Stabilizing blood-derived extracellular vesicles: Evaluation of Nucleic Acid Plus BCT™ for EV and RNA integrity

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Introduction

Extracellular vesicles (EVs) present in blood carry biomarkers potentially implicated in clinical diagnosis, prognosis, and disease monitoring. However, blood is a complex biological matrix that undergoes substantial ex vivo changes after collection. Delayed blood processing can induce platelet activation, hemolysis, and cellular breakdown, resulting in time-dependent increases in blood cell–associated EVs and contamination of EV-associated RNA signals. These preanalytical artifacts represent a major barrier to reproducibility in blood-based EV studies. In this study, we investigated the impact of blood collection tube chemistry on EV population stability and EV-associated RNA integrity during delayed processing, with a focus on Nucleic Acid Plus BCT (formerly known as Protein Plus BCT™) compared to EDTA, ACD-A and RNA Complete BCT™.

MATERIALS AND METHODS

Blood collection and plasma preparation

Whole blood from self-declared healthy volunteers was collected into EDTA, ACD-A, RNA Complete BCT, or Nucleic Acid Plus BCT for EV flow cytometry, EV RNA, or EV ELISA analyses. Plasma was isolated by double spin protocols at room temperature. For EV flow cytometry analysis, plasma was prepared following the ISTH recommended centrifugation condition (2500 ×g, 15 min, twice, no brake) at draw time and at 8 and 72 hours post-collection. For EV RNA and EV ELISA analyses, plasma was prepared using a Streck inhouse protocol (1800 ×g, 15 min, followed by 2800 ×g, 15 min, full brake) at draw time and 24, 72, or 144 hours post-collection. Plasma samples were aliquoted and frozen prior to downstream analyses.

EV and residual cell flow cytometry

Single particle EV flow cytometry was performed by EV Count (Amsterdam, Netherlands) using an Apogee A60Micro flow cytometer (Apogee Flow Systems). Particles were detected by side scatter (SSC) triggering, and analysis was restricted to particles >200 nm. A refractive index (RI) cutoff of <1.42 was applied for platelet- and leukocyte-derived EVs, and <1.45 for erythrocyte-derived EVs, to exclude lipoprotein contaminants. Platelet, erythrocyte, and leukocyte derived EVs were quantified using lineage specific markers CD61, CD235a, and CD45, respectively. Residual platelets and erythrocyte ghosts were assessed by flow cytometry using the Northern Lights™ flow cytometer (Cytek Biosciences).

EV-associated RNA analysis

EVs were immunomagnetically enriched using EasySep™ Human EV Positive Selection Kits (STEMCELL Technologies) targeting CD9/CD63/CD81. RNA was extracted from enriched EVs using the Maxwell® RSC miRNA Plasma and Serum Kit (Promega Corporation). mRNAs were quantified by ddPCR using (Bio-Rad) iScript and ddPCR Supermix and TaqMan Gene Expression Assays. miRNAs were quantified by qRT-PCR using the miRCURY® LNA® RT and SYBR™ Green PCR Kits and miRCURY LNA miRNA PCR Assays (QIAGEN).

EV ELISA

EV concentrations were quantified using Atlas Human EV Lumi ELISA Kit (Everest Biolabs). All plasma samples were diluted to 1/40 to fall within the linear range of the assay, and EV concentrations were reported as particles per mL plasma by comparing the luminescence signals against a serial dilution of the provided EV standard.

Nucleic Acid Plus BCT minimizes time-dependent changes in blood cell–derived EV populations during delayed blood processing

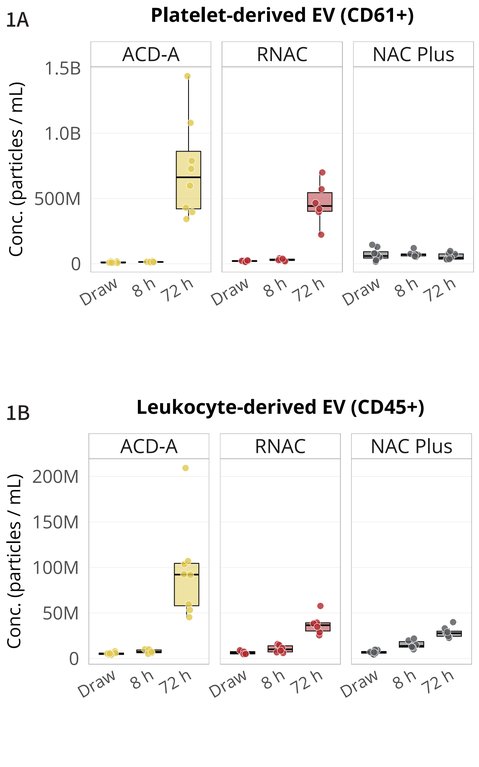

Figure 1. Delayed blood processing induces progressive increases in platelet-, erythrocyte-, and leukocyte-derived EV populations across all tubes. Nucleic Acid Plus BCT substantially limited ex vivo increases compared to ACD-A and RNA Complete BCT, consistent with reduced blood cell activation and improved preanalytical stabilization. (**A**) Platelet-derived EVs increased ~3× in Nucleic Acid Plus BCT versus ~21× (RNA Complete BCT) and ~72× (ACD-A) at 72 hours. (**B**) Erythrocyte-derived EVs increased ~15× in Nucleic Acid Plus BCT compared to ~26× (ACD-A) and ~232× (RNA Complete BCT). (**C**) Leukocyte-derived EVs showed the smallest increase in Nucleic Acid Plus BCT (~4×), demonstrating broad stabilization across blood cell types. (**D**) Wright-Giemsa stained blood smears showing erythrocyte crenation and platelet activation in samples drawn into ACD-A and RNA Complete BCT, but Nucleic Acid Plus BCT at 72 hours post-collection.

N = 6–8 donors

Nucleic Acid Plus BCT maintains near baseline residual platelet levels following storage

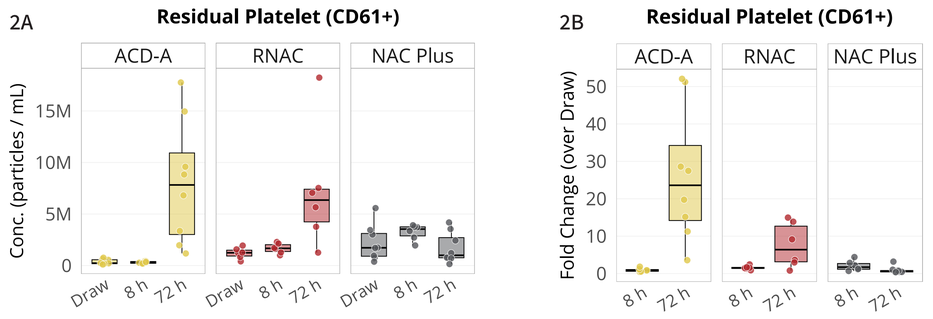

Figure 2. Residual platelets were detected in plasma prepared using the ISTH-recommended double-spin protocol across all collection tubes. (**A**) Platelet concentrations remained near baseline in Nucleic Acid Plus BCT throughout delayed processing, while ACD-A and RNA Complete BCT showed marked increases by 72 hours. (**B**) Fold-change analysis confirmed near-baseline residual platelet levels in Nucleic Acid Plus BCT (~0.9×), compared to marked increases in ACD-A (~26×) and RNA Complete BCT (~7.6×) at 72 hours.

N = 6–8 donors

Nucleic Acid Plus BCT stabilizes EV-associated mRNA and miRNA during storage

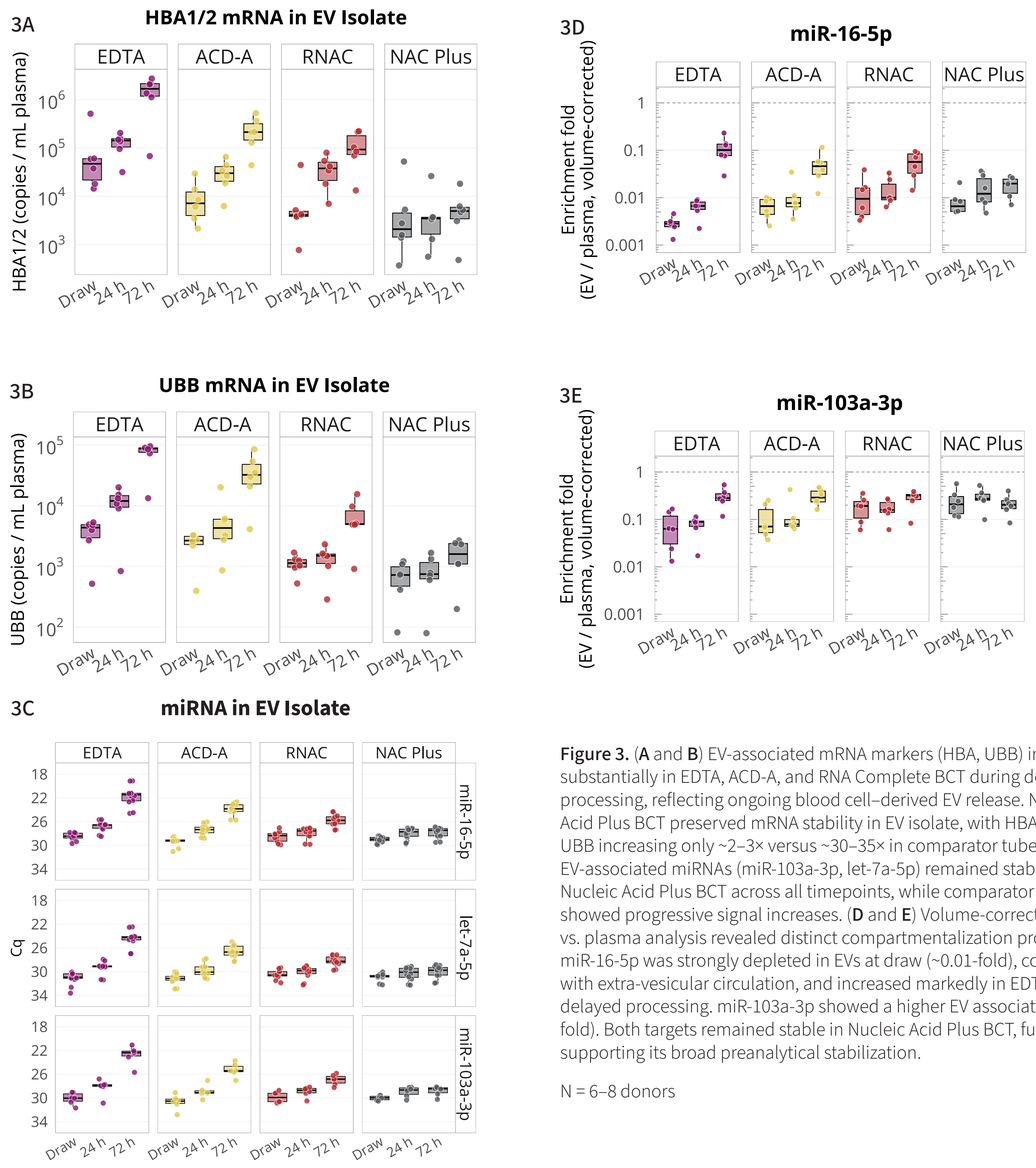

Figure 3. (**A** and **B**) EV-associated mRNA markers (HBA, UBB) increased substantially in EDTA, ACD-A, and RNA Complete BCT during delayed processing, reflecting ongoing blood cell–derived EV release. Nucleic Acid Plus BCT preserved mRNA stability in EV isolate, with HBA and UBB increasing only ~2–3× versus ~30–35× in comparator tubes. (**C**) EV-associated miRNAs (miR-103a-3p, let-7a-5p) remained stable in Nucleic Acid Plus BCT across all timepoints, while comparator tubes showed progressive signal increases. (**D** and **E**) Volume-corrected EV vs. plasma analysis revealed distinct compartmentalization profiles: miR-16-5p was strongly depleted in EVs at draw (~0.01-fold), consistent with extra-vesicular circulation, and increased markedly in EDTA during delayed processing. miR-103a-3p showed a higher EV association (~0.2-fold). Both targets remained stable in Nucleic Acid Plus BCT, further supporting its broad preanalytical stabilization.

N = 6–8 donors

Nucleic Acid Plus BCT minimizes time-dependent changes in whole EV concentrations during delayed blood processing

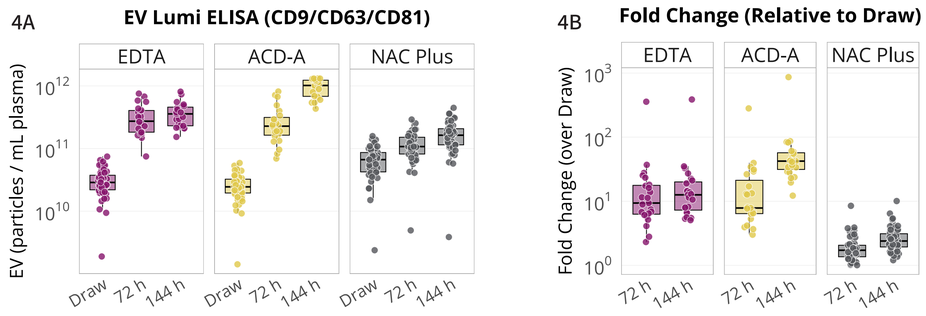

Figure 4. (**A**) Whole EV concentrations, measured by CD9/CD63/CD81-based ELISA, increased progressively during delayed blood processing in EDTA and ACD-A, while Nucleic Acid Plus BCT maintained near-baseline levels across all timepoints. (**B**) At 72 hours, EV concentrations increased ~10.4× (EDTA) and ~8.0× (ACD-A) relative to draw, rising to ~12.6× and ~41.1× by 144 hours, respectively. In contrast, Nucleic Acid Plus BCT showed minimal increases at both 72 hours (~1.7×) and 144 hours (~2.4×), remaining in the low single-digit fold-change range with markedly less variability.

N = 23 or 53 donors (EDTA, ACD-A); N = 53 donors (Nucleic Acid Plus BCT)

KEY TAKEAWAYS

- Nucleic Acid Plus BCT minimizes time-dependent ex vivo expansion of platelet and erythrocyte-derived EVs as well as residual platelet contamination during delayed processing.
- Nucleic Acid Plus BCT better preserves EV-associated RNA signals compared to EDTA, ACD-A, and RNA Complete BCT.
- Whole EV concentrations measured by CD9/CD63/CD81-based ELISA are better maintained during delayed processing in Nucleic Acid Plus BCT.
- Nucleic Acid Plus BCT stabilizes EV-associated miRNA compartmentalization, maintaining the EV vs. plasma distribution of both extra-vesicular and EV-enriched (miR-103a-3p) targets across delayed processing intervals.
- Nucleic Acid Plus BCT supports standardized preanalytical workflows for blood-based EV biomarker studies.

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