, from IDT. The probe for the short fragment was purchased from Applied Biosystems (Foster City, CA) and the long fragment probe was purchased from IDT. A PCR master mix, 2× ddPCR™ Supermix for Probes, was purchased from Bio-Rad Laboratories (Hercules, CA). Final concentrations of primers and probe in PCR reactions were 900 nM and 250 nM, respectively, in a final volume of 20 μL. The DNA template input volume was 5 μL and some samples required 10-fold dilutions to bring the cfDNA concentrations into the dynamic range of the ddPCR system. A Bio-Rad QX100 Droplet Digital™ PCR System was used as described by Hindson and colleagues [13]. Thermal cycling was performed with a Bio-Rad C1000 Touch Thermal cycler. The following PCR conditions were used: 98 °C for 10 min, 40 cycles of 30 s at 95 °C, 30 s at 54 °C and 30 s at 72 °C. A final extension was done at 72 °C for 10 min followed by a heating step at 98 °C for 10 min to inactivate the polymerase. Data analysis was done using Bio-Rad QuantaSoft software version 1.3.2.

Effect of storage on total pDNA and gDNA concentration in blood samples

This study was conducted using blood from 12 donors. Blood was drawn from each donor into one 10 mL K_3EDTA tube and one 10 mL BCT. All tubes were stored at RT. Aliquots of blood were taken from both tube types on days 0, 1, 2, 3, 7 and 14, the plasma separated and gDNA and pDNA was quantified.

Effect of shipping on total pDNA and gDNA concentrations in blood samples

For a shipping study, blood was drawn from 6 donors. From each donor blood was drawn into two 10 mL K_3EDTA tubes and two 10 mL BCTs. One K_3EDTA tube and one BCT from each donor were packed in an insulated box equipped with a temperature monitoring device (Omega Nomad, Omega Engineering, Stamford CT) and shipped from Omaha, NE round trip via overnight air freight to a laboratory in Lincoln, NE (elapsed time 48 h). Upon return, plasma was separated as noted above. A control set of tubes was not shipped and left at RT. Aliquots of control blood were removed from tubes on days 0 and 2, the plasma separated and the gene targets quantified by ddPCR.

Statistical analysis

Statistical analysis was carried out using Microsoft Excel for Office 2010. Analysis was performed using paired, two-tailed Student's *t*-test and $p < 0.05$ was considered statistically significant.

Results

Effect of storage on pDNA and contaminating gDNA concentrations in blood samples

Samples were stored at RT for up to 14 days in order to investigate the effect of storage on pDNA and contaminating gDNA concentrations in blood drawn into K_3EDTA tubes and BCTs. Fig. 1A shows that blood drawn into K_3EDTA tubes had statistically significant increases in pDNA concentration from day 2 onwards with a 2-fold increase on day 2, 12-fold increase on day 3, 49-fold increase on day 7 and 204-fold increase on day 14 ($p = 0.0092$, 0.0021 and <0.0001, respectively). As shown in Fig. 1B, blood drawn into BCTs showed no significant increases in pDNA concentration up to day 14 at which point there was a slight, but statistically significant increase ($p < 0.0001$) of 4-fold. Fig. 2A illustrates the effect of storage on ontaminating gDNA concentration in plasma obtained from blood

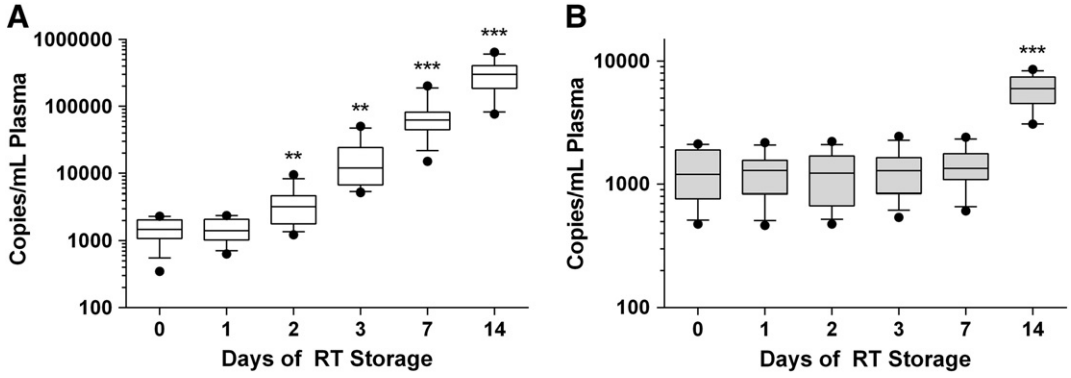

Fig. 1. Effect of RT storage of blood samples on pDNA concentration. Blood samples were drawn into either K_3EDTA tubes or BCTs and stored at RT. Aliquots of blood were removed at indicated times and the plasma was separated. After the DNA was isolated from plasma, pDNA concentration was determined by ddPCR using a short β-Actin gene target (136 bp). The pDNA concentration in K_3EDTA tubes (panel A) showed statistically significant increases when compared to the initial time point. Beginning on day 2, pDNA copy number continued to increase up until day 14 when it was >200-fold as compared to the initial time point. In BCTs (panel B), a statistically significant change was not observed until day 14, when a 4.5-fold change in pDNA copy number was found. For both panels: The line inside of the box indicates median value. The limits of the box represent the 75th and 25th percentiles. The whiskers indicate the 10th and 90th percentiles. The dots indicate maximum and minimum values. Panels A and B are in logarithmic scale. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p \leq 0.001$ n = 12.

drawn into K_3EDTA tubes, showing a statistically significant increase in gDNA concentration over time. There were significant sequential fold increases of 1.2, 5, 13, 80 and 456 in gDNA concentration, respectively ($p = 0.037$, 0.009, 0.008, 0.0011 and <0.0001). The blood samples drawn into BCTs (Fig. 2B) showed no such increases in gDNA concentration except for day 14, which showed a small but statistically significant 1.9-fold increase ($p = 0.02$).

Effect of shipping on pDNA and contaminating gDNA concentrations in blood samples

In order to study the stresses of shipment on whole blood samples from a collection point to an analytical site, blood was drawn from each donor into two 10 mL K_3EDTA tubes and two 10 mL BCTs. One 10 mL K_3EDTA and one 10 mL BCT from each donor were shipped via air freight, to a laboratory in Lincoln, NE and back over the course of two days in an insulated foam cooler. A control set of samples comprised of a K_3EDTA tube and BCT from each donor was not shipped, left at RT and aliquots of blood removed on day 0 and day 2. The internal temperature monitoring device registered an average temperature of 17 °C with a range of 13–23 °C over the course of the 47.5 h of shipping. Fig. 3 illustrates that statistically significant increases in pDNA concentration occurred in shipped K_3EDTA tubes (Fig. 3A) compared to the initial pDNA concentration of control K_3EDTA tubes ($p = 0.0011$) after they had returned. There was also statistically significant increases in gDNA levels in shipped K_3EDTA blood samples ($p = 0.0157$) compared to control values (Fig. 3B). In the BCT samples, there were no statistically significant changes in gDNA concentration (Fig. 3B) between the initial and non-shipped blood samples or the non-shipped and shipped tubes ($p = 0.7561$ and 0.1355, respectively). There were also no statistically significant changes in pDNA concentration in BCTs (Fig. 3A) between initial and non-shipped or shipped and non-shipped ($p = 0.1549$ and 0.0675 respectively).

Discussion

Circulating fetal cfDNA in maternal blood is currently being used in non-invasive prenatal diagnosis of trisomy [15], fetal sex determination [16] and detecting RHD genes in RhD negative mothers [17]. Recent findings have shown that tumor-derived cfDNA present in the blood of cancer patients can be used as biomarkers for non-invasive cancer diagnosis and prognosis [18,19]. One of the major technical challenges to the effective use of cfDNA targets as biomarkers is quantifying scarce targets. For example, the proportion of fetal cfDNA in maternal plasma is <10% [4]. Therefore, any additional DNA released from nucleated cells after blood is drawn further decreases the proportion of fetal or tumor derived cfDNA in blood. Previous studies have shown that increasing the time interval between blood draw and pDNA extraction or shipment of blood samples increases pDNA concentration [20,21]. In order to maintain the proportion of rare target cfDNA in a blood

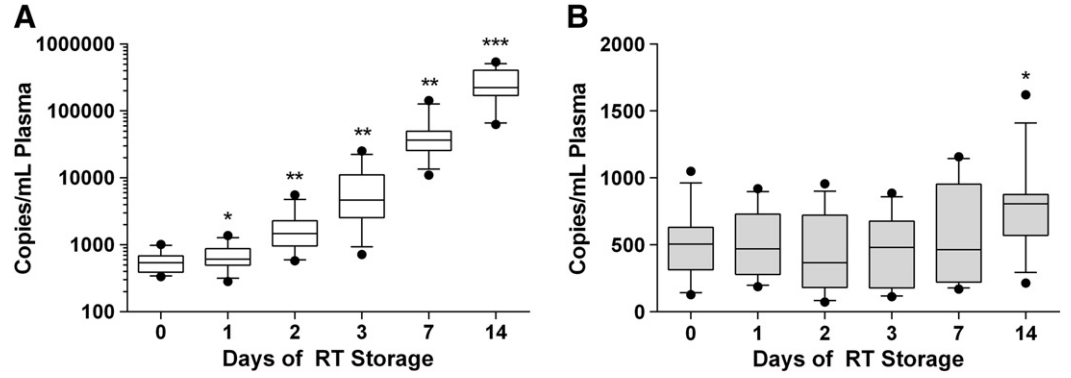

Fig. 2. Effect of RT storage of blood samples on gDNA concentration. Blood was drawn into K_3EDTA and BCTs and stored as whole blood at RT for up to 14 days. At the noted times, aliquots were removed, plasma was isolated, and DNA was extracted. A long β-Actin gene target (420 bp) was quantified using ddPCR. In blood drawn into K_3EDTA tubes (panel A), plasma samples showed statistically significant increases in gDNA concentration compared to the initial time point. These increases continued up until day 14, when a 456-fold increase in gDNA copy number was observed. In blood samples drawn into BCTs (panel B), a statistically significant increase was not observed until day 14, at which point gDNA had increased <2-fold. For both panels: The line inside of the box indicates median value. The limits of the box represent the 75th and 25th percentiles. The whiskers indicate the 10th and 90th percentiles. The dots indicate maximum and minimum values. Panel A is in logarithmic scale $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p \leq 0.001$ n = 12.

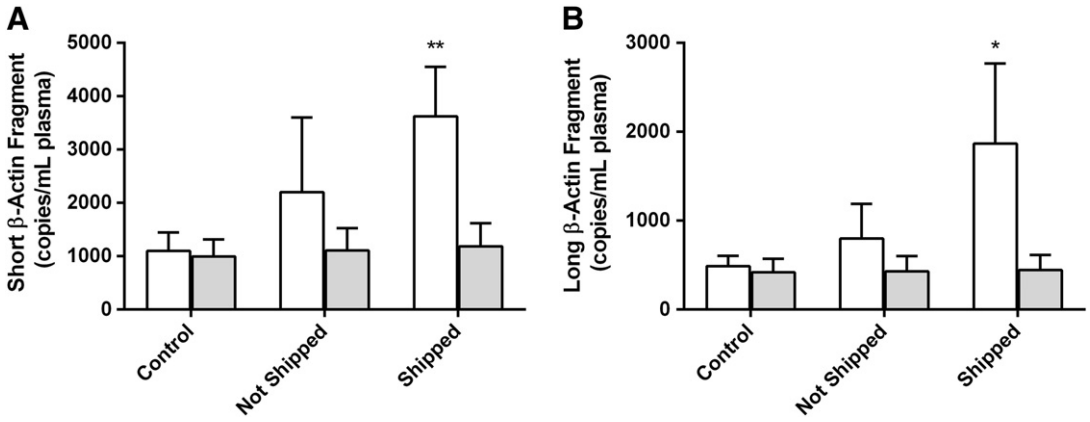

Fig. 3. Effect of shipping on pDNA and gDNA concentrations in blood. Blood was drawn into K_3EDTA and BCTs. Whole blood samples were either retained and stored at RT to serve as controls or shipped via air freight over the course of 48 h to another location and back. For the controls, initial aliquots were removed at day 0 and another aliquot was harvested at day 2 when the shipped samples had returned. From all of the samples, plasma was isolated and DNA was extracted. Concentrations of pDNA (panel A) and gDNA (panel B) were determined by ddPCR. In samples drawn into K_3EDTA tubes (white bars) there were statistically significant increases in both gDNA and pDNA in shipped samples. When the same samples were drawn into BCTs (gray bars) there were no statistically significant changes in either fragment population. For both panels: Error bars indicate SD, $^{*}p < 0.05$, $^{**}p < 0.01$ n = 6.

sample, it is necessary to minimize WBC disruption. Previously, Zheng and colleagues reported that naturally occurring cfDNA is comprised of shorter DNA fragments <200 bp [8]. From the work of Chan and colleagues, we presume that DNA fragments longer than >300 bp are WBC-derived gDNA [9]. Therefore, developing a PCR-based assay to quantify both small and large DNA fragments may help to determine the degree of gDNA contamination of cfDNA.

In a previous study, we used qPCR to quantify cfDNA in plasma [7]. We then attempted to develop a qPCR assay to amplify a DNA fragment >300 bp but were not successful. In this study we developed an assay capable of amplifying a 420 bp fragment of β-actin gene using ddPCR technology. Droplet Digital PCR has several advantages over qPCR. With ddPCR, it is possible to discriminate small differences as low as 1.25-fold, with great accuracy. Unlike qPCR, standard curves or references are not required in ddPCR to get absolute copy numbers. Poor amplification efficiencies sometimes caused by amplicon targets >150 bp do not effect ddPCR DNA copy number estimation as compared to qPCR as sequestration of the PCR reaction into tens of thousands of nanoliter reactions further assists in the quantification of difficult targets [13].

Equipped with the longer fragment ddPCR assay to measure contaminating gDNA in plasma, we then used a shorter DNA fragment assay and ddPCR to evaluate pDNA integrity. Our data show that pDNA and gDNA levels significantly increased during RT storage in blood collected into K_3EDTA tubes (Figs. 1A and 2A). Here, statistically significant increases in gDNA concentration in K_3EDTA tubes, indicating nucleated cell disruption, occurred within 24 h storage at RT. However, pDNA and gDNA concentrations in BCTs did not increase significantly with RT storage for up to 7 days (Figs. 1B and 2B). On day 14, BCTs showed a slight but statistically significant increase in both pDNA and gDNA concentrations. Even though there were statistically significant increases, samples stored in BCTs only showed a 4.5-fold increase in pDNA and a 2-fold increase in gDNA concentrations on day 14 while the corresponding increases in pDNA and gDNA concentrations in K_3EDTA tubes were 204-fold and 456-fold, respectively. Our study determined that storage of whole blood in BCTs prevented increases in both long and short fragment forms of DNA, as seen in paired samples drawn into K_3EDTA collection devices.

Often, advanced molecular diagnostic testing requires that patient blood samples be shipped to a centralized laboratory for analysis. Previous studies have shown that in blood samples collected into K_3EDTA tubes pDNA levels increased following sample storage and/or shipping because of the time elapsed between draw and analysis. Over-handling of samples may induce cellular damage. Due to these problems, it is generally recommended that blood samples be centrifuged to isolate plasma and the resultant plasma cryopreserved and shipped cold to prevent cfDNA adulteration by gDNA [22]. However, our results indicate that when blood is drawn into BCTs, no prior plasma separation or freezing is necessary before shipment or sample storage to prevent changes in either pDNA or gDNA (Fig. 3). Traditional reagents used in cell stabilization, such as formaldehyde and glutaraldehyde, are known to damage DNA and RNA by chemical modification. By promoting nucleic acid–protein cross-link formation, these reagents make the extraction of nucleic acids difficult [23]. In previous studies, the proprietary stabilization cocktail in BCTs had been shown by ^{13}C NMR to be formaldehyde-free [24] and the treatment of samples with the BCT reagent did not measurably alter extraction or amplification [24]. In this study we utilized next generation ddPCR technology to show the advantage of a cell stabilizing reagent cocktail that prevents the release of gDNA into plasma after blood draw and while preserving the original cfDNA population. Using this blood collection device, blood samples can be shipped at RT, which allows flexibility for offsite blood sample analysis at specialized laboratories or collected and processed en masse for downstream analysis of the cfDNA without preliminary centrifugation or cryopreservation while preserving the integrity cfDNA in samples as determined by a novel ddPCR assay.

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