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Unlocking Multi-Analyte Plasma Testing with Nucleic Acid Plus BCT™

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NUCLEIC ACID PLUS BCT **Nucleic Acid Plus BCT is for Research Use Only (RUO). Not for use in diagnostic procedures.**

Introduction

Traditional liquid biopsies have historically focused on measuring a single analyte, such as circulating tumor DNA (ctDNA) or a specific protein marker in blood. While these approaches have had a transformative impact on precision medicine, with applications extending beyond oncology to include prenatal testing, transplantation medicine, infectious disease, inflammatory and autoimmune disorders, and neurodegenerative diseases [1-3], single-analyte strategies provide a limited representation of complex disease biology. As a result, there has been a shift in focus toward multi-analyte liquid biopsy approaches, in which multiple classes of biomarkers are assessed simultaneously to provide a more holistic readout of disease biology.

Although the sample input – a simple blood draw – appears straightforward, multi-analyte testing introduces substantial challenges related to pre-analytical variables including sample collection, handling, processing, and storage [4, 5]. For each analyte of interest, including cell-free DNA (cfDNA), cell-free RNA (cfRNA), plasma proteins, and Extracellular Vesicles (EVs), the measured signal is profoundly influenced by the relative contributions of different blood cellular sources (**Table 1**) [6-9]. Conventional blood collection tubes like EDTA can effectively prevent coagulation but are not designed to stabilize blood cells or preserve multiple analytes under conditions of delayed processing or other pre-analytical stressors; as a result, they are more prone to generate artifacts when sample handling is not strictly controlled in real world applications.

Here, we demonstrate the use of Nucleic Acid Plus BCT (formerly known as Protein Plus BCT) as a specialized blood collection tube that maintains plasma integrity and minimizes pre-analytical variables for multi-analyte analysis.

Table 1. Blood cell contribution to interference of measured cfDNA, cfRNA, protein or EV signal during delayed processing or as a result of other pre-analytical stressors.

	cfDNA	cfRNA	Protein	EVs
White Blood Cells	Primary	Significant	Significant	Limited
Red Blood Cells	None	Significant	Significant	Significant
Platelets	Limited	Limited	Significant	Limited

Results

NUCLEIC ACID PLUS BCT MAINTAINS PLASMA INTEGRITY DURING AMBIENT TEMPERATURE WHOLE BLOOD STORAGE

Hemolysis and platelet activation are key *ex vivo* changes in blood that can compromise plasma sample integrity for downstream multi-analyte analysis. In contrast to blood drawn into EDTA, whole blood collected into Nucleic Acid Plus BCT showed no visible hemolysis at the time of blood draw or after 7 days of ambient temperature whole blood storage (**Figure 1A-B**). Blood collected into Nucleic Acid Plus BCT displayed no loss in plasma volume recovery, a critical parameter for multi-analyte applications, after 7 days of whole blood storage.

Conversely, blood collected into EDTA and stored for 7 days exhibited significant plasma volume loss, retaining only 49–81% of draw-time plasma volume depending on the donor (**Figure 1C**). Flow cytometry analysis demonstrated minimal *ex vivo* platelet activation in blood collected into Nucleic Acid Plus BCT, whereas blood collected into EDTA showed substantial platelet activation with pronounced donor-to-donor variability, a characteristic verified using Scanning Electron Microscopy (SEM) (**Figure 1D-E**).

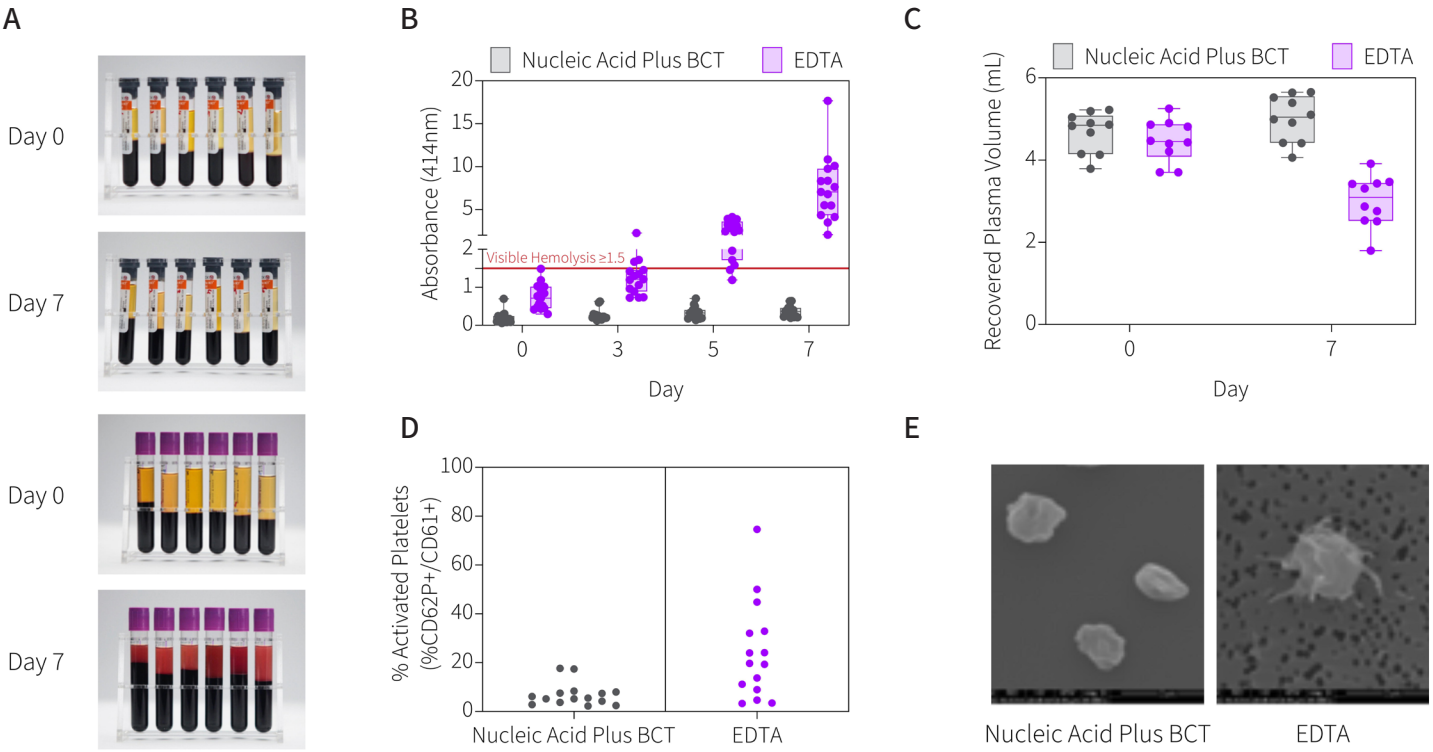

Figure 1. (**A**) Post-spin images of blood collected into Nucleic Acid Plus BCT or EDTA from matching donors at draw (day 0) and 7 days of ambient temperature whole blood storage (n=6). (**B**) Absorbance at 414 nm of isolated plasma (n=15). (**C**) Volume of plasma recovered from blood drawn into two 5 mL Nucleic Acid Plus BCTs or one 10 mL EDTA tube at draw (day 0) or after 7 days of ambient temperature whole blood storage (n=10). (**D**) Draw-time semiquantitative platelet activation analysis of blood collected into Nucleic Acid Plus BCT and EDTA (n=15). (**E**) Representative SEM images of platelets in blood collected into Nucleic Acid Plus BCT or EDTA from the same donor at draw time. (day 0).

NUCLEIC ACID PLUS BCT MAINTAINS CFDNA INTEGRITY AND DRAW-TIME CONCENTRATION DURING AMBIENT TEMPERATURE WHOLE BLOOD STORAGE

To evaluate whether Nucleic Acid Plus BCT stabilizes cfDNA concentrations during ambient temperature whole blood storage, DNA was extracted from plasma isolated from blood collected into Nucleic Acid Plus BCT or EDTA at days 0, 2, and 7 post collection. At draw time (day 0), cfDNA concentration, percentage of cfDNA, and PCR-amplified β-actin copy numbers were comparable between blood collected into Nucleic Acid Plus BCT and EDTA. During blood storage, cfDNA levels in blood collected into Nucleic Acid Plus BCT remained stable, with minimal change through day 7 In contrast, blood collected into EDTA showed a pronounced increase in both cfDNA and β-actin copy numbers, consistent with progressive cellular lysis and genomic DNA (gDNA) release into plasma, which lowers the percentage of cfDNA in the sample (**Figure 2A-C**). Fragment size profiling confirmed preservation of the characteristic nucleosomal cfDNA fragment pattern in blood collected into Nucleic Acid Plus BCT at day 7, while blood collected into EDTA displayed a broader size distribution indicative of gDNA contamination (**Figure 2D**). Together, these data demonstrate that Nucleic Acid Plus BCT maintains the draw-time concentration and integrity of plasma cfDNA for up to 7 days during ambient temperature whole blood storage.

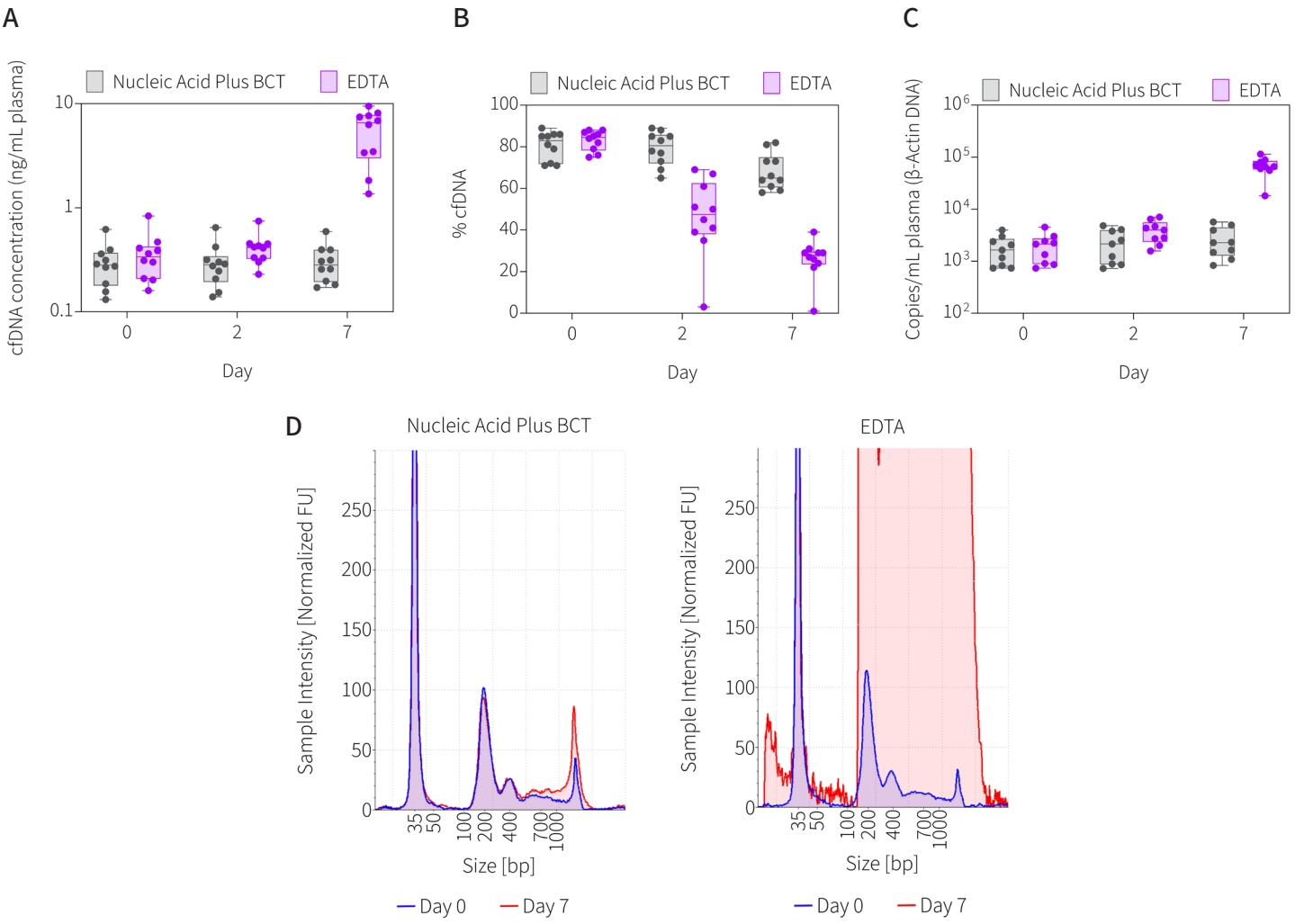

Figure 2. (**A, B**) Cell-free DNA ScreenTape analysis of cfDNA concentration (**A**) and % cfDNA (**B**) in blood collected into Nucleic Acid Plus BCT or EDTA at draw and after 2 and 7 days of ambient temperature whole blood storage (n=10). (**C**) dPCR quantification of target cfDNA in blood collected into Nucleic Acid Plus BCT or EDTA at draw and after 2 and 7 days of ambient temperature whole blood storage (n=10). (**D**) Fragment size profiling (ScreenTape) of blood collected into Nucleic Acid Plus BCT and EDTA at draw and after 7 days of ambient temperature whole blood storage (representative donor shown).

NUCLEIC ACID PLUS BCT MAINTAINS CFRNA INTEGRITY AND DRAW-TIME CONCENTRATION DURING AMBIENT TEMPERATURE WHOLE BLOOD STORAGE

To evaluate whether Nucleic Acid Plus BCT stabilizes cfRNA concentrations during ambient temperature whole blood storage, we extracted cfRNA from plasma isolated from blood collected into Nucleic Acid Plus BCT or EDTA at draw and after 7 days of ambient temperature blood storage and analyzed using automated electrophoresis. Blood collected into Nucleic Acid Plus BCT showed limited *ex vivo* changes in the cfRNA size profile after 7 days of ambient temperature whole blood storage compared to those collected into EDTA (**Figure 3A**). Analysis of specific transcript levels were assessed using UBB (ubiquitin B) and HBA (hemoglobin alpha) as markers of cellular RNA contamination from blood cell lysis, demonstrated transcripts remain stable in blood collected into Nucleic Acid Plus BCT during ambient temperature whole blood storage (**Figure 3 B-C**). Conversely, blood collected into EDTA tubes exhibited a dramatic increase in both transcripts (**Figure 3B-C**). These data illustrate the preservation of cfRNA concentration for up to 7 days of ambient temperature whole blood storage when using Nucleic Acid Plus BCT.

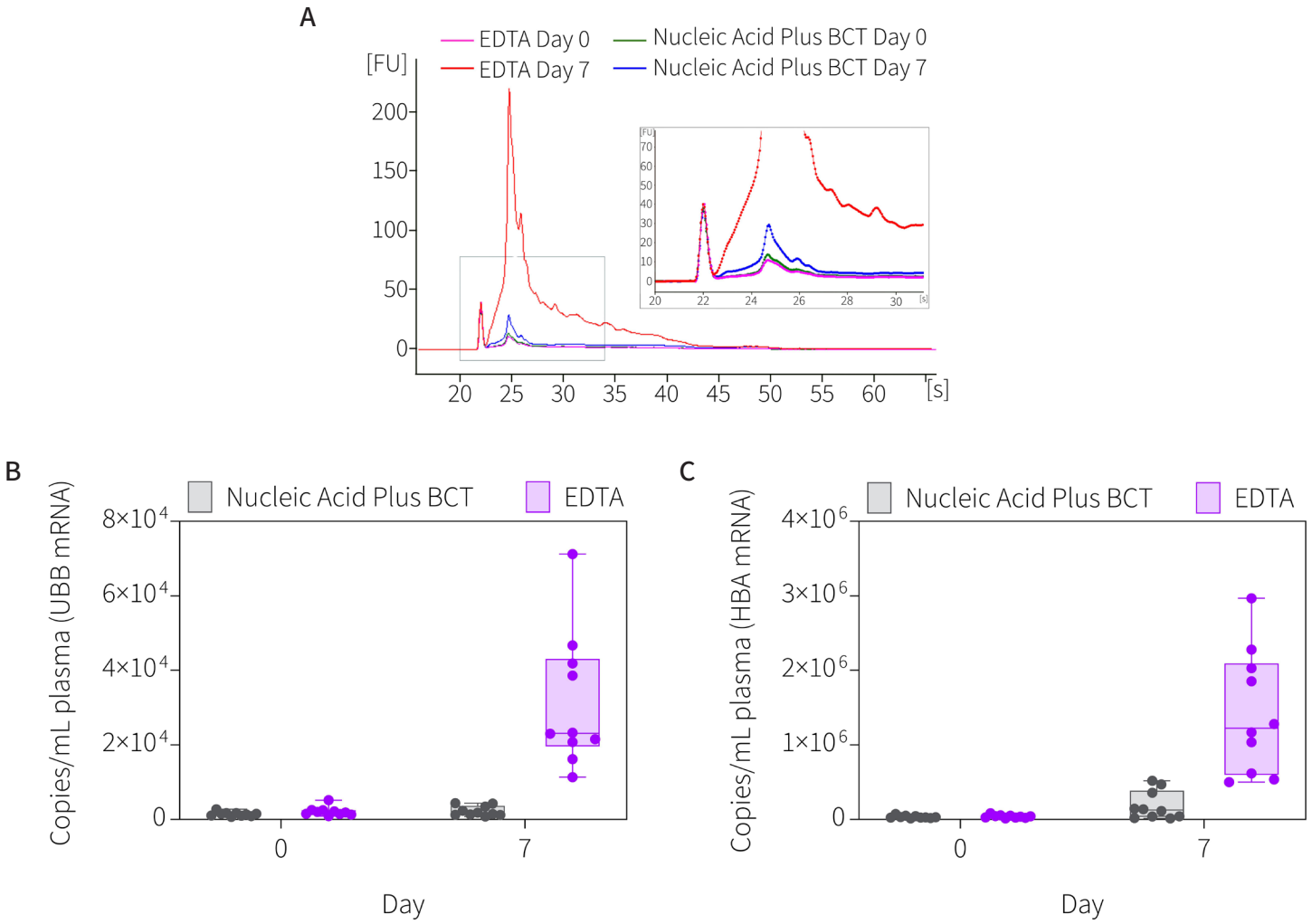

Figure 3. (**A**) Electropherogram overlay of cfRNA extracted from blood collected into Nucleic Acid Plus BCT and EDTA at draw time and after 7 days of ambient temperature whole blood storage (representative donor). (**B, C**) dPCR quantification of UBB (**B**) and HBA (**C**) in plasma isolated from blood collected into Nucleic Acid Plus BCT and EDTA at draw and after 7 days of ambient temperature whole blood storage (n=10).

NUCLEIC ACID PLUS BCT MAINTAINS DRAW-TIME PLASMA PROTEOME DURING AMBIENT TEMPERATURE WHOLE BLOOD STORAGE

To evaluate whether Nucleic Acid Plus BCT maintains the plasma proteome and draw-time concentrations of circulating proteins during ambient temperature whole blood storage, concentrations of plasma protein biomarkers were measured with multiple proteomic platforms, including Ella™ Simple Plex and Luminex® xMAP assays, Olink® PEA technology and mass spectrometry-based analysis. Plasma isolated from blood collected into Nucleic Acid Plus BCT showed stable concentrations of a broad panel of plasma protein biomarkers for up to five days of ambient temperature whole blood storage, while plasma isolated from blood collected into EDTA displayed marked increases in concentrations over time (**Figure 4A-C**). Deep proteome profiling using mass spectrometry coupled with the Seer Proteograph XT™ protein enrichment platform also demonstrated that the number of identified proteins in blood collected into Nucleic Acid Plus BCT remained similar during ambient temperature whole blood storage, but increased significantly in blood collected and stored in EDTA due to plasma contamination from degrading blood cells (**Figure 4D**).

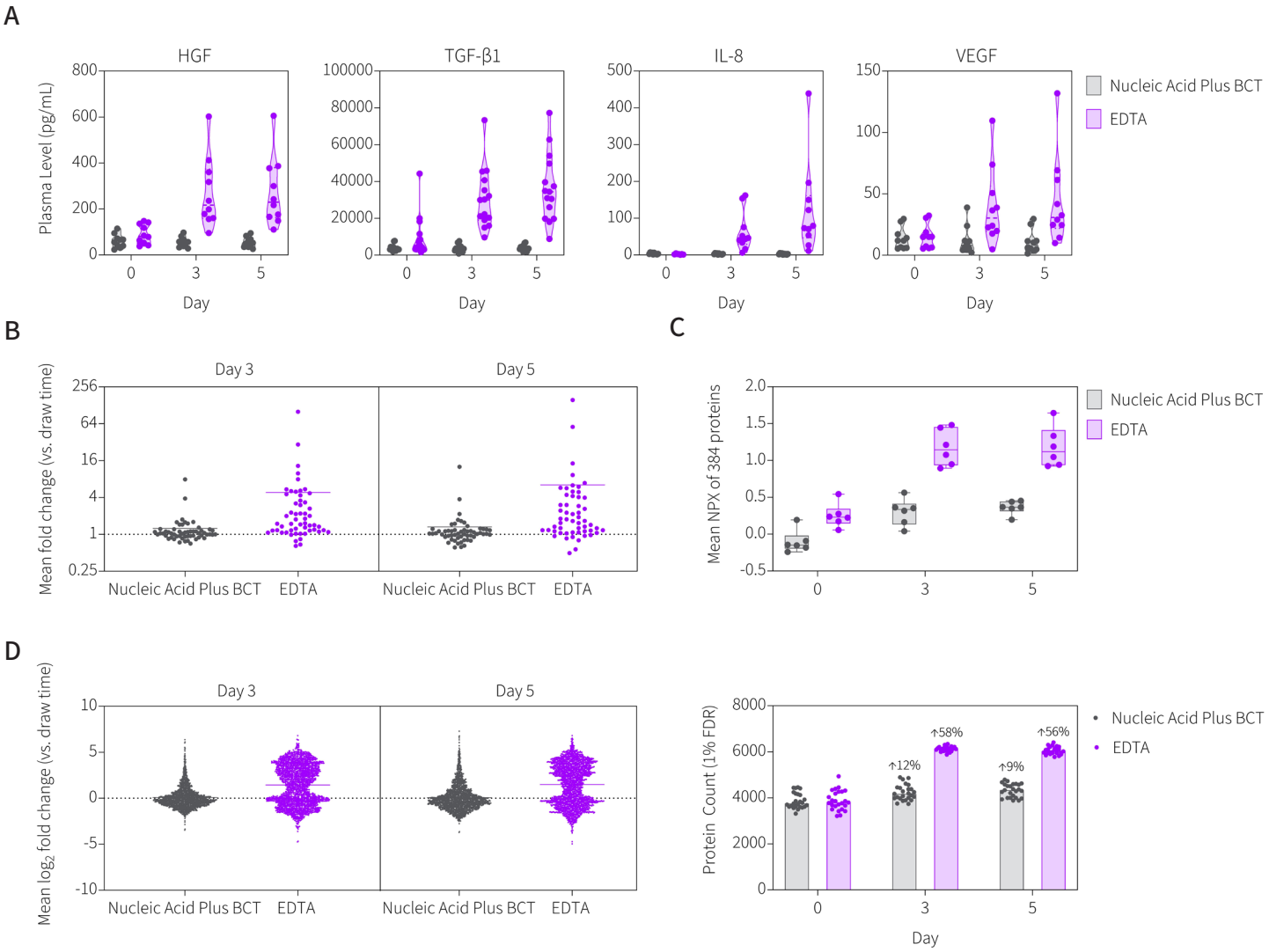

Figure 4. (**A**) Plasma protein levels in blood collected into Nucleic Acid Plus BCT and EDTA at collection or after up to 5 days of ambient temperature whole blood storage (representative biomarkers shown; n=10 or 15). (**B**) Mean protein fold change relative to draw-time abundance for 55 plasma protein markers of interest in blood collected into Nucleic Acid Plus BCT and EDTA after 5 days of ambient temperature whole blood storage (n=10). (**C**) Mean Normalized Protein eXpression (NPX) of 384 proteins from Olink Explore 384 Cardiometabolic panel in plasma isolated from blood drawn into Nucleic Acid Plus BCT and EDTA at draw and after 3 and 5 days of ambient temperature whole blood storage (donor-matched samples compared, n=6). (**D**) Mean log2 fold change in abundance of quantifiable proteins and protein identification count relative to draw time in blood collected into Nucleic Acid Plus BCT and EDTA after 3 and 5 days of ambient temperature whole blood storage (Mass spectrometry analysis paired with nanoparticle-based protein enrichment, n=5 x 5 technical replicates).

NUCLEIC ACID PLUS BCT MAINTAINS DRAW-TIME EV CONCENTRATION AND EV-RNA SIGNAL INTEGRITY DURING AMBIENT TEMPERATURE WHOLE BLOOD STORAGE

To asses EV levels in whole blood collected into Nucleic Acid Plus BCT during ambient temperature whole blood storage, EV particle concentration and EV-associated RNA targets (miR-103a-3p and let-7a-5p (miRNA) and HBA and UBB (mRNA)) were analyzed. EV particle concentration in plasma was quantified using an EV ELISA assay. For blood collected into EDTA, EV concentrations increased markedly following blood storage (**Figure 5A**). Conversely, blood collected into Nucleic Acid Plus BCT exhibited minimal changes during ambient temperature whole blood storage for up to 5 days and showed reduced donor-to-donor variability compared to EDTA (**Figure 5A**). For RNA analysis, EVs were enriched from plasma by targeting tetraspanin-positive particles (CD9, CD63 and CD81), prior to RNA extraction and analysis. Blood collected into Nucleic Acid Plus BCT demonstrated minimal changes in EV-RNA levels during ambient temperature whole blood storage, whereas blood collected into EDTA showed pronounced increases in RNA targets, presumably due to blood cell-derived EV release (**Figure 5B**). These results indicate that Nucleic Acid Plus BCT preserves both EV abundance and the integrity of downstream RNA signals.

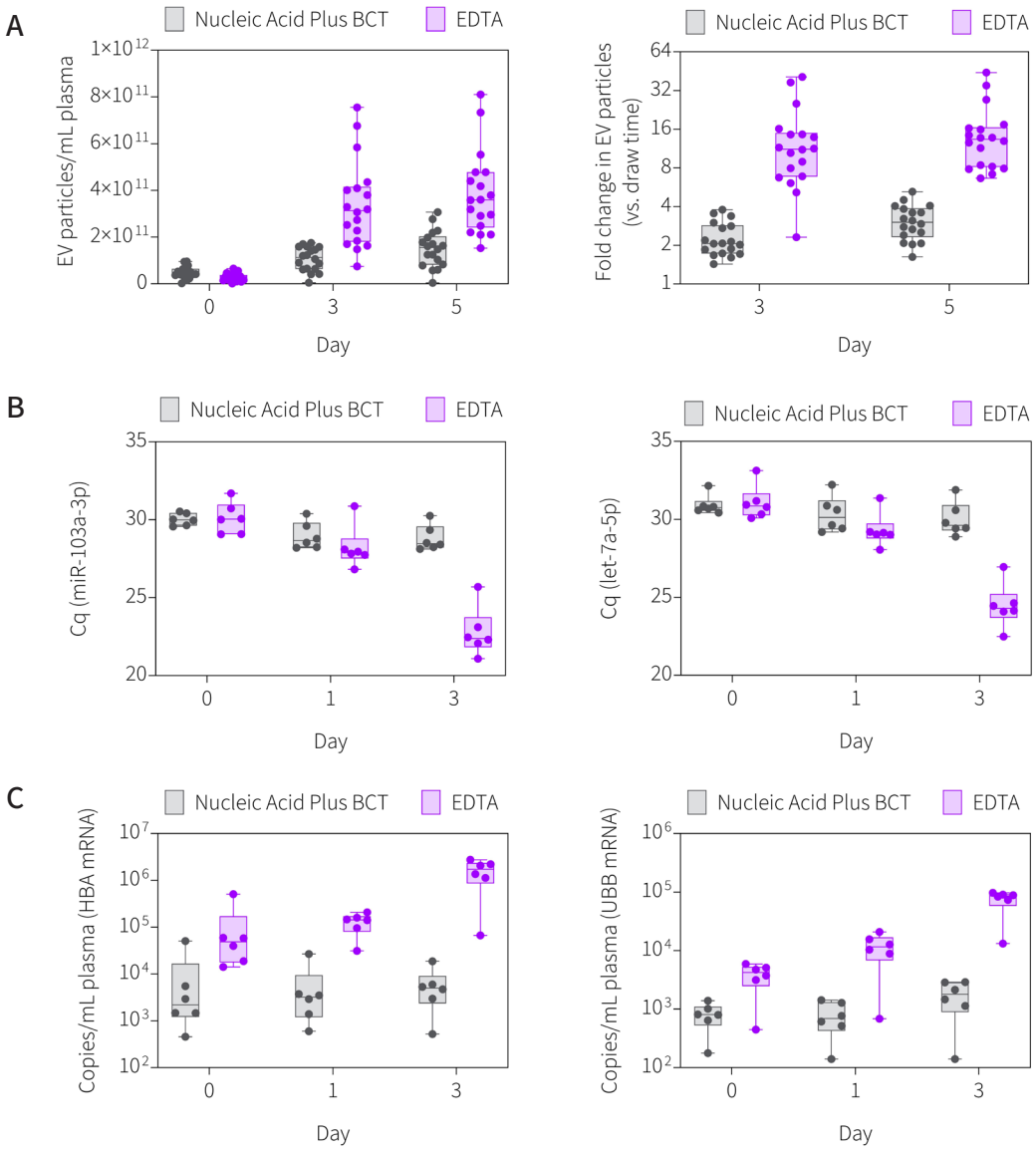

Figure 5. (**A**) Change in plasma EV concentration in blood collected into Nucleic Acid Plus BCT and EDTA after 3 or 5 days of ambient temperature whole blood storage relative to draw time (n=18). (**B, C**) Concentration of EV-associated miRNAs (miR-103a-3p, let-7a-5p) (**B**) and mRNA markers (HBA, UBB) (**C**) in plasma isolated from Nucleic Acid Plus BCT and EDTA at draw and after 1 or 3 days of ambient temperature whole blood storage (n=6).

Conclusion

Here, we demonstrate that Nucleic Acid Plus BCT effectively maintains draw-time plasma levels of cfDNA, cfRNA, EV and circulating proteins during extended whole blood storage at ambient temperature by limiting *ex vivo* platelet activation, hemolysis, and blood cell degradation. As the future of liquid biopsy increasingly depends on integrated, multianalyte profiling approaches to advance in multiple directions (longitudinal disease monitoring, early detection, and integrated omics analysis), pre-analytical variability introduced during blood collection, storage, and processing remains a fundamental barrier to reliable data interpretation. Nucleic Acid Plus BCT ensures blood sample integrity, minimizes pre-analytical variables and reduces confounding artifacts, supporting robust downstream workflows for discovery, translational research, and clinical assay development.

Nucleic Acid Plus BCT is For Research Use Only. Not for use in diagnostic procedures. Nucleic Acid Plus BCT should only be used for research or the development of new assays.

Methods

BLOOD COLLECTION

Blood from self-proclaimed healthy donors was drawn into Nucleic Acid Plus BCT and EDTA tubes. All whole blood samples were processed immediately (within 4 hours after draw time, day 0) or stored at ambient temperature for up to 7 days depending on analyte. Plasma was isolated using a double-spin protocol (1800 ×g for 15 min and 2800 ×g for 15 min, both at room temperature per IFU) and frozen at -80 °C until use.

PLATELET ACTIVATION ANALYSIS

Platelet activation was assessed by (i) flow cytometry (BD FACSCanto® II), using whole blood samples stained with a cocktail of antibodies: CD61-PerCP (a marker for platelets, BD® Biosciences) and CD62P-PE (a marker for activated platelets, BioLegend®). Acquisition and analysis were performed by scatter gating (forward scatter [FSC] and side scatter [SSC]) followed by fluorescent gating. Activated platelets (%) were quantified by the CD61+CD62P+ platelet population of all gated platelets (FlowJo v10.7.2), and (ii) SEM images of platelet-rich plasma, taken by the University of Nebraska Medical Center Electron Microscopy Core Facility.

CELL-FREE NUCLEIC ACID EXTRACTION AND ANALYSIS

Frozen plasma was thawed and total plasma nucleic acid extracted using the QIAamp Circulating Nucleic Acid Kit (Qiagen) following the manufacturer's miRNA protocol. Eluted DNA was assayed using the cfDNA ScreenTape Assay (Agilent) and QIAcuity dPCR for the housekeeping gene β-actin (QIAGEN). A fraction of the eluate was reversed transcribed using the iScript™ gDNA Clear cDNA Synthesis Kit (Bio-Rad) followed by dPCR to quantify both Ubiquitin B and red blood cell-specific Hemoglobin A1/2 transcripts. Bioanalyzer High Sensitivity RNA 6000 Pico assay (Agilent) was used to characterize size profile of cfRNA following an RNA cleanup and concentration step.

PLASMA PROTEIN ANALYSIS

Plasma levels of protein biomarkers were measured by (i) Ella™ Simple Plex™ Assays (Bio-Techne®), (ii) Luminex® xMAP® Technology Assays (Bio-Techne, samples tested by LuminexPLORE Lab (Diasorin, S.p.A.)), (iii) Olink® Proximity Extension Assay (Olink® Explore 384 Cardiometabolic panel, samples tested by Vanderbilt University Medical Center High-Throughput Biomarker Core Lab), following manufacturers' dilution and processing recommendations, (iv) proteome enrichment via Seer Proteograph XT™ workflow followed by data-independent acquisition (DIA) analysis on Orbitrap Astral™ mass spectrometer (Thermo Scientific).

EV AND EV-RNA ANALYSIS

EV particle concentration was measured using the Atlas Human EV Lumi ELISA Kit (Everest Biolabs). Plasma samples were diluted at 1:40 ratio to fall within the linear range of the assay. Results were reported as EV particles/mL plasma by interpolating luminescence signals against a serial dilution of the manufacturer-provided EV standard. EV-RNA analysis was conducted following EV isolation using EasySep™ Human EV Positive Selection Kits (STEMCELL Technologies) targeting tetraspanins CD9, CD63, and CD81. RNA was then extracted from the enriched EVs using the Maxwell® RSC miRNA Plasma and Serum Kit (Promega Corporation). mRNA targets (HBA and UBB) were quantified by droplet digital PCR (ddPCR) using iScript reverse transcriptase, ddPCR Supermix (Bio-Rad Laboratories), and TaqMan Gene Expression Assays (ThermoFisher Scientific). miRNA targets (miR-103a-3p and let-7a-5p) were quantified by qRT-PCR using the miRCURY® LNA® RT Kit for reverse transcription and miRCURY® LNA® SYBR™ Green PCR Kit with miRCURY LNA miRNA PCR Assays (QIAGEN). Results were reported as copies/mL plasma (mRNA) or quantification cycle (Cq) values (miRNA).

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