A Comprehensive Assessment of the Impact of Preanalytical Variables on Cell-Free DNA and Circulating Tumor Cells in Blood

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INTRODUCTION

Liquid biopsy continues to gain traction as a minimally invasive method to monitor biomarkers associated with malignancy and metastasis. A simple blood draw can reveal key biomarkers such as cell-free DNA (cfDNA, including circulating tumor DNA from cancer patients) and circulating tumor cells (CTCs). However, these analytes are present in low quantities and are prone to rapid degradation and contamination, which presents challenges for the detection of rare CTCs and cfDNA targets. Higher molecular weight genomic DNA from poorly stabilized leukocytes can contaminate plasma, compromising liquid biopsy assay results by increasing the total amount of extracted DNA while diluting rare cfDNA targets. Preservation of CTCs and cfDNA over time is thus essential for the accuracy of downstream tests, and preanalytical variables that impact plasma volume, cfDNA yield and quality, and cell recovery in ex *vivo* blood must be understood. Here, we identified several preanalytical variables, including type of blood collection tube, shipping conditions, and storage time and temperature. We then carried out a series of studies to better understand the interplay between these preanalytical variables and plasma recovery, cell recovery, and cfDNA preservation in healthy donor blood, contrived samples, and cancer patient samples.

We found that choice of blood collection tube, in particular, has a significant impact on preservation of plasma volume, CTCs, and cfDNA, particularly under "real-world" stress conditions including temperature excursions, package shipping, and storage time post-draw. The work presented here demonstrates the importance of assessing preanalytical variables for the preservation of low-abundance biomarkers in whole blood without compromising sample integrity. This will ultimately help researchers make informed decisions on how best to stabilize their analytes of interest without introducing unintended variables due to blood tube selection, shipping and storage conditions, and plasma isolation conditions.

METHODS

Blood Samples. Blood from healthy donors and Stage IV colorectal cancer patients was collected in K_2EDTA tubes, LBgard® Blood Tubes, and other commercially available tubes that preserve cfDNA. Blood samples were subjected to temperature and shipping conditions as indicated. At each time-point, plasma was fractionated from whole blood per manufacturers' recommended protocols and stored at -80°C, or cells (CTCs and WBCs) were isolated and stained for flow cytometry.

Plasma DNA Extraction and Analysis. Plasma DNA was extracted using the QIAamp Circulating Nucleic Acid Kit or as described previously (Allawi HT et. al., 2017 AACR poster #712). Plasma DNA was quantified and characterized using the ThermoFisher Quant-iT dsDNA Assay Kit (high sensitivity) and β-actin QuARTS® Assay (Exact Sciences Corp., Madison WI). Size distribution profiles of DNA peaks were characterized using the High Sensitivity DNA Analysis kit and the Agilent 2100 Bioanalyzer.

Droplet Digital PCR. A DNA fragment bearing the Horizon KRAS G12D mutation (cfDNA mimic) 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 KRAS G12D Mutation Assay and BioRad QX200 ddPCR instrument.

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

RESULTS

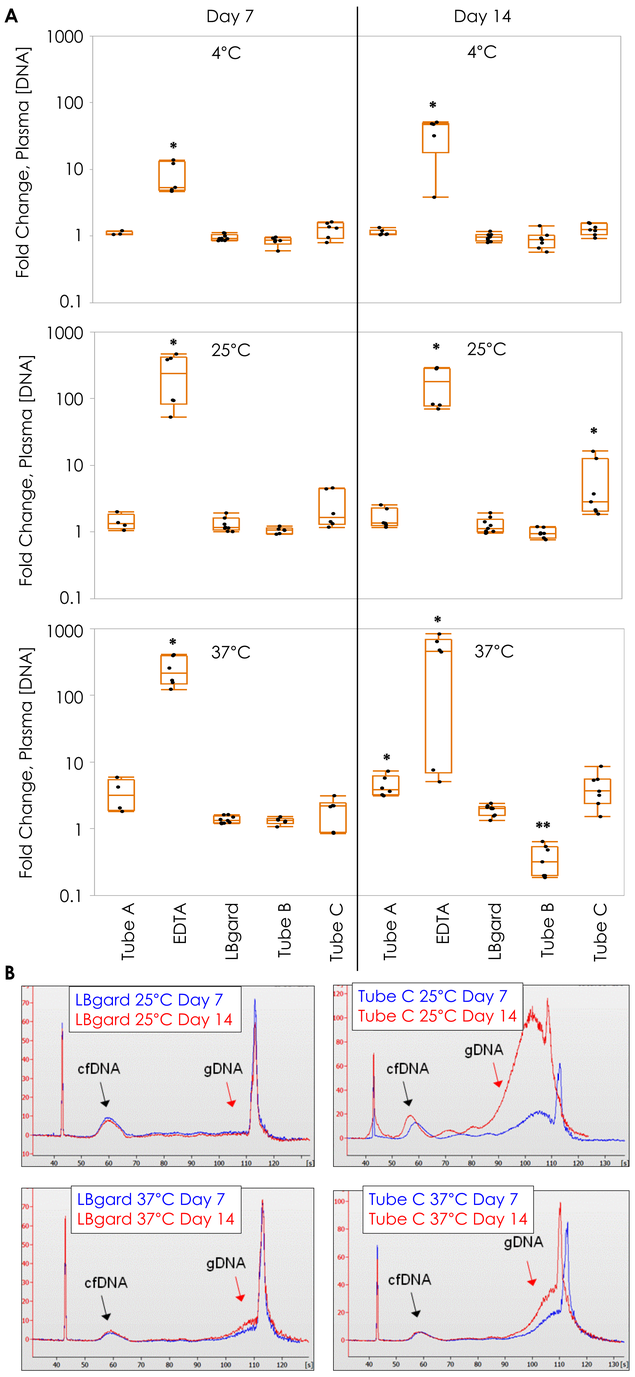

Figure 1. cfDNA stabilization in healthy donor blood collected in commercially available tubes and stored at 4°C, 25°C, and 37°C. A) Change in plasma DNA concentrations from Day 0 post-draw to Day 7 or Day 14 post-draw. A fold change > 1 indicates genomic DNA contamination from cell lysis, and a fold change < 1 indicates loss of cfDNA due to degradation. EDTA samples had significantly higher fold changes at all temperatures. Tube C samples had significantly higher fold changes when stored at 25°C for 14 days. Tube A samples had significantly higher fold changes when stored at 37°C for 14 days, while Tube B samples had significantly lower fold changes at 37°C. for 14 days (* denotes $p < 0.05$ and ** denotes $p < 0.01$, Steel-Dwass non-parametric comparison for all pairs). **B)** Representative Bioanalyzer traces depicting cfDNA and contaminating gDNA peaks. Y axis values are relative fluorescence units.

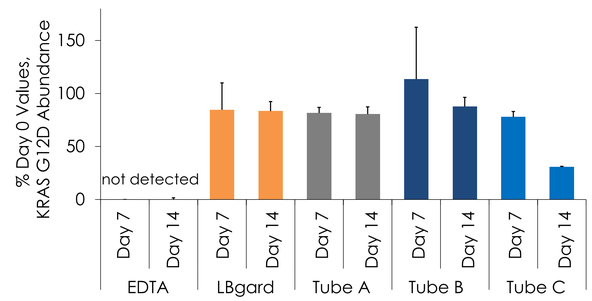

Figure 2. Stabilization of cancer allele spiked into healthy donor blood, as quantified by ddPCR. Contrived samples comprising healthy donor blood spiked with a 170 bp fragment bearing the cancer marker KRAS G12D were incubated for 7 or 14 days at 25°C. KRAS G12D recovery is reported as % of Day 0 copy number for KRAS G12D.

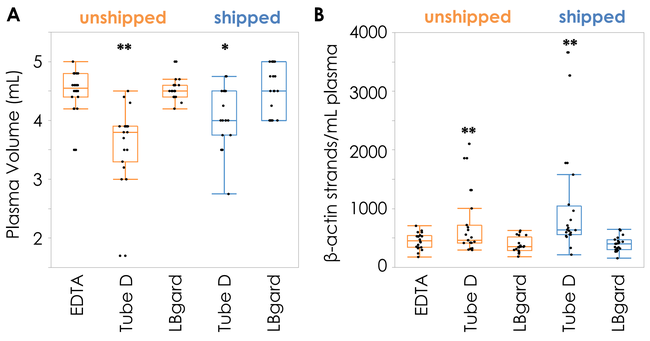

Figure 3. cfDNA stabilization in blood collected from 20 healthy donors in commercially available tubes and subjected to overnight shipping stress. A) Recovered plasma volumes for samples shipped overnight from Madison, WI to Mayo Clinic, Rochester, MN versus unshipped controls. All samples were 6 days post-draw except EDTA samples, which were processed within 4 hours post-draw. Plasma volumes were significantly less for Tube D samples, shipped and unshipped, than for LBgard samples (* denotes $p < 0.05$ and ** denotes $p < 0.01$, Steel-Dwass non-parametric comparison for all pairs). **B)** QuARTS® Assay β-Actin quantification results of extracted plasma DNA, where an increase in signal corresponds to an increase in genomic DNA contamination from cell lysis. Plasma β-Actin was significantly higher in Tube D samples than in unshipped EDTA samples and both shipped and unshipped LBgard samples (** denotes $p < 0.01$, Steel-Dwass non-parametric comparison for all pairs).

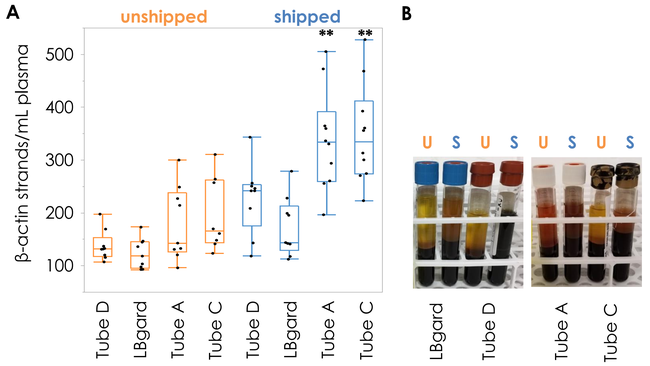

Figure 4. cfDNA stabilization in blood from 19 healthy donors in commercially available tubes and subjected to 6 day shipping stress. A) β-Actin QuARTS® Assay quantification of extracted plasma DNA (processed 6 days post-draw), where an increase in signal corresponds to an increase in genomic DNA contamination from cell lysis. Tube A and Tube C shipped samples had significantly more plasma β-Actin than unshipped samples of the same tube type and shipped LBgard samples (** denotes $p < 0.01$, Steel-Dwass non-parametric comparison for all pairs). **B)** Representative images of unshipped (U) and shipped (S) tubes post-centrifugation showing hemolysis and significant loss of plasma in select tubes. LBgard tubes exhibited no plasma loss and minimal hemolysis.

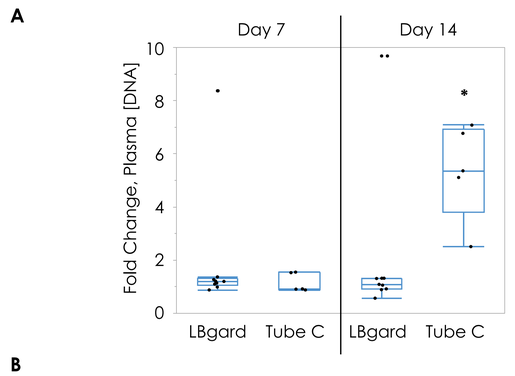

Figure 5. cfDNA stabilization in blood from patients subjected to overnight shipping stress and 25°C storage. A) Change in plasma DNA concentrations from Day 1 post-draw to Day 7 or Day 14 post-draw. A fold change > 1 indicates an increase in genomic DNA contamination from cell lysis, and a fold change < 1 indicates loss of cfDNA due to degradation. The fold change is significantly higher for Tube C samples on day 14 than for LBgard samples (* denotes $p < 0.05$, Wilcoxon signed-rank test). **B)** Representative Bioanalyzer traces depicting cfDNA and contaminating gDNA peaks.

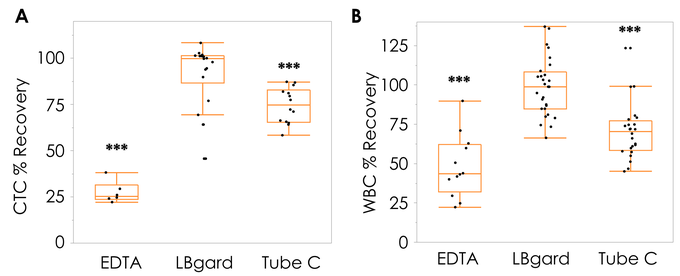

Figure 6. Circulating tumor cell and white blood cell recovery from blood collected in commercially available tubes and stored for up to 4 days at 25°C. A) Recovery of CTCs from spiked healthy donor blood collected in EDTA tubes, LBgard tubes, or Tube C after storage for 4 days at 25°C. **B)** Recovery of WBCs from the same healthy donor blood samples after storage for 4 days at 25°C. CTC and WBC % recoveries are significantly lower for EDTA samples and Tube C samples than for LBgard samples (*** denotes $p < 0.005$, Steel-Dwass non-parametric comparison for all pairs).

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 targets. Through a series of experiments using "real-world" blood tube handling and processing for downstream measurements of low-abundance biomarkers, we show that key preanalytical variables including type of blood collection tube, blood storage/shipment conditions, and time post-blood draw have pronounced effects on successful analyte measurements. Our results show that LBgard tubes stabilize cfDNA, CTCs, and WBCs, as well as preserve plasma volume, withstanding prolonged "real-world" environmental stresses. We conclude that the LBgard tube is superior to the other commercially available tubes tested in these studies for the preservation or cells and cell-free DNA in blood.

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