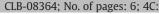
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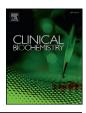
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Optimizing blood collection, transport and storage conditions for cell **Q4**1 free DNA increases access to prenatal diagnostic testing $\stackrel{ ightarrow}{=}$ 2

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ABSTRACT

Objectives: Fetal mutations and fetal chromosomal abnormalities can be detected by molecular analysis 24 of circulating cell free fetal DNA (ccffDNA) from maternal plasma. This comprehensive study was aimed to 25 investigate and verify blood collection and shipping conditions that enable Noninvasive Prenatal Diagnostic 26 Testing. Specifically, the impact of shipping and storage on the stability and concentration of circulating 27 cell-free DNA (ccfDNA) of blood in Streck® Cell-Free DNA™ Blood Collection Tubes (Streck BCTs, Streck, 28 Omaha NE), designed to minimize cellular degradation, and thus effectively prevent dilution of ccffDNA by 29 maternal genomic DNA, was evaluated.

Design and methods: Peripheral venous maternal blood was collected into Streck BCTs to investigate 31 four aspects of handling and processing conditions: (1) time from blood draw to plasma processing; (2) storage 32 temperature; (3) mechanical stress; and (4) lot-to-lot tube variations. 33

Results: Maternal blood stored in Streck BCTs for up to 7 days at ambient temperature provides stable con- 34 centrations of ccffDNA. The amount of fetal DNA did not change over a broad range of storage temperatures 35 (4 °C, 23 °C, 37 °C, 40 °C), but the amount of total (largely maternal) DNA increased in samples stored at 36 23 °C and above, indicating maternal cell degradation at elevated temperatures. Shipping maternal blood in 37 Streck BCTs, did not affect sample quality.

Conclusions: Maternal plasma DNA stabilized for 0 to 7 days in Streck BCTs can be used for non-invasive 39 prenatal molecular applications, when temperatures are maintained within the broad parameters assessed in 40 this study. 41

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Q547 Introduction

Chromosomal, genetic and biochemical abnormalities of the fetus 48 are routinely detected by analyzing fetal cells obtained through invasive 49procedures such as amniocentesis and chorionic villus sampling [1]. A 5051small but definite risk of injury to both mother and fetus is imparted by invasive sampling procedures [2]. An alternate source of genetic

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material that accurately represents fetal status is circulating cell-free 53 fetal DNA (ccffDNA) found in maternal plasma. The placenta is thought 54 to be the main source for ccffDNA, and consequent clearance of ccffDNA 55 from maternal plasma occurs within hours after birth [3,4]. Recently the 56 use of ccffDNA has enabled the introduction of non-invasive prenatal 57 testing (NIPT) methods such as fetal RHD genotyping from maternal 58 plasma [5] and fetal aneuploidy detection in high risk women [6]. How- 59 ever, care must be taken to prevent an increase of circulating maternal 60 DNA after phlebotomy (through maternal white cell lysis), because 61 quantitative applications, such as non-invasive aneuploidy detection, 62 are impaired by a relative decrease in fetal fraction (FF). Typical fetal 63 fractions range from 2% to 40% with a mean of 10% of total ccfDNA 64 across varying gestation ages [6,10]. The minimum fetal fraction for ac- 65 curate determination of Trisomy 21 is 4% as measured by the Fetal 66 Quantifier Assay (FQA) and set as QC cut-off [6]. Thus a small increase 67 in maternal DNA could reduce the fetal fraction below the 4% QC cut 68 off, particularly in cases of low initial fetal fraction. Below 4% FF no 69 test results would be reported. Enabling the access of a broad popula-70 tion of mothers to non-invasive prenatal testing methods, requires ro-71 bust and validated blood collection devices and processing protocols. 72

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Abbreviations: ccf, circulating cell-free; ccffDNA, circulating cell-free fetal DNA; BCT, blood collection tube; NIPT, non-invasive prenatal testing; IRB, Institutional Review Board; FF, fetal fraction; FQA, Fetal Quantifier Assay; MALDI-TOF MS, matrixassisted laser desorption/ionization time-of-flight mass spectrometer; NSC, no storage control; MPS, massively parallel sequencing; NTC, no template control.

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To maintain high fetal fraction, processing protocols for maternal blood 73 74 collected into standard EDTA Tubes require cold storage of the blood samples followed by plasma preparation within 6 h. Plasma prepara-7576 tion initiates with a low speed centrifugation of maternal blood for fractionation of the plasma from blood cells. The plasma layer is subse-77 quently removed and centrifuged at a higher speed to pellet any resid-78 79ual debris from the plasma. Plasma processing is cumbersome and thus 80 only performed at collection sites when absolutely necessary due to lo-81 cation or other circumstances. The requirement for immediate process-82 ing to plasma for blood collected in standard EDTA Tubes, and the associated costs of processing at collection sites, would unnecessarily 83 limit the availability of NIPT to a broad population. To overcome these 84 challenges, an ideal blood collection device would enable shipment of 85 whole blood at ambient temperatures (6 °C-37 °C) and extend the 86 timeframe for plasma processing. Such a device would facilitate central-87 ized processing and analysis. Several alternatives exist to EDTA Tubes 88 for collecting blood and preparing plasma for molecular diagnostic 89 tests. Three potential tube types for maintaining fetal fractions can be 90 found: tubes that are designed to create a physical barrier (gel plug or 91 mechanical separator) between cellular and non-cellular blood compo-92nents, tubes providing reagents for keeping maternal blood cells intact 93 and active for a defined period of time, and tubes with cell-preserving 94 95reagents. A tube with cell-preserving reagents to prevent white blood cell degradation (which would release maternal DNA) and inhibit 96 nuclease mediated DNA degradation for up to 14 days at ambient tem-97 perature was recently introduced by Streck and marketed as Streck 98 Cell-Free DNA[™] BCT (Streck BCTs)[7]. 99

100 Barrett and colleagues revealed that the concentrations of "short-fragment" ccffDNA from blood collected Streck BCTs did not 101 change when stored at ambient temperature over a period of 72 h. 102 However, when blood was stored in EDTA Tubes for 72 h, an increase 103 in "long-fragment" (maternal) plasma DNA was evident [8]. Results 104 by Hidestrand et al. indicated that in blood collected into EDTA 105Tubes that were shipped either with or without frozen ice packs 106 had an increased level of total plasma DNA after 72 h. When whole 107 blood in Streck BCTs is shipped without ice packs (72 h) the level of 108 total DNA is unchanged, while when shipped with frozen ice packs, 109110 the level of total DNA is reported to increase [9].

This study was aimed to characterize the ability of the Streck BCTs 111 to maintain ccff DNA concentrations and inhibit nuclease mediated 112 DNA degradation under conditions that are pragmatic for routine 113 use for clinical applications. Our evaluation of the utility of the Streck 114 BCT to preserve the integrity of the fetal DNA fraction focused on four 115 key variables: influence of storage time (up to 14 days), storage tem-116 perature (for 24 h of storage), mechanical stress (ASTM Standard 117 D5276-98) and lot-to-lot consistency (3 lots). 118

Materials, methods and experimental 119

- Blood samples 120
- 121 Blood collection protocols

Patients were selected based on the following inclusion criteria: 1221) Subject must be pregnant carrying a singleton fetus of 10–16 weeks 123of gestational age inclusive. 2) Subject must be 18 years of age or older 124125or an emancipated minor. 3) Subject has consented to have up to 50 mL of whole blood collected at one or more monthly (\geq 25 days) 126scheduled clinic visits. 127

Blood from patients, up to 5×10 mL of peripheral blood, was col-128lected by venipuncture in varying combinations of two types of evac-129uated blood collection tubes, Vacutainers® - K2EDTA Tubes (BD # 130366643, referred to as EDTA Tubes in text; Becton, Dickinson and 131 Company Franklin Lakes, NJ) and Cell-Free DNA Streck™ BCT (Streck 132# 218962, referred to as Streck BCTs in text; Streck Omaha, NE). 133 134 Streck BCTs contain K₃EDTA as anticoagulant.

EDTA Tubes

Blood collected in K₂EDTA Tubes was transported chilled, with re- 136 frigerated ice packs, to a processing center and plasma was isolated 137 within 6 h of blood draw unless otherwise indicated. The first centrifu- 138 gation was conducted at 2500 \times g for 10 min at 4 °C, after which the 139 plasma was carefully removed and transferred to a fresh tube. The sec- 140 ond centrifugation was conducted at $15,500 \times g$ for 10 min at 4 °C. The 141 resultant plasma was collected and stored frozen at \leq -70 °C until 142 analysis. 143

Streck BCTs

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Blood collected in Streck BCTs was shipped at ambient tempera- 145 ture to a processing center and plasma was isolated within 24 h of 146 blood draw, unless otherwise indicated. The first centrifugation was 147 at 1600 \times g for 15 min at room temperature, after which the plasma 148 was carefully removed and transferred to a fresh tube. The second 149 centrifugation was at 2500×g for 10 min at room temperature. The 150 resultant plasma was collected and stored frozen at \leq -70 °C until 151 analysis. 152

DNA extraction

EDTA and Streck BCT plasma samples were thawed and 154 centrifuged at 1600 \times g for 10 min at 4 °C. Four mL of the supernatant 155 was removed and circulating DNA was extracted with the QIAamp® 156 DSP Circulating NA Kit (QIAGEN Cat# 61504, Hilden, Germany). The 157 manufacturer's protocol was followed for EDTA plasma; Streck plas- 158 ma extraction utilized an extended Proteinase K digestion time of 159 60 min. DNA was eluted with 55 µL of Qiagen Buffer AVE (elution 160 buffer) from the QIAamp kit. 161

DNA quantitation

Fetal and total circulating DNA was detected using the FQA, a 163 methylation-sensitive, single nucleotide extension MALDI-TOF MS 164 assay [10]. This assay is capable of determining the gender of the 165 fetus, and of quantifying fetal DNA (regardless of gender). FQA was 166 run as indicated with 10 or 12.5 µL of extracted circulating DNA in 167 quadruplicate PCR and quadruplicate MS reactions (16 reactions 168 total). Data were analyzed as described [10]; total and fetal copies 169 per mL plasma are provided by dedicated scripts, from which the 170 fetal fraction was calculated. For total copies the dynamic range of 171 the FQA assay is between 450 and 7500 [10]. Samples with total 172 DNA copy numbers that were above the maximum dynamic range 173 of the assay were adjusted to 7500 copies before normalization. 174

Study variables

Storage time

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Blood was stored in EDTA Tubes and in Streck BCTs for 1 to 14 days 177 and compared to EDTA or Streck no-storage-control (NSC), respectively. 178 From each donor (20 pregnant female, 10 non-pregnant female, or 10 179 male subjects per time point, respectively) blood was collected into 2 180 EDTA Tubes and 2 Streck BCTs (10 mL per tube). EDTA NSC and Streck 181 NSC were generated by processing blood into plasma within 6 h post 182 blood draw. Blood in remaining EDTA Tubes and Streck BCTs was stored 183 for either 1 day, 2 days, 3 days, 4 days, 7 days, or 14 days, after which 184 plasma was prepared and stored frozen at \leq -70 °C until use. 185

Before extracting ccfDNA, all plasma samples were thawed and 186 centrifuged at 1600 ×g for 10 min to pellet debris. FQA reactions 187 were performed in quadruplicate using 10 µL of extracted DNA [10]. 188 Data were analyzed using either: i) ANOVA for EDTA and Streck NSC 189 for Day 1 to Day 14, and ii) comparing EDTA NSC of a specific subject 190 (e.g. Subject 1 of Day 1) to the matched samples stored in EDTA Tubes 191 and Streck BCTs. 192

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