Nucleic Acid BCT™ maintains draw-time concentration of cell-free DNA, extracellular vesicles, and associated cell-free RNA

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Background

A major issue in blood analysis is sample degradation during storage and shipping. Examples of this include 1) breakdown of white blood cells (WBCs), which leads to release of fragmented genomic DNA and 2) deterioration of both WBCs and erythrocytes (reticulocytes) which release extracellular vesicles (EVs) and EV-associated cfRNA. In both cases, DNA and/or RNA emanating from cellular breakdown lead to nonspecific increases of these in the plasma fraction, obscuring the true draw-time concentrations of cfDNA and/or cfRNA. Use of such samples could result in an analysis that is not reflective of the sample at draw-time. To address this issue, cell-stabilization tubes, such as the Streck Cell-Free DNA BCT® (cfDNA BCT) and RNA Complete BCT® (RNAC) are used to preserve draw-time concentrations of cfDNA or EVs and EV-associated cfRNA, respectively. While these tubes effectively preserve sample concentration for up to 7 days, they are specific to particular analytes. With the Nucleic Acid BCT™ (NAC), a single tube stabilizes draw-time concentrations of cfDNA, EVs and cfRNA for up to 7 days.

Supporting Data

Nucleic Acid BCT maintains draw-time plasma volume while limiting hemolysis

Whereas non-stabilizing tubes, such as EDTA, or even current tubes intended for cfDNA usage, suffer from storage time-dependent increases in hemolysis and related decreases in recoverable plasma volume, Nucleic Acid BCT is designed to limit both (Figure 1). This is accomplished via an optimized stabilization solution that maintains the integrity of erythrocytes and WBCs. Blood samples stabilized in Nucleic Acid BCT have decreased hemolysis compared to equivalent samples stored in other stabilization tubes or EDTA and better maintain draw-time plasma volume during room temperature sample storage (Figure 1B, C). This is critical for those assays containing a plasma volume requirement for their analyte extraction workflows. At the same time, retention of draw-time plasma volume directly results in increased extractable analyte yield (e.g., cfDNA yield).

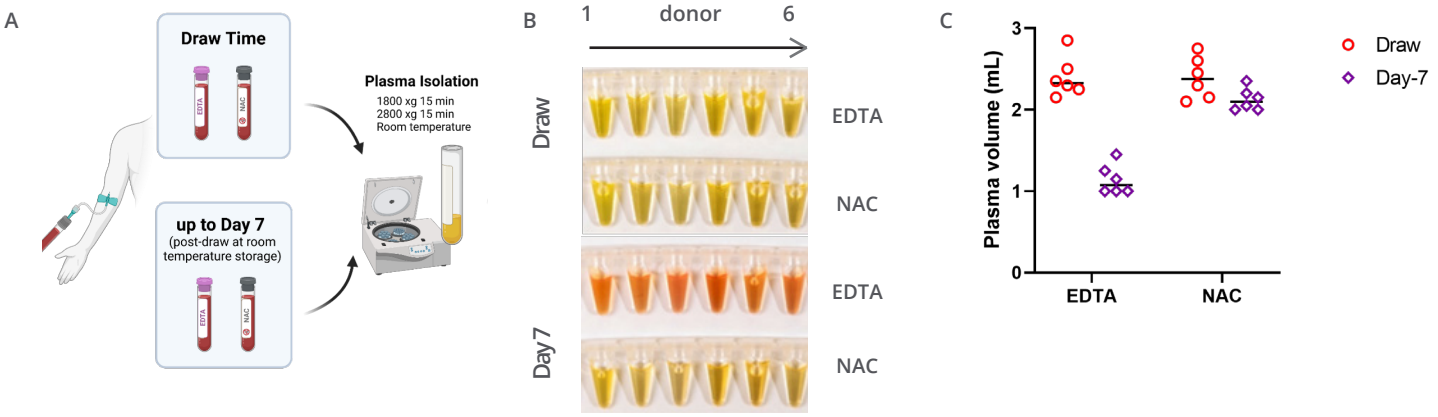

Figure 1. Nucleic Acid BCT maintains draw-time plasma characteristics.. (A) *Blood was collected from self-declared healthy donors into EDTA or Nucleic Acid BCT. Plasma was isolated immediately after draw (Draw) or after 7 days (Day-7) of ambient temperature storage using a generic double spin centrifugation protocol and immediately frozen at -80 °C.* ***(B)*** *Hemolysis of blood samples collected into EDTA or Nucleic Acid BCT immediately after draw or after 7 days of ambient temperature storage.* ***(C)*** *Plasma volume is maintained to near draw-time levels in Nucleic Acid BCT.*

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Nucleic Acid BCT™ maintains draw-time plasma cfDNA levels during storage

Total nucleic acid was isolated from plasma using the QIAamp® Circulating Nucleic Acid Kit (Qiagen) according to the manufacturer's "3 mL Plasma" protocol, with the exception that the 60 °C incubation was extended to 60 minutes (Figure 1A). Resultant cfDNA concentration was measured using the Qubit™ dsDNA HS Assay according to kit-included instructions (Thermo-Fisher). Sample quality was assayed using Cell-Free DNA ScreenTape analysis following the manufacturer's protocol (Agilent Technologies). While blood collected into non-stabilizing EDTA tubes demonstrates robust time-dependent increases in plasma DNA levels, equivalent donor samples collected into the Nucleic Acid BCT maintain draw-time plasma cfDNA concentration for up to 7 days when stored at room temperature (Figure 2B). Further, Day-7 cfDNA size and calculated %cfDNA remain similar to draw time for blood collected into Nucleic Acid BCT, but not donor-matched EDTA (Figure 2C, D). Together, these data demonstrate that Nucleic Acid BCT maintains the draw-time characteristics and concentration of plasma cfDNA for up to 7 days of ambient storage.

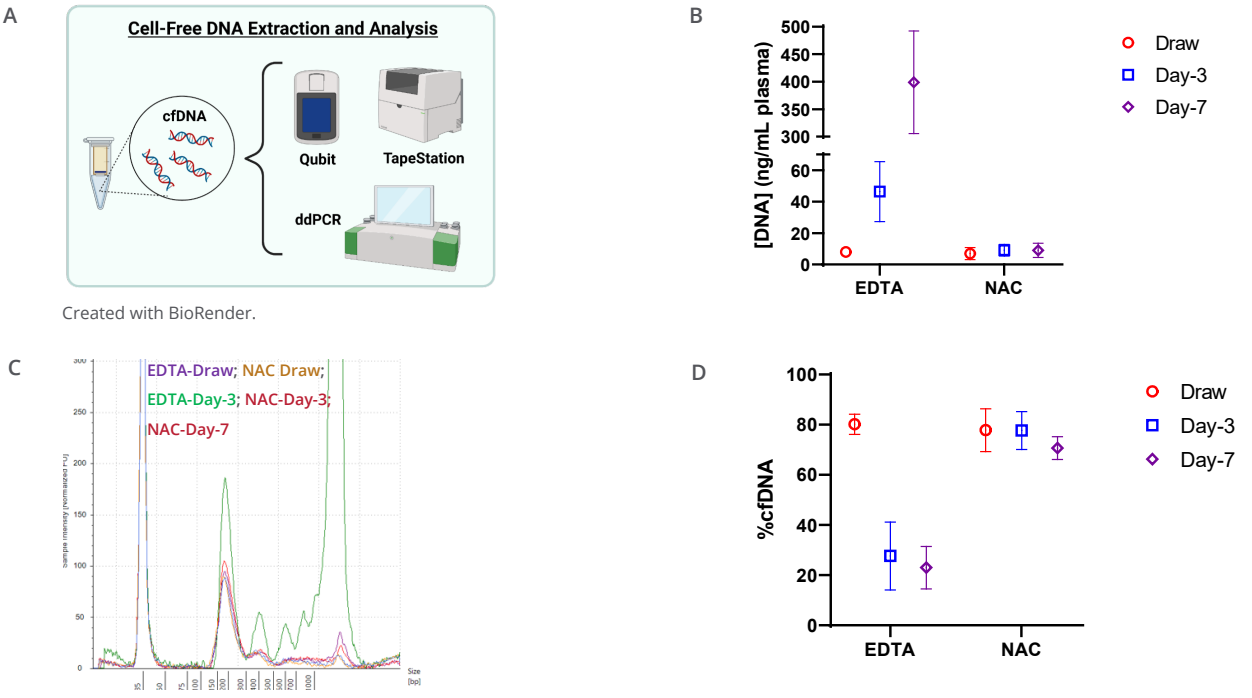

Figure 2. Plasma cfDNA levels are stabilized by Nucleic Acid BCT for up to 7 days of ambient storage. (A) *Workflow of cfDNA collection and analysis. Cell-free DNA isolated from plasma was assayed for concentration, size and purity using a combination of Qubit™ dsDNA HS Assay, Cell-Free DNA ScreenTape analysis, and Bio-Rad ddPCR. Cell-free DNA concentration* ***(B)****, size* ***(C)****, and purity* ***(D)*** *in plasma collected into EDTA or Nucleic Acid BCT immediately after draw (Draw) or after 3 (Day-3) or 7 days (Day-7) of ambient temperature storage. [DNA] and %cfDNA are graphed as mean ± STDEV for 6 donors. The single donor electropherogram shown in* ***(C)*** *is representative of what is normally observed for all donors.*

To demonstrate the effectiveness of Nucleic Acid BCT in stabilizing draw-time levels of cfDNA in a clinically relevant example, we turned to contrived sample generation using cancer cell-line DNA. As background, without proper cell stabilization, plasma disease-specific cfDNA (e.g., circulating tumor DNA) becomes contaminated with fragmented genomic DNA originating from degrading WBCs. This, in turn, may result in false low mutant allele fractions for a given mutated gene. In order to exemplify this, $EGFR^{L858R}$ DNA was isolated from the NCI-H1975 cell line, fragmented to ~200 nucleotides, and then used in spike-in experiments with the cfDNA isolated in Figure 2. Concentration of the mutant $EGFR^{L858R}$ allele cannot be contaminated by fragmented genomic DNA (no endogenous mutation for these donors), whereas wild-type (WT) EGFR can. Therefore, only the quantity of WT EGFR should be affected by storage condition and time. The amount of mutated and WT EGFR in samples was quantified using a multiplexed ddPCR reaction that amplifies both versions of the gene. While the concentration of spike-in $EGFR^{L858R}$ remains constant during storage, levels of WT EGFR increase markedly in a storage time-dependent manner when samples are stored in EDTA (Figure 3A,B). In contrast, WT EGFR levels are maintained to baseline for up to 7 days for blood collected into the Nucleic Acid BCT

(Figure 3A,B). When comparing the mutant to WT in ratio, the Nucleic Acid BCT™ maintains draw-time mutant allele frequency (MAF) out to 7 days of blood storage (Figure 3C). This finding is critical for assays of MAF where the detected frequency is already at or near the limit of detection of most next-generation sequencing-based assays.

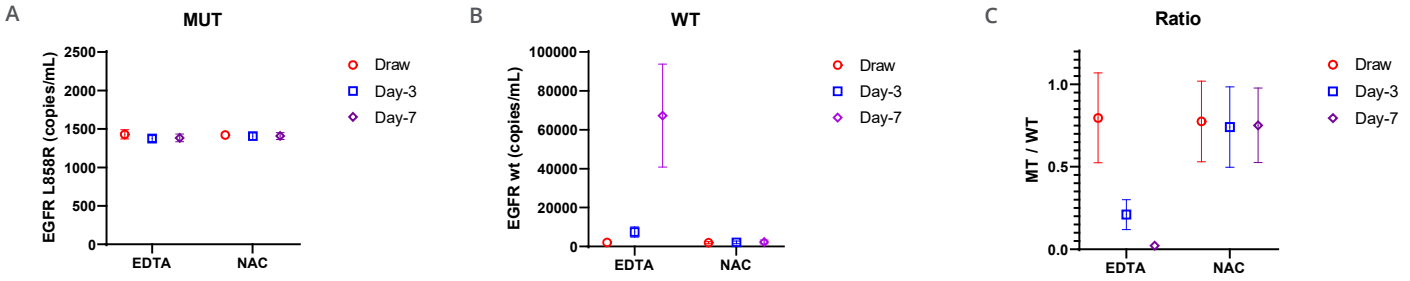

Figure 3. Nucleic Acid BCT enables detection of circulating mutant alleles following blood sample storage. *Amount of mutant EGFR^{L858R}* ***(A)*** *or wild type EGFR* ***(B)*** *in plasma collected from EDTA or Nucleic Acid BCT over the course of 7 days ambient blood sample storage.* ***(C)*** *Calculated frequency of mutant EGFR^{L858R} versus the concentration of the unmutated wild type EGFR gene. Values are displayed as mean ± STDEV for 6 donors.*

Draw-time concentration of EVs is maintained in Nucleic Acid BCT

Non-stabilized blood samples exhibit increased EV concentration as storage time increases. To examine the ability of the Nucleic Acid BCT to maintain EV concentrations in blood samples, plasma was isolated from samples stored up to 7 days at ambient temperature. 70 nm qEVsingle size exclusion columns were used to purify EVs from plasma according to manufacturer's instructions (Izon Science). The resultant EV eluent was diluted in PBS and particles were counted using the NanoSight NS300 (Malvern Pananalytical). Once EV concentration was measured, fold change was calculated by dividing the EV concentration at initial draw by the concentration after 3 or 7 days (sample dilution factor was included). When samples were collected into Nucleic Acid BCT, draw-time EV concentration was maintained for up to 7 days of ambient temperature storage (Figure 4B). In contrast, EV concentrations increased markedly at days 3 and 7 for equivalent samples collected in EDTA (Figure 4B). These data demonstrate that Nucleic Acid BCT is effective in maintaining draw-time EV concentration for up to 7 days ambient temperature storage.

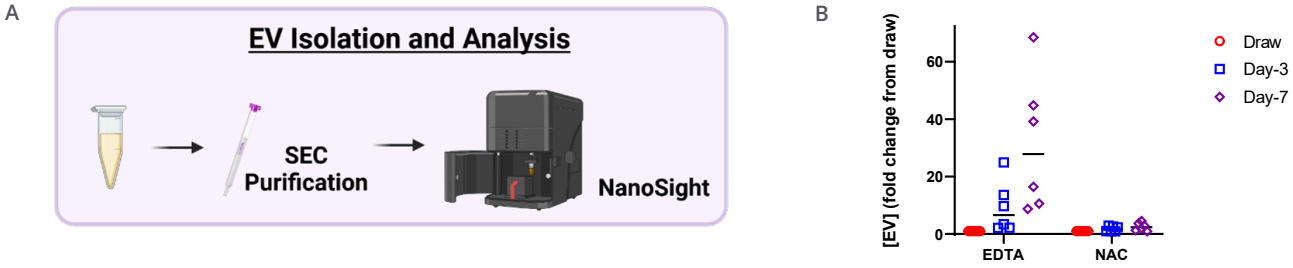

Figure 4. Nucleic Acid BCT stabilizes EV concentration for up to 7 days of ambient storage. ***(A)*** *Workflow of EV purification and analysis. EVs were purified from plasma using 70 nm qEVsingle size exclusion columns, diluted in PBS, and then counted using the NanoSight NS300.* ***(B)*** *Concentration of EVs from plasma collected into EDTA or Nucleic Acid BCT immediately after draw (Draw) or after 3 (Day-3) or 7 days (Day-7) of ambient temperature storage. n=6 self-declared healthy donors.*

Draw time cfRNA concentration is maintained in Nucleic Acid BCT

EVs contain important cellular information, including and most specifically, cfRNA. As such, the ability of Nucleic Acid BCT to maintain plasma cfRNA concentrations was measured. Blood was collected and plasma processed as in Figure 2. Total nucleic acid was then isolated using the QIAamp Circulating Nucleic Acid Kit (Qiagen) according to manufacturer's "3 mL miRNA" protocol (60 °C incubation was extended to 60 minutes). The final eluent was subsequently DNase1-digested (Qiagen DNase1 Kit) and cfRNA purified with the RNeasy MinElute Clean-up Kit (Qiagen). Resultant cfRNA concentration was determined with the Qubit™ miRNA Assay according to kit-included instructions (Thermo-Fisher)

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(Figure 5B). To qualitatively compare cfRNA between samples collected in EDTA or Nucleic Acid BCT™, the Bioanalyzer RNA Pico Assay was used per kit included instructions (Agilent). Samples collected into the Nucleic Acid BCT maintained cfRNA concentration for up to 7 days, whereas cfRNA levels increased substantially with time for samples collected into EDTA (Figure 5B). Further, samples collected in the Nucleic Acid BCT maintained a draw-time cfRNA size profile for up to 7 days of ambient temperature storage (Figure 5C). These data demonstrate that Nucleic Acid BCTs effectively maintain draw-time cfRNA concentration during blood sample storage.

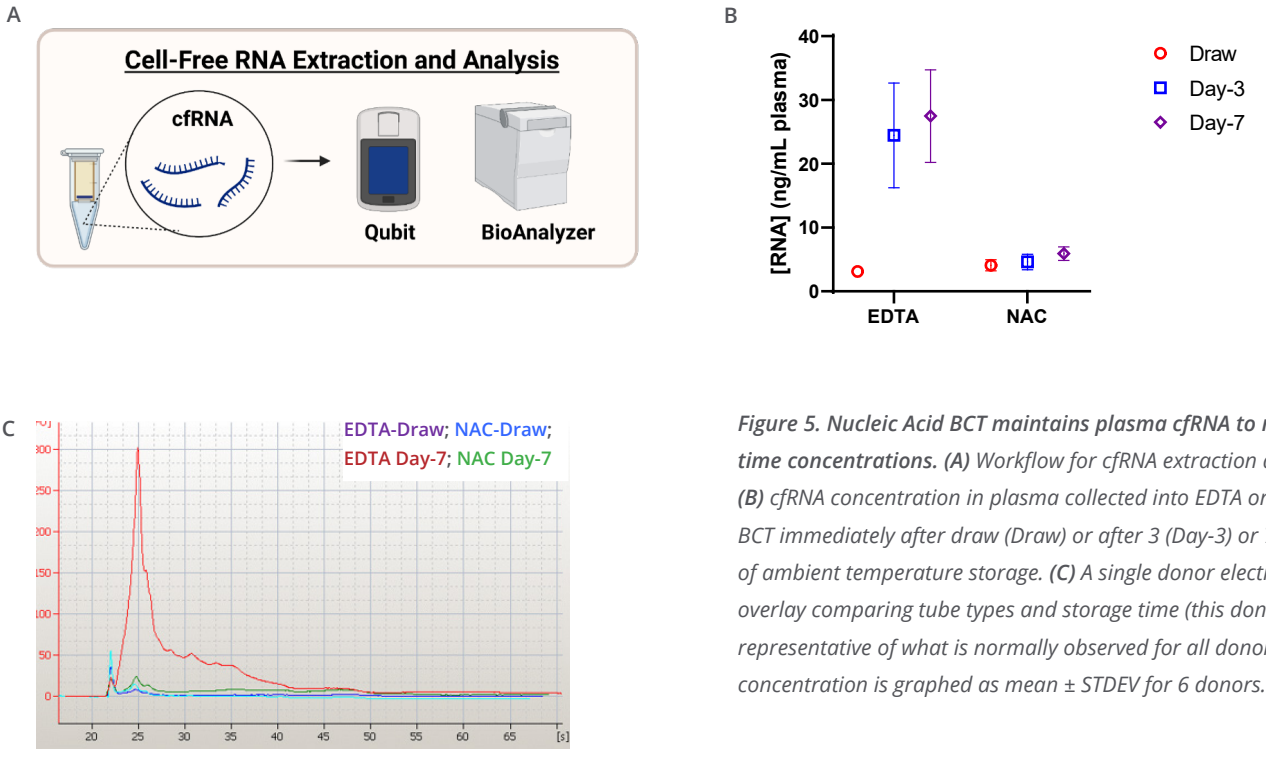

*Figure 5. Nucleic Acid BCT maintains plasma cfRNA to near draw-time concentrations. **(A)** Workflow for cfRNA extraction and analysis. **(B)** cfRNA concentration in plasma collected into EDTA or Nucleic Acid BCT immediately after draw (Draw) or after 3 (Day-3) or 7 days (Day-7) of ambient temperature storage. **(C)** A single donor electropherogram overlay comparing tube types and storage time (this donor is representative of what is normally observed for all donors assayed). RNA concentration is graphed as mean ± STDEV for 6 donors.*

Conclusion

Nucleic Acid BCT is a novel Streck blood collection tube that stabilizes all plasma nucleic acids (cfDNA AND cfRNA) and extracellular vesicles for up to 7 days at room temperature. This tube is ideal for labs seeking to reduce hemolysis in their samples, maximize plasma yield, and maintain draw-time concentrations of cfDNA, extracellular vesicles, and cfRNA. Nucleic Acid BCT is a powerful addition to the liquid biopsy toolkit allowing for combined interrogation of both cfDNA (mutation profiling) and cfRNA (transcriptome analysis and expressed fusion gene detection) in addition to downstream analysis of EVs.