

Research Article

Preservation of Circulating Cell-Free Fetal RNA in Maternal Blood Using a Blood Collection Device Containing a Stabilizing Reagent

Jianbing Qin¹, Craig Bassett² and MR Fernando^{1*}

¹Research and Development Division, Streck, Inc., LaVista, NE 68128, USA ²Methodist Physicians Clinic Women's Center, Omaha, NE 68022, USA

Abstract

Objective: To investigate the ability of Cell-Free RNA BCT (BCT) blood collection device to stabilize fetal cell-free RNA in maternal plasma when compared to K₃EDTA collection tubes.

Design and methods: Blood samples were drawn from healthy pregnant donors into K_3 EDTA tubes and BCTs and kept at ambient temperature (22°C). Plasma was separated by centrifugation and cell-free RNA was extracted. Circulating fetal RNAs from plasma were quantified by digital droplet polymerase chain reaction (ddPCR).

Results: Blood drawn into K₃EDTA tubes showed a decrease in fetal mRNA concentration for human placental lactogen (hPL), β subunit of human chorionic gonadotropin (β hCG) and placenta-specific 4 (PLAC4) over three days of ex vivo incubation at 22°C. Blood drawn into BCTs, however, showed no significant change in mRNA concentrations over the same period of time.

Conclusion: Cell-Free RNA BCT blood collection device preserve scirculating fetal RNA in maternal blood for at least three days at 22°C, which enhances the potential clinical utility of circulating fetal RNA in maternal blood for noninvasive prenatal diagnostic assay development.

Keywords: Fetal cell-free RNA; Blood collection tubes; Stability of fetal cell-free RNA in blood

Introduction

The presence of circulating fetal cell-free RNA in maternal blood was initially reported in 2000 by Poon and colleagues [1]. Following this discovery, many other clinically important fetal/placental cell-free mRNA molecules were detected in maternal blood [2,3]. Using realtime quantitative RT-PCR assays, Ng and colleagues have detected and quantitated the mRNAs for human placental lactogen (hPL) and the β subunit of human chorionic gonadotropin (β hCG) in maternal blood samples [4]. Quantitation of β hCG mRNA concentration in maternal blood is clinically important because it is elevated in trisomy 21 pregnancies, but drastically reduced in trisomy 18 pregnancies [5]. Fetal cell-free mRNAs for hif1a, p21 and corticotrophin-releasing hormone (CRH) are also clinically important because they are elevated in preeclampsia [6,7]. In current clinical practice, fetal genetic material required for prenatal diagnosis of fetal genetic disorders are obtained by amniocentesis or chorionic villus sampling, but are highly invasive procedures because of the risk they pose to the pregnancy. The above new discoveries have presented us with opportunities to develop noninvasive prenatal diagnostic tools based on fetal cell-free RNA in maternal blood.

However, there are some prerequisites for successful utilization of fetal cell-free RNA in maternal blood for the development of noninvasive prenatal diagnostic tests. One such prerequisite is the controlling of pre-analytical variables that might impact the utility of fetal cell-free RNA in the development of noninvasive prenatal diagnostic tests. Delays in blood processing may have profound effects on test outcomes. Some fetal cell-free RNA molecules may not be stable during blood processing and storage. Previously, it has been demonstrated that cell-free β -actin mRNA concentration decreases in blood plasma with time after phlebotomy, probably due to RNA degradation [8]. Another pre-analytical variable that might affect the test outcome is the release of non-target background RNA from blood cells during sample processing and storage. Salway et al. [9] have shown

that cell-free mRNA concentrations for glyceraldehyde-3-phosphate dehydrogenase and beta-2-microglobulin increase with time after phlebotomy. This kind of background increase may adversely affect the detection of rare mRNA targets. In a previous study by our laboratory, we compared the stability of cell-free RNA in whole blood collected into a regular collection device (K₃EDTA) and a device with a stabilizing reagent (BCT). According to the results of that study, BCT device could stabilize cell-free RNA in whole blood samples for 3 days at room temperature compared to K₃EDTA collection tubes [10]. In another study, we have also shown that BCT device could minimize background RNA increase caused by agitation and temperature fluctuations that can occur during blood sample storage and shipping [11]. In this study, we compared BCT device against traditional K₃EDTA tubes for its ability to preserve fetal cell-free RNA in maternal plasma.

Materials and Methods

Pregnant donor recruitment

Two groups of healthy pregnant women, a first trimester group and a second trimester group, were recruited for this study from Methodist Hospital, Omaha NE, USA. This study was approved by the Institutional Review Board (IRB Number 1090) of the Methodist Hospital, Omaha NE and informed consent was obtained from all donors prior to blood draw.

*Corresponding author: M Rohan Fernando, Research and Development Division, Streck Inc., 7002 S 109 Street, Omaha, NE 68128, USA, Tel: 402-537-5282; Fax: 402-537-5352; E-mail: rfernando@streck.com

Received November 11, 2013; Accepted January 13, 2014; Published January 20, 2014

Citation: Qin J, Bassett C, Fernando MR (2014) Preservation of Circulating Cell-Free Fetal RNA in Maternal Blood Using a Blood Collection Device Containing a Stabilizing Reagent. J Mol Genet Med 7: 097 doi: 10.4172/1747-0862.1000097

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Blood collection

Two 10 ml blood samples were drawn into two different blood collection tubes from each donor. One tube was a K₃EDTA collection tube (BD vacutainer[®], Becton Dickinson, Franklin Lakes, NJ) and the other was Cell-Free RNA[™] BCT (Streck catalog, part numbers 218975 or 218976) a vacuum collection tube developed by Streck[®], Inc., La Vista, NE, that contains proprietary chemicals to stabilize cell-free RNA in whole blood. Blood was mixed well immediately after the draw by inverting the tube 10 times. Blood samples collected at the Methodist Hospital were sent to Streck[®], Inc. for analysis within 2 hrs of collection. Samples were kept at ambient temperature during the entire experimental period.

Sample processing

Plasma was separated from blood two hours post collection (day 0) or after 1, or 3 days as noted. To separate plasma, sample tubes were centrifuged at 300xg for 20 minutes at room temperature. The upper plasma layer was removed without disturbing the buffy coat and transferred to a new tube that was then centrifuged at 5000xg for 10 minutes to assure cell-free plasma. The clarified plasma was then transferred to a new tube for storage at -80°C pending RNA extraction.

Cell-free RNA isolation from plasma

The cell-free RNA was extracted and purified from plasma using the QIAamp^{*} Circulating Nucleic Acid Kit (Qiagen, Santa Clarita, CA). The manufacturer's recommended protocol was modified slightly by increasing the duration of the Proteinase K treatment at 60°C from 30 minutes to one hour. An on-column DNase treatment step was included to remove DNA and cell-free RNA was eluted in 30 μ l nuclease-free water that was passed over the column twice.

Droplet digital PCR

Primers and probes for the reverse transcriptase digital PCR quantitation of mRNAs for β hCG (92 bp), hPL (97 bp) and hPLAC4 (80 bp) were prepared as described previously [4,12]. All primers and probes were purchased from Integrated DNA Technologies's (Coralville, IA), except for the probe for hPLAC4 which was purchased from Applied Biosystems (Foster City, CA). The RT-PCR was performed using the QX100 Droplet Digital PCR system (Bio-Rad, Hercules, CA). One-Step RT-ddPCR^{∞} Kit for probes was purchased from Bio-Rad and manufacturer's instructions were followed. Data analysis was done using Bio-Rad QuantaSoft analysis software.

Statistical analysis

Statistical analysis was performed using paired Student's t-test. p<0.05 was considered statistically significant.

Results

Effect of storage on cell-free β hCG mRNA concentration in maternal blood samples

To study the effect of storage on cell-free $\beta hCG\,mRNA$ concentration



Figure 1: Effect of storage of maternal blood samples on circulating fetal cell-free β hCG mRNA concentration in K₃EDTA tubes or BCTs. Blood from first trimester (A) and second trimester (B) pregnant donors were drawn into K₃EDTA tubes and BCTs and stored at room temperature. Aliquots were removed on days 0, 1, and 3, plasma RNA was isolated, and mRNA for β hCG was quantified by ddPCR. Over time, decrease in the concentration of β hCG was observed in K₃EDTA samples while there was no significant change in BCT samples. *p<0.02, n=8.

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in maternal blood drawn into K₂EDTA tubes and BCTs, blood samples were stored at 22°C and aliquots of blood removed from each tube on days 0, 1 and 3 and processed for the quantitation by digital PCR. Figure 1A illustrates the effect of storage on βhCG mRNA concentration in first trimester maternal blood drawn into K3EDTA tubes and BCTs. In K EDTA blood, initial βhCG mRNA concentration (day 0) was 2569 copies/mL plasma which was stable only up to day 1 and at day 3 it was 1543 copies/mL plasma which shows a 40% decrease (p = 0.12)compared to day 0 value. Day 0 βhCG mRNA concentration in BCT blood was 2109 copies/mL plasma and at day 3 it was 2213 copies/mL plasma which shows that β hCG mRNA concentration is stable in BCT blood during the 3 day storage period. Figure 1B shows the effect of storage on βhCG mRNA concentration in second trimester maternal blood samples. According to the results shown in Figure 1B, there is a significant decrease (~18-fold) in βhCG mRNA concentration in second trimester maternal blood compared to first trimester maternal blood. Initial BhCG mRNA concentration in second trimester K, EDTA blood was 143 copies/mL plasma and by day 3 it dropped to 63 copies/ mL plasma which shows a 56% decrease ($p \le 0.02$) compared to initial concentration. However, βhCG mRNA concentration was stable in second trimester maternal blood drawn into BCTs for 3 days at 22°C with 115 and 136 copies / mL plasma at day 0 and day 3, respectively.

Effect of storage on cell-free PLAC4 mRNA concentration in maternal blood samples

Figure 2A shows the effect of storage on PLAC4 mRNA concentration in first trimester maternal blood drawn into K_x EDTA

tubes and BCTs. In K₃EDTA blood, initial (day 0) PLAC4 mRNA concentration was 233 copies/mL plasma and at day 3 it was 188 copies/mL plasma which is a19% decrease (p = 0.61) compared to day 0 concentration. Blood drawn into BCTs showed no change in PLAC4 mRNA concentration during storage at 22°C for 3 days. Figure 2B shows the effect of storage on PLAC4 mRNA concentration in second trimester maternal blood samples. According to Figure 2B, PLAC4 mRNA concentration in second trimester maternal blood. In K₃EDTA blood initial PLAC4 mRNA concentration was 958 copies/mL plasma and it was stable only up to day 1 and at day 3 it was 391 copies/mL plasma which shows a statistically significant 59% decrease (p \leq 0.01). However, blood drawn into BCTs showed no change in PLAC4 mRNA concentration during storage at 22°C for 3 days.

Effect of storage on cell-free hPL mRNA concentration in maternal blood samples

Figure 3A illustrates the storage effect on hPL mRNA concentration in first trimester maternal blood samples drawn into K₃EDTA tubes and BCTs. The hPL mRNA concentration in K₃EDTA tubes showed a gradual decrease over time. At days 0, 1 and 3, hPL mRNA concentrationswere483, 309 and 218 copies/mL plasma, respectively. Compared to day 0 concentration, at day 1 and 3 there was a 36% and 55% statistically significant decrease ($p \le 0.03$; $p \le 0.02$) in hPL mRNA concentration, respectively. However, hPL mRNA concentration was stable in maternal blood drawn into BCTs at 22°C for 3 days. Figure 3B shows the effect of storage on hPL mRNA concentration in second



Figure 2: Effect of storage of maternal blood samples on circulating fetal cell-free PLAC4mRNA concentration in K_3 EDTA tubes or BCTs. Blood from first trimester (A) and second trimester (B) pregnant donors were drawn into K_3 EDTA tubes and BCTs and stored at room temperature. Aliquots were removed on days 0, 1, and 3, plasma RNA was isolated, and mRNA for PLAC4 was quantified by ddPCR. PLAC4mRNA concentration decreased over time in K_3 EDTA samples while there was no significant change in BCT samples. *p<0.01, n=8.

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trimester maternal blood samples. As shown in Figure 3B, initial hPL mRNA concentration in second trimester maternal blood showed a 10-fold increase compared to initial hPL mRNA concentration in first trimester maternal blood. Initial hPL mRNA concentration in second trimester K₃EDTA blood was 4805 copies/mL plasma and by day 3 it dropped to 1973 copies/mL plasma which shows a 59% decrease ($p \le 0.01$) compared to initial concentration. However, hPL mRNA concentration was stable in second trimester maternal blood drawn into BCTs for 3 days at 22°C.

Discussion

Fetal genetic material required for prenatal diagnosis of fetal genetic disorders are obtained from invasive procedures such as amniocentesis and chorionic villus sampling [13]. Circulating fetal cell-free RNA in maternal blood has the potential to be used for the development of noninvasive prenatal diagnostic tests. Current clinical practice requires blood samples to be stored at the site of collection and/or transported to a central facility for analysis. This may expose the blood sample for long delays, temperature fluctuations and shaking which may enhance the cell-free RNA background increase as well as RNA degradation. In a previous study, we have shown that BCT device could minimize postphlebotomy cell-free RNA background increase and RNA degradation at 22°C for 3 days in blood samples [10]. It has also been demonstrated that BCT device could minimize cell-free RNA background increase caused by temperature fluctuations and agitation that may occur during blood sample storage and shipping [11]. In the current study, we investigated the ability of BCT device to stabilize circulating fetal cell-free RNA in maternal blood samples when stored at 22°C for 3 days. Three fetal cell-free mRNA molecules, βhCG, PLAC4 and hPL were used as markers to compare the stability of circulating fetal cellfree RNA in maternal blood samples drawn into K₂EDTA tubes and BCTs. Considering the clinical significance of early prenatal diagnosis, we used first and second trimester maternal blood samples in this study. According to Figure 1A and B, there was a significant decrease in βhCG mRNA concentration in first and second trimester maternal blood collected into EDTA tubes at day 3. The other two mRNA molecules quantitated in this study, PLAC4 and hPL also showed statistically significant decreases in K₂EDTA blood at day 3 when stored at 22°C, except for the first trimester PLAC4 concentration According to Ng and colleagues, a large proportion of circulating fetal cell-free RNAs are particle associated thus protected from plasma nuclease [4]. However, storage and transportation of blood samples in blood collection tubes without cell stabilizers and nuclease inhibitors for periods longer than 24 h at room temperature may compromise the integrity of circulating fetal cell-free RNA molecules. This may be due to the maternal blood cell lysis and resultant protease and other degrading enzyme release which may damage membrane of the particles exposing cell-free fetal RNA molecules to plasma nucleases. Concentrations of all three mRNA molecules in maternal blood drawn into BCTs were stable for 3 days at 22°C. The chemical cocktail in BCTs contains cell stabilizing reagents and nuclease inhibitors [10]. Stabilizing reagents in BCTs stabilize maternal blood cells preventing release of proteases and other degrading enzymes during blood sample storage and transportation. RNase inhibitor in BCTs inhibits plasma RNase and protects non-



Figure 3: Effect of storage of maternal blood samples on circulating fetal cell-free hPL mRNA concentration in K_3 EDTA tubes or BCTs. Blood from first trimester (A) and second trimester (B) pregnant donors were drawn into K_3 EDTA tubes and BCTs and stored at room temperature. Aliquots were removed on days 0, 1, and 3, plasma RNA was isolated, and mRNA for hPL was quantified by ddPCR. hPLmRNA concentration decreased over time in K_3 EDTA samples while there was no significant change in BCT samples. *p<0.03,**p<0.02, n=8.

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particle associated circulating fetal cell-free RNA from degradation [10].

In conclusion, we provide evidence that circulating fetal cell-free RNAs in whole blood samples drawn into K_3 EDTA tubes are unstable when stored at 22°C for 3 days. However, it is possible to store whole blood samples collected into BCT device for 3 days at 22°C without compromising the integrity of circulating fetal cell-free RNA molecules. Therefore, BCT device provide flexibility for whole blood samples to be stored at 22°C for 3 days without centrifugation and cryopreservation thus supporting the development of new noninvasive prenatal diagnostic tests based on fetal cell-free RNA in maternal blood.

Acknowledgement

We the authors thank Paul Dye for help with figure preparation, Bradford A. Hunsley and Patty Alvarado for coordinating the IRB process and pregnant donor blood sample transportation from Methodist Hospital, Omaha NE, to Streck Inc., Omaha NE.

Conflict of Interests

CB declares that no conflicts of interest exist. MRF and JQ are full time employees of Streck Inc.

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