Comparative Analysis of Liquid Biopsy Blood Collection Tubes

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INTRODUCTION

Liquid biopsy has gained traction as a minimally invasive method to monitor biomarkers associated with malignancy and metastasis. A simple blood draw can reveal key biomarkers such as circulating tumor cells (CTCs) and cell-free DNA (cfDNA). These analytes are present in low quantities and are prone to rapid degradation and contamination. Preservation of CTCs and cfDNA over time is essential for the accuracy of downstream tests. Preanalytical variables that affect cfDNA yield and quality in *ex vivo* blood are incompletely defined and understood. Higher molecular weight (HMW) DNA from leukocytes can contaminate plasma, compromising liquid biopsy assay results by increasing the total amount of extracted DNA while diluting rare cfDNA. cfDNA quality can be assessed using size distribution analysis, where a high proportion of the ~170 bp cfDNA peak and low levels of HMW DNA indicate the presence of high-quality, informative cfDNA. cfDNA yield and quality are known to be sensitive to preanalytical variables including type of blood collection tube, storage time and conditions post-phlebotomy, and centrifugation conditions used to fractionate plasma.

We carried out a range of studies to better understand the interplay between preanalytical variables and cfDNA yield and quality. We compare three commercial liquid biopsy blood collection tubes—the common industry anticoagulant tube (EDTA), Streck's Cell-Free DNA BCT® (Streck), and Biomatrica's LBgard® Blood Tube (LBgard)—with respect to preservation of high-quality cfDNA and CTCs, recovery of these key analytes, and compatibility of these isolated analytes with downstream molecular tests and automated platforms.

METHODS

Blood Samples. Blood from healthy donors and from Stage IV colorectal cancer (CRC) patients was collected in EDTA, Streck, or LBgard tubes. Blood samples were incubated as indicated. At each time-point, plasma was isolated and stored at -80°C or -20°C or cells (CTCs and WBCs) were isolated and stained for flow cytometry.

Plasma DNA Extraction and Analysis. Plasma was fractionated from whole blood using a variety of double-spin protocols based on tube manufacturers' recommendations and published protocols. DNA from plasma was extracted using commercially available kits, including the NEXTprep-Mag™ cfDNA Isolation Kit (Bioo Scientific), either manually or on the chemagic-360™ automation platform (Chemagen). Plasma DNA was quantified by Quant-iT (ThermoFisher), by qPCR using RPS18 primer probes (ThermoFisher), or by Agilent Bioanalyzer software. Size distribution profiles of DNA peaks were characterized using the High Sensitivity DNA Analysis kit and the 2100 Bioanalyzer (Agilent).

Droplet Digital PCR (ddPCR). A DNA fragment bearing the KRASG12D mutation (cfDNA mimic; Horizon) was spiked into blood samples and incubated for up to 14 days at 25°C. Plasma DNA was isolated and analyzed by ddPCR using the KRASG12D Mutation Assay and QX200 ddPCR instrument (BioRad).

Next Generation Sequencing (NGS). For Figure 4, targeted methylation-specific libraries were constructed from extracted DNA by bisulfite conversion, enrichment and amplification. Libraries underwent targeted NGS using Ion Torrent PGM™. For Figure 5, whole-genome libraries were prepared using the NEXTflex cfDNASeq library kit for Illumina sequencing (Bioo Scientific), with 12 cycles of PCR amplification. Libraries were diluted 1:10 and analyzed by Agilent Bioanalyzer.

Cell Recovery. VCaP cells (CTC mimics) were spiked into healthy donor blood and incubated at 25°C for 4 days. EpCAM+ VCaP cells and CD45+ WBCs were quantified by flow cytometry (Novocyte; ACEA). CTC and white blood cell (WBC) recoveries were normalized to Day 0 counts.

RESULTS

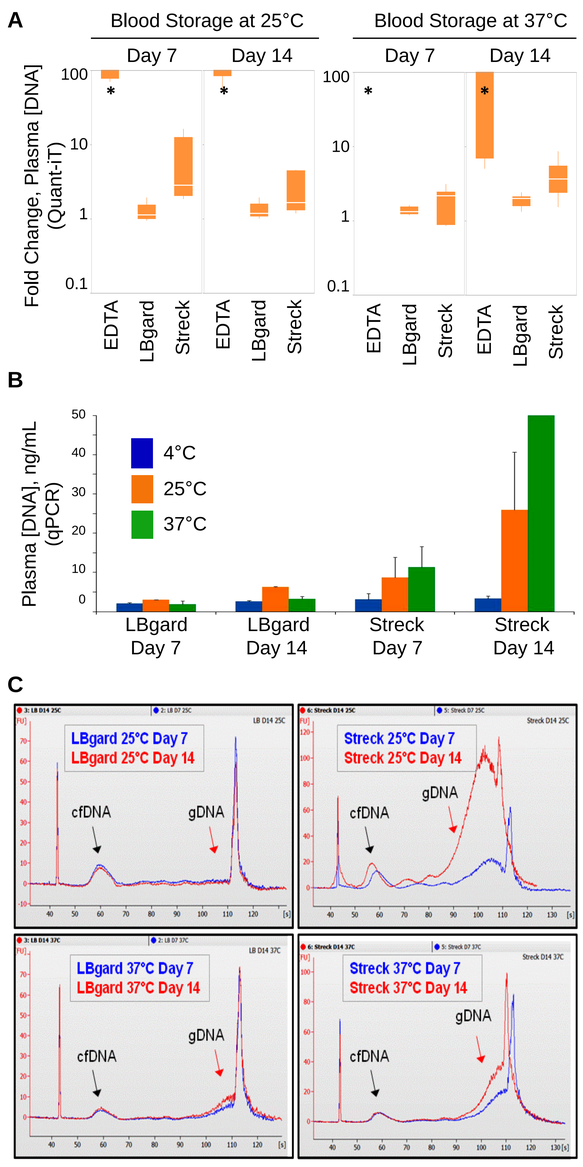
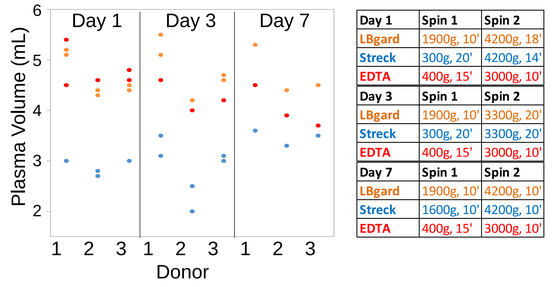
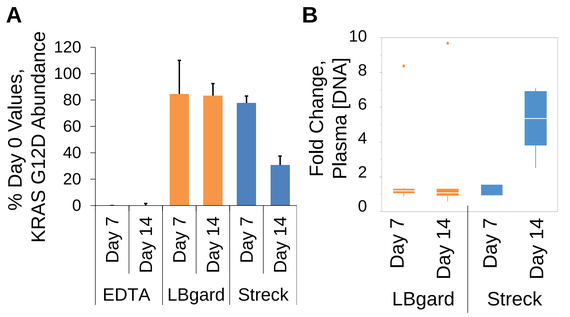


Figure 1. **Plasma DNA from healthy donor blood samples stored at 4°C, 25°C, 37°C, and subjected to temperature excursions. A)** Blood was collected in EDTA, LBgard, or Streck tubes and incubated at 25°C or 37°C for up to 14 days. Plasma DNA was extracted and quantified by Quant-iT. Fold change = Day 7 [DNA] / Day 0 [DNA] or Day 14 [DNA] / Day 0 [DNA]. **B)** Blood was collected in LBgard or Streck tubes, incubated at 4°C, 25°C, 37°C for up to 14 days. Plasma DNA was extracted and quantified by qPCR with RPS18 primers. **C)** Plasma DNA was characterized for cfDNA vs gDNA by Bioanalyzer. **D)** Blood samples were subjected to temperature cycling, representing temperature excursions experienced during shipping/storage. Plasma DNA was analyzed as in A). *asterisks (*) = off-scale values.*



Day 1	Spin 1	Spin 2
LBgard	1900g, 10'	4200g, 18'
Streck	300g, 20'	4200g, 14'
EDTA	400g, 15'	3000g, 10'
Day 3	**Spin 1**	**Spin 2**
LBgard	1900g, 10'	3300g, 20'
Streck	300g, 20'	3300g, 20'
EDTA	400g, 15'	3000g, 10'
Day 7	**Spin 1**	**Spin 2**
LBgard	1900g, 10'	4200g, 10'
Streck	1600g, 10'	4200g, 10'
EDTA	400g, 15'	3000g, 10'

Figure 2. Plasma volume recovery. Blood was collected in EDTA (●), LBgard (●), or Streck (●) tubes and stored for 1, 3, or 7 days at ambient temperature. Tubes were centrifuged according to manufacturer's recommended double spin protocol(s) for each tube type (see tables), and recoverable plasma volume was measured.



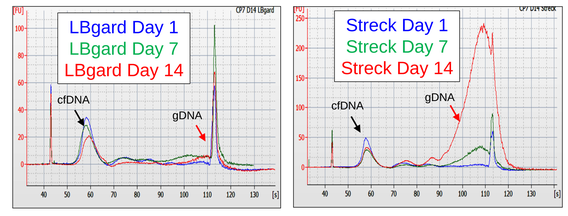


Figure 3. **Rare cancer allele abundance and Stage IV colorectal cancer patient cfDNA stabilization over 14 days at 25°C**. **A)** Healthy blood samples were collected in EDTA, LBgard, or Streck tubes and spiked with a ~170 bp DNA fragment bearing KRASG12D. Blood samples were incubated at 25°C for up to 14 days. DNA was extracted from plasma, and the fractional abundance of KRASG12D was determined by ddPCR. **B)** Blood was collected from Stage IV CRC patients in LBgard or Streck tubes and incubated at 25°C for up to 14 days. Plasma DNA was extracted and quantified by Quant-iT. Fold change = Day 7 [DNA] / Day 1 [DNA] or Day 14 [DNA] / Day 1 [DNA]. **C)** Representative Bioanalyzer traces.

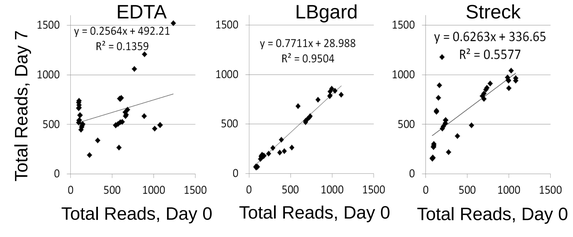


Figure 4. cfDNA stabilization over 7 days for NGS. Total reads (unique reads) for each tube type, comparing Day 0 to Day 7 samples. An R^2 value of 1 and a slope of 1 indicate no change in number of reads over time.

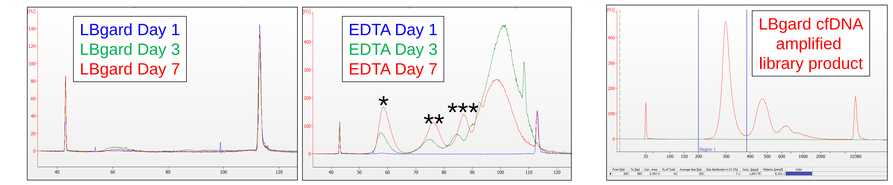


Figure 5. cfDNA profiles from low plasma volume input and representative whole genome libraries. A) Blood was collected from healthy donors in LBgard and EDTA tubes and stored for 1, 3, or 7 days at ambient temperature. Plasma was isolated, and DNA was extracted from a low volume of plasma (0.8 mL) using the NextPrep-Mag™ cfDNA Isolation Kit. Bioanalyzer traces show an increase over time in DNA covering multiple size distributions for samples collected and stored in EDTA tubes but not LBgard tubes. Representative traces for one donor are shown. Note that the mono- (*), di- (**), and tri- (***) nucleosome peaks, as well as the HMW DNA peak, show dramatic increases over time for blood collected in EDTA tubes. **B)** DNA extracted from LBgard tubes was used as input for whole genome library preparation using the NEXTflex cfDNASeq library kit for Illumina sequencing (Bioo Scientific). High yield and quality cfDNA libraries were obtained from low cfDNA inputs (from 0.8 mL plasma) from LBgard tubes. Bioanalyzer quantitation of a representative trace indicates that 62% of library product was derived from the mono-nucleosome peak, with the remaining derived from di- and tri-nucleosomes.

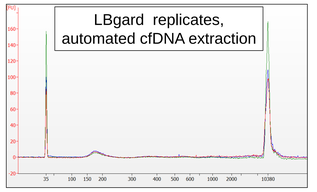


Figure 6. Reproducible mono-nucleosomal cfDNA recovery using automated extraction. Blood was collected in LBgard tubes from several healthy donors. Plasma was fractionated and pooled, and cfDNA was extracted from duplicate 5 mL samples using the NextPrep-Mag™ cfDNA Isolation Kit on the chemagic™ 360 platform. The cfDNA was characterized for mono-nucleosomal cfDNA by Bioanalyzer. Representative Bioanalyzer traces for replicates are shown, which highlight reproducible mono-nucleosomal (~170 bp) cfDNA yield and quality.

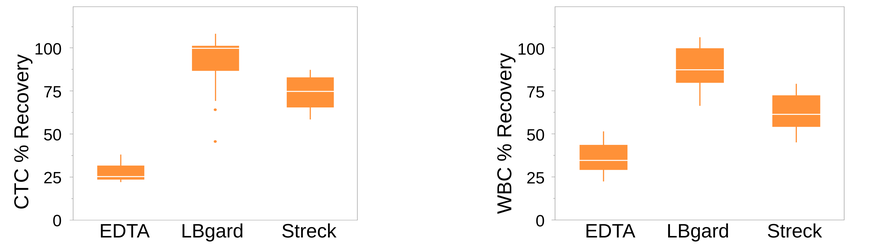


Figure 7. CTC and WBC Recovery from blood samples stored for up to 4 days at 25°C. CTC-spiked healthy blood samples collected in LBgard, Streck, or EDTA tubes were incubated for 4 days at 25°C. CTCs (left) and WBCs (right) were stained and quantified by flow cytometry on Day 0 and Day 4. Day 4 percent recoveries are normalized to Day 0 absolute cell counts.

CONCLUSION

The potential of liquid biopsies to allow non-invasive monitoring of mutations associated with malignancy and metastasis is exciting, but challenges exist in achieving the required sensitivity for detecting rare CTCs and cfDNA variants. Preanalytical variables including type of blood collection tube, blood storage/shipment conditions, time post-blood draw, and plasma recovery have pronounced effects on analyte yield and quality.

Leukocytes in unstabilized blood samples release DNA that dilutes cancer alleles. We show that this contaminating leukocyte content comprises not only HMW DNA as expected from WBC lysis, but also DNA derived from mono-, di-, and tri-nucleosomes. Increased levels of nucleosomal peaks from unstabilized (EDTA tube) blood samples may be generated as *ex vivo* leukocytes gradually succumb to hypoxia-induced apoptosis or, potentially, as HMW gDNA is released from necrotic leukocytes and subsequently cleaved by still-active blood nucleases (References 1-3).

Overall, our results show that LBgard tubes stabilize cfDNA, preserving its yield and quality and preventing contamination from leukocyte DNA for up to 14 days at 25°C and at 37°C. Significantly more gDNA is released in Streck and EDTA tubes than in LBgard tubes in both healthy donor blood and CRC patient blood by Day 14 post-draw. LBgard tubes provide the highest recoverable plasma volume, and the fractional abundance of a circulating tumor DNA mimic (spiked mutant DNA) is maintained in LBgard tubes but not in Streck or EDTA tubes. LBgard tubes are compatible with downstream automated extraction and library prep, as demonstrated using the chemagic-360 platform, and yield high-quality whole genome sequencing libraries even from low plasma inputs. Finally, cell recovery (CTC mimics and WBCs) is significantly higher after 4 days at 25°C from blood stored in LBgard tubes than in Streck tubes.

References

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