Use of Nucleic Acid BCT™ with Plasma Nucleic Acid Next-Generation Sequencing Workflows

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Introduction

Liquid biopsies, which interrogate body fluids in search of analytes released by cancer cells, are becoming increasingly common in clinical oncology. Foremost among the technologies used are those that employ nucleic acids, such as cell-free DNA (cfDNA). Because many current next generation sequencing (NGS)-based assays perform near the limit of assay detection, identifying critical mutations, fusions, or expression patterns are analogous to finding a needle in a haystack. Compounding successful assay development are preanalytical variables such as blood collection, transport and storage, which influence analyte availability and detectability for downstream use. Here, we provide workflows for NGS-based sequencing of cfDNA isolated from Nucleic Acid BCT.

Nucleic Acid BCT is For Research Use Only. Not for use in diagnostic procedures.

Materials and Methods

Sample Collection and Preparation

Blood from consenting, self-proclaimed healthy donors was drawn into EDTA and Nucleic Acid BCT and processed immediately (Draw) or stored at ambient temperature for up to 7 days (Day 7). Plasma preparation followed the double centrifugation protocol described in the Nucleic Acid BCT IFU and was subsequently frozen at -80 °C.

Synthetic cfDNA spike-in oligos

Four different synthetic 170bp DNA duplexes (EGFR^{L858R}, EGFR^{E19del}, KRAS^{G12D}, and PIK3CA^{E545K}) were synthesized by Integrated DNA Technologies (Coralville, IA). All synthesized cfDNA-like duplexed nucleotides were complementary to TruSight™ Tumor 15 Library Prep Kit (Illumina®) reagents. Pooled oligos were spiked to an anticipated mutant frequency of 0.10 or as indicated.

Plasma DNA Isolation

To isolate plasma DNA, the MAGicBead™ cfDNA Kit (Zymo Research) was used in combination with the KingFisher™ Apex System (Thermo Scientific™). Isolation was carried out using 4 mL of thawed plasma as input and followed manufacturer recommendations specific to Streck blood collection tubes (digestion for 30 minutes at 55 °C). Eluant DNA concentration was determined using the Qubit™ dsDNA HS Assay (Thermo Scientific) per kit recommendations.

ddPCR analysis

EGFR and EGFR^{L858R} copy number was analyzed using previously published primer/probe sets that recognize both the wild type allele (expressed endogenously) and the synthetic mutant copy. Droplet generation, PCR, and analysis were performed using the QX600™ AutoDG™ Droplet Digital™ PCR System (Bio-Rad).

Library preparation, sequencing and data analysis

DNA sequencing libraries were generated according to instructions provided in the TruSight Tumor 15 Reference Guide. Library size was determined using the High Sensitivity d1000 Screentape run on the TapeStation 4200 (Agilent). 2 x 150bp sequencing was performed using the Illumina NextSeq® 500 system with mid-output sequencing kits. All data were saved in Illumina BaseSpace™ prior to analysis with the TruSight Tumor 15 application.

Results

While many users of stabilizing blood collection tubes assume that these devices stabilize cfDNA, cfRNA, and other important analytes, this is not entirely the case. Rather, these blood collection tubes are formulated to stabilize blood cells and prevent the deleterious effect of cellular breakdown on draw-time plasma analyte concentrations. To demonstrate that Nucleic Acid BCT limits changes to draw-time cfDNA concentrations, two concentrations (high and low) of synthetic mutant cfDNA-like duplexed oligonucleotides ($EGFR^{L858R}$) were spiked into whole blood samples collected into EDTA or Nucleic Acid BCT and concentration was measured at draw-time or after 7 days of whole blood storage at ambient temperature. $EGFR^{L858R}$ mutant concentrations in whole blood collected into EDTA tubes markedly decreased following 7 days of ambient temperature storage (Figure 1). This coincided with a dramatic increase in endogenous wildtype EGFR allele concentration, presumably due to WBC breakdown and release of fragmented genomic DNA (Figure 1). In contrast, $EGFR^{L858R}$ spike-in and endogenous EGFR allele concentrations remained stable in donor-matched samples collected into Nucleic Acid BCT and stored for the same amount of time, suggesting that Nucleic Acid BCT both stabilizes cfDNA and maintains cellular integrity to prevent release of blood cell-associated DNA during storage (Figure 1).

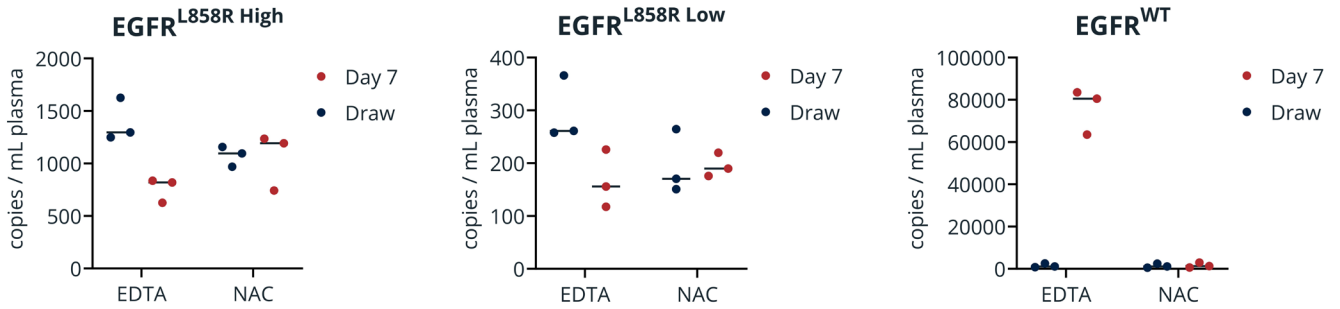

Figure 1: Nucleic Acid BCT stabilizes cfDNA during ambient whole blood storage. EGFR concentrations in donor-matched whole blood samples drawn into EDTA or Nucleic Acid BCT at draw time or after 7 days of ambient temperature storage. $EGFR^{L858R}$ cfDNA-like duplex oligonucleotides were spiked into whole blood samples in two different dilutions to target the anticipated wild-type plasma concentration (L858R High) and 10% of the anticipated wild-type plasma concentration (L858R Low). (n=3).

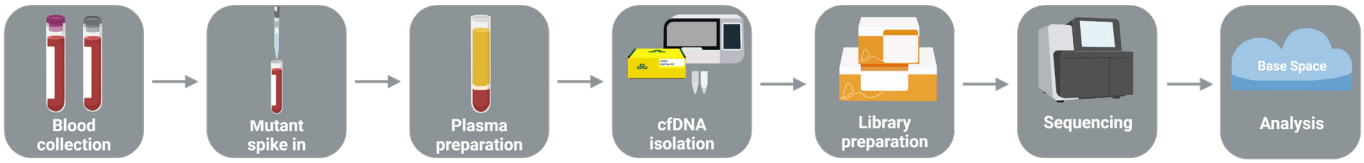

Figure 2: End-to-end workflow from blood collection to sequencing result analysis. Mutant cfDNA was spiked into blood from 10 donors collected into EDTA or Nucleic Acid BCT. At draw time or after 7 days of ambient temperature storage, plasma was prepared and cfDNA was isolated. The TruSight™ Tumor 15 Library Prep Kit was used to generate libraries from isolated cfDNA. This library was then sequenced with the NextSeq550® and analyzed. Figure created with BioRender.com.

To further illustrate the capability of Nucleic Acid BCT to maintain target analyte concentration during whole blood storage, we developed an end-to-end workflow for NGS-based mutant allele detection (Figure 2). In brief, blood was drawn into EDTA or Nucleic Acid BCT, spiked with a pool of four mutant duplex oligonucleotides ($EGFR^{L858R}$, $EGFR^{E19del}$, $KRAS^{G12D}$, and $PIK3CA^{E545K}$) and stored at room temperature for up to 7 days. Following whole blood storage, plasma was collected and cfDNA was purified, sequenced using the Illumina® TruSight Tumor 15 Kit and analyzed with Illumina-based BaseSpace™ applications.

While plasma DNA yield was similar for samples collected into EDTA and Nucleic Acid BCT and extracted at draw time, dramatic differences in yield were observed after 7 days of blood storage. For samples collected into Nucleic Acid BCT, plasma DNA yield after 7 days room temperature storage were similar to that at draw time, whereas matched samples collected into EDTA tubes demonstrated up to 800-fold increases of plasma DNA between draw time and day 7 extractions. Diluted plasma DNA (≤2.0ng/uL) was used for library preparation according to the instructions provided in the TruSight Tumor 15 manual. When resultant library size and concentration across samples was verified, we observed that all samples demonstrated the expected ~350bp size, regardless of blood collection tube type or storage time point (Figure 3B). Once pooled and sequenced, each of the tested samples passed the yield >500x coverage on >93.5% of bases targeted by the TruSight Tumor 15 assay (Figure 3C).

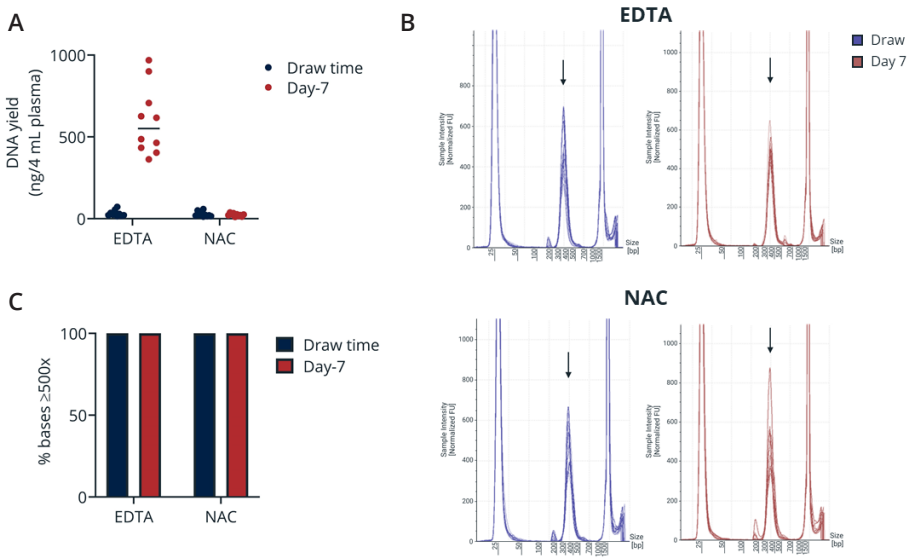

Figure 3: Nucleic Acid BCT maintains draw-time concentrations of plasma DNA over time. (**A**) DNA yield obtained from blood collected into EDTA or Nucleic Acid BCT at draw time or after 7 days of ambient temperature storage. (**B**, **C**) Screentape analysis (**B**) and sequencing read coverage (**C**) for libraries generated from samples collected into EDTA or Nucleic Acid BCT at draw time or after 7 days of ambient temperature storage. Blue traces, draw time; red traces, Day 7. Input DNA is normalized to ≤ 2 ng/µL during the library prep process, therefore resultant library concentrations should be similar for all samples. (n=10).

All samples were run through the BaseSpace™ TruSight™ Tumor 15 application to determine variant allele frequency. Because draw-time samples provided plasma DNA at the ~0.1 target mutant allele frequency for all four synthetic mutant spike-in controls (range 0.035-0.20), we were able to examine the effect of storage in stabilizing blood collection tube (Nucleic Acid BCT) in relation to matched donor samples collected into a non-stabilizing tube (EDTA). While mutant allele frequency for all four mutants was similar between tube types at draw-time, after 7 days of storage, there was a substantial shift in detectability after 7 days of storage, with none of the mutants detected in samples collected into EDTA tubes but 100% of the mutants detected in matched samples collected into Nucleic Acid BCT (Figure 4). These data suggest that Nucleic Acid BCT maintains the draw-time mutant allele frequency of important biomarkers for up to 7 days of ambient temperature storage.

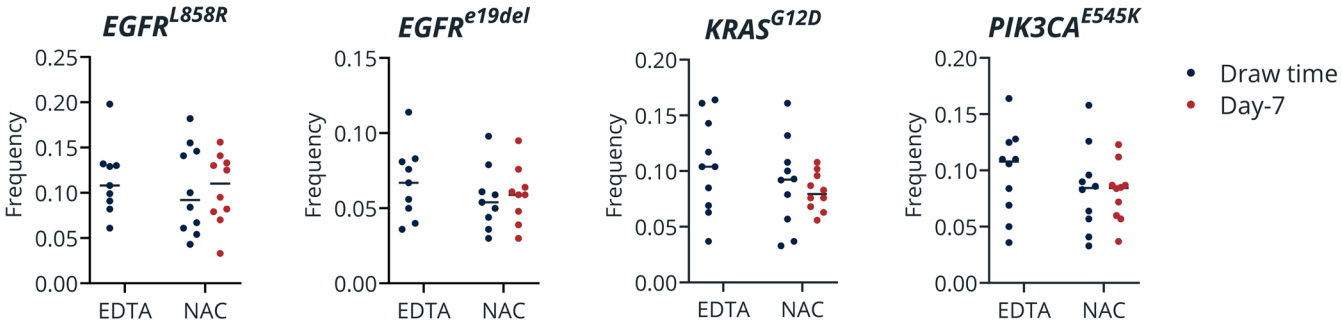

Figure 4: Mutant allele frequency is retained during whole blood storage in Nucleic Acid BCT. Mutant allele frequency in samples collected into EDTA or Nucleic Acid BCT at draw time or after 7 days* of ambient temperature storage. (n=10). *No variants detected in samples collected into EDTA at day 7.

Conclusions

Nucleic Acid BCT maintains draw-time plasma DNA concentrations during whole blood transport and storage at ambient temperature. This ensures precious sample integrity is maintained and offers laboratories and assay developers reduced preanalytical variability for NGS-based applications.