Capture and Concentrate Cell-Free DNA From K2EDTA Plasma

James Erickson, Stephanie Barksdale, and Chamodya Ruhunusiri
Application Scientists, Ceres Nanosciences

Introduction

Cell-free DNA (cfDNA) constitutes extracellular fragmented genetic material released by cells and is found in many body fluids, including blood, cerebrospinal fluid, and urine. All cells shed cfDNA, but conditions such as cancer and pregnancy can elevate the presence of it in these fluids due to increased cellular turnover. Notably, cfDNA derived from cancer cells may carry specific mutations, enabling mutation detection through minimally invasive fluid collections (1, 2). Tests that utilize this approach are referred to as liquid biopsies.

FDA approval has been granted for cfDNA tests for specific cancer types, such as non-small cell lung cancer, however, challenges for these types of tests persist (3). One challenge is that actionable mutations are frequently present at very low concentrations, potentially diminishing their detectability below the thresholds set by the employed assays. Another challenge is that large amounts of genomic DNA (gDNA) can be present in these samples, which can also affect assays' limits of detection (4, 5). Addressing these challenges is crucial for improving the reliability and applicability of liquid biopsy in cancer diagnostics and monitoring.

Ceres Nanosciences' hydrogel Nanotrap® Extraction Advanced Technology (NEAT) Liquid Biopsy Kit uses the Nanotrap hydrogel particle technology to capture and concentrate cfDNA from plasma samples while reducing gDNA contamination. The result is a purer, more concentrated cfDNA product for downstream analysis. The NEAT Liquid Biopsy Kit is compatible with multiple types of blood collection tubes, including cfDNA stabilization tubes, with automated and manual methods available for a range of plasma volumes.

Key Benefits

- **Rapid capture and concentration of cell-free DNA, 50 – 800 base pair fragments**
- **Reduced genomic DNA contamination, 2000 base pair fragments, and larger**
- **Improved detection of multiple low-abundance mutant allele gene targets from a single plasma sample**
- **Concentrated cfDNA eluant compatible with PCR, dPCR, ddPCR, or NGS analysis**
- **Automated and manual methods available for a range of plasma volumes with no required centrifugation steps**

Materials

Product	Company	SKU
NEAT Liquid Biopsy Kit	Ceres Nanosciences	7730
Seraseq ctDNA Mutation Mix v2 0.125%	Seracare	0710-0143
Seraseq ctDNA Mutation Mix v2 0.5%	Seracare	0710-0141
Seraseq ctDNA Mutation Mix v2 WT	Seracare	0710-0144
Invitrogen 50 bp DNA Ladder	Thermo Fisher Scientific	10416014
KingFisherTM Apex with 96 DW Head	Thermo Fisher Scientific	5400930
KingFisher Apex 24 Combi Head	Thermo Fisher Scientific	24079940
KF Apex 24 DW Heating Block	Thermo Fisher Scientific	24075940

(Materials continued)

Product	Company	SKU
KingFisher 24 Deep-well Plate, Barcoded	Thermo Fisher Scientific	95040470B
KingFisher 24 Deep-Well Tip Comb & Plate, Barcoded	Thermo Fisher Scientific	97002610B
KingFisher 96 Deep-well Plate, Barcoded	Thermo Fisher Scientific	95040450B
KingFisher 96 Plate (200 µL), Barcoded	Thermo Fisher Scientific	97002540B
KingFisher 96 Deep-well Tip Comb, Barcoded	Thermo Fisher Scientific	97002534B
TapeStation 4200	Agilent Technologies	G2991BA
TapeStation Cell-free DNA ScreenTape	Agilent Technologies	5067-5630
TapeStation Cell-free DNA ScreenTape	Agilent Technologies	5067-5633
QIAcuity One	QIAGEN	5plex Digital PCR system
dPCR Mutation Assay EGFR 6224 Human	QIAGEN	DMH0000386-B
dPCR Mutation Assay EGFR 6240 Human	QIAGEN	DMH0000085-A
Molecular Biology Grade Water	Corning	46-000
Basematrix Negative Diluent	Seracare	1805-0075
Molecular Biology Grade Ethanol	Decon Labs	71006-01
Human Plasma K2EDTA Individual Donor Not Filtered	BioIVT	HUMANPLK2

Methods

Sample Preparation for TapeStation 4200 analysis

Human plasma (gender unspecific; individual donor) was purchased from BioIVT. Whole blood from healthy human donors was drawn into collection bags containing K2EDTA anticoagulant. The whole blood was centrifuged once, collected with a plasma extractor, then frozen prior to shipment. Plasma samples were used with the NEAT Liquid Biopsy Kit without any additional centrifugation. Plasma samples for Kit 1 and Kit 2 workflows were centrifuged once more at 16,000 g for 10 min, per the kit manufacturers' recommendations.

Sample Preparation for dPCR analysis

Contrived samples for dPCR were made by spiking plasma with 0.125% mutant allele frequency Seraseq ctDNA Mutation Mix at 5 ng/mL. Seracare's Seraseq standards were used because they emulate the nature of cfDNA in plasma encapsulated within exosomes. Samples were gently inverted to mix. Single spun plasma samples were used with the NEAT Liquid Biopsy Kit without any additional centrifugation. Plasma samples for Kit 1 and Kit 2 workflows were centrifuged once more at 16,000 g for 10 min, per the kit manufacturers' recommendations.

NEAT Liquid Biopsy Kit Automated Extraction of cfDNA from Plasma on the KingFisher Apex System

Plasma samples were processed using the NEAT Liquid Biopsy Kit with 4 mL automated protocols on a KingFisher Apex System. All plates were filled with reagents as indicated in the protocol and loaded onto the KingFisher Apex System for cfDNA concentration and extraction. The final elution volume was 20 – 25 µL and was used directly with quantification instruments and downstream analysis platforms.

Competitor Extractions of cfDNA from Plasma

The same plasma samples were processed using commercially available cfDNA extraction kits from competitors. Kit 1 (manual processing using silica column method) and Kit 2 (automated on a KingFisher Apex System using silica magnetic bead method) were used to process samples to compare to the NEAT Liquid Biopsy Kit. Instructions from the manufacturers were followed for all extraction procedures.

Fragment Analysis using TapeStation 4200

The Agilent TapeStation 4200 is an automated capillary electrophoresis instrument that can be used with the Cell-free DNA ScreenTape Assay [50-800 bp]. This instrument separately quantifies the amount of cfDNA and the amount of gDNA in a sample, offering an advantage over instruments like spectrophotometers and fluorometers that quantify the entire amount of DNA (cfDNA plus gDNA) in a sample. TapeStation analysis was completed following manufacturer procedures. Briefly, 2 µL of Cell-free DNA Sample Buffer and 2 µL of eluted DNA

sample were loaded into each well of a 96-well sample plate for the TapeStation 4200 system. The plate was mixed, centrifuged, and then subjected to analysis on the Agilent TapeStation 4200. The resulting data from the assay includes total DNA concentration, percent cfDNA, and cfDNA concentration.

Digital PCR Analysis

Digital PCR (dPCR) analysis was performed using QIAGEN QIAcuity along with the QIAcuity OneStep Advanced Probe Kit. EGFR mutations T790M (COSM6224) and L858R (COSM6240) primer/probe sets obtained from QIAGEN were used as directed by the manufacturer. A reaction mix comprising 30 µL of reagents/water and 10 µL of the sample was prepared for each well. After sealing the plate with a Nanoplate seal, it was loaded onto the QIAcuity instrument and processed according to the specified amplification and detection settings. The data were analyzed using QIAGEN software and GraphPad Prism 9.0.

Results

NEAT Liquid Biopsy Kit improves cfDNA concentration

Plasma samples from three individual donors were processed (4 mL per sample) using the NEAT Liquid Biopsy Kit and two competitor kits (Kit 1 and Kit 2). The elution products from all three extraction kits were analyzed for quantification and purity data with the Agilent TapeStation 4200, Cell-Free DNA ScreenTape. The NEAT Liquid Biopsy Kit provided an average of 3-fold improvement in cfDNA concentration when compared to Kit 1. The NEAT Liquid Biopsy Kit workflow provided an average of 1.3-fold improvement in cfDNA concentration when compared to Kit 2 **[Figure 1 and Table 1]**.

DNA Concentration from 4 mL Plasma

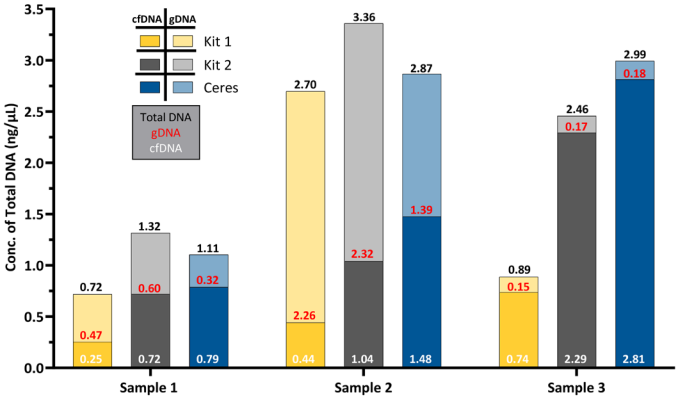


Figure 1: Bar graph representing total DNA concentration, cfDNA concentration and gDNA concentration for extraction of cfDNA from 4 mL of plasma. K2EDTA plasma was collected from three healthy donors and processed using three cfDNA extraction methods. Kit 1 was a manual column-based extraction method. Kit 2 and NEAT Liquid Biopsy Kit were processed using the KingFisher Apex. Samples were run in triplicate. The elution was analyzed on the Agilent Tapestation 4200 using the Cell-Free DNA Screentape. cfDNA concentration and large fragment exclusion from the Tapestation analysis shows the NEAT Liquid Biopsy Kit outperforming the competitor kits.

Percentage increase in cfDNA with NEAT Liquid Biopsy Kit

Sample	NEAT vs Kit 1	NEAT vs Kit 2
Sample 1	216%	9.72%
Sample 2	236%	42.3%
Sample3	280%	22.7%

Table 1: Table showing the percent increase in cfDNA concentration between the NEAT Liquid Biopsy Kit and Kit 1 and Kit 2, respectively.

NEAT Liquid Biopsy Kit Improves cfDNA Purity

Plasma samples from three individual donors were processed (4 mL per sample) using the NEAT Liquid Biopsy Kit and two competitor kits (Kit 1 and Kit 2). The elution products from all three extraction kits were analyzed using the Agilent TapeStation 4200 with Cell-Free DNA ScreenTape. The NEAT Liquid Biopsy Kit provided equivalent or higher cfDNA purity, with overall less gDNA than Kit 1 and Kit 2 **[Figure 1 and 2]**.

DNA Fragment Sizes from Different Extraction Kits

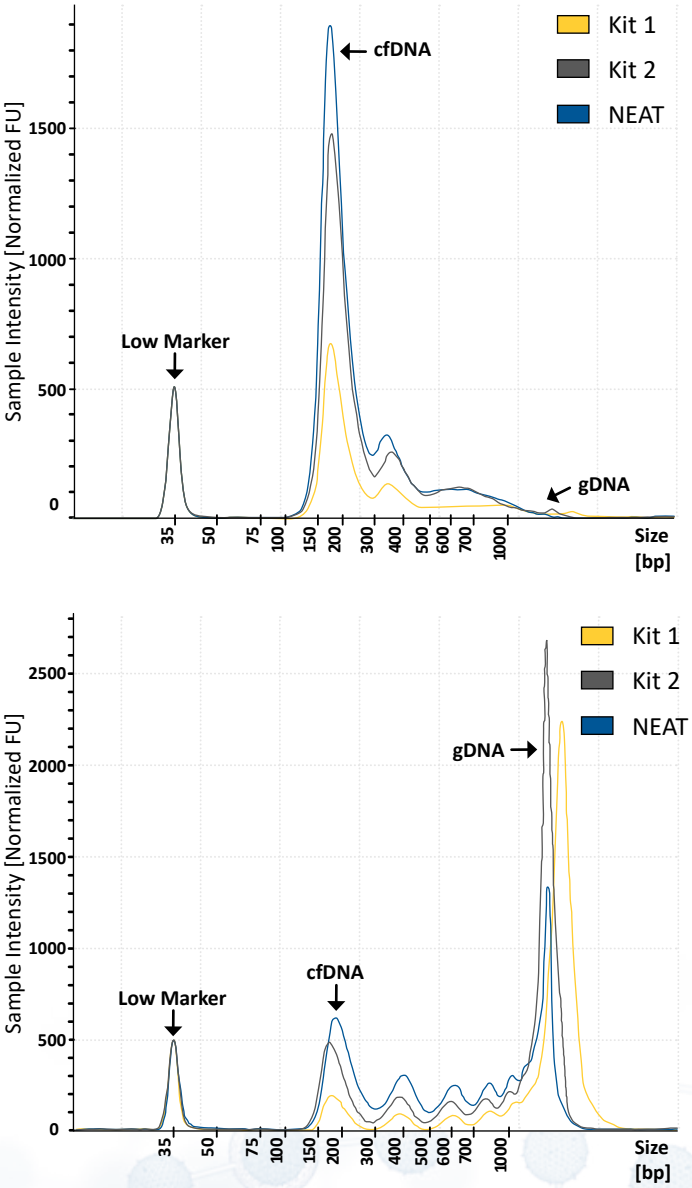


Figure 2: Electropherogram showing intensity and fragment size for extraction of cfDNA from 4 mL of plasma. K2EDTA plasma was collected from two healthy donors (Sample 2, top; Sample 3, bottom) and processed using three cfDNA extraction methods. Kit 1 was a manual column-based extraction method. Kit 2 and NEAT Liquid Biopsy Kit were processed using the KingFisher Apex. Samples were run in triplicate, and a representative trace is shown. Eluates were analyzed on the Agilent Tapestation 4200 using the Cell-Free DNA Screentape. Trace from the Tapestation software shows the NEAT Liquid Biopsy Kit yields a higher concentration of cfDNA and low gDNA contamination compared to competitor kits.

NEAT Liquid Biopsy Kit Improves Detection of Mutations in Contrived Samples

Plasma samples from two healthy donors, spiked with 0.125% mutant allele frequency Seraseq ctDNA Mutation Mix at 5 ng/mL were processed in triplicate (4 mL per replicate per sample) using the NEAT Liquid Biopsy Kit and two competitor kits and were analyzed using dPCR. The results indicate that the NEAT Liquid Biopsy Kit provides higher concentrations of rare mutant alleles in the eluate as compared to competitor kits.

In Sample 1, for the L858R mutation, extraction using both Kit 1 and Kit 2 resulted in similar copies per microliter (0.29 and 0.22 copies/µL), whereas the NEAT Liquid Biopsy Kit resulted in a concentration of 0.36 copies/µL, representing a more than 24% increase. For the T790M mutation in the same sample, the NEAT Liquid Biopsy Kit shows a 107% increase in concentration compared to Kit 1, with a yield of 0.51 copies/µL versus Kit 1's 0.21 copies/µL; in samples processed with Kit 2, the mutation was not even detectable.

In Sample 2, mutant allele concentrations are doubled in samples extracted with the NEAT Liquid Biopsy Kit compared to both Kit 1 and Kit 2. Notably, the NEAT Liquid Biopsy Kit demonstrates a 143% increase in the detection of the T790M mutation in Sample 2, yielding 0.51 copies/µL, while Kit 1 yielded 0.21 copies/µL, and in samples processed with Kit 2, the mutation was not even detectable **[Figure 3]**.

Mutation Concentrations in cfDNA

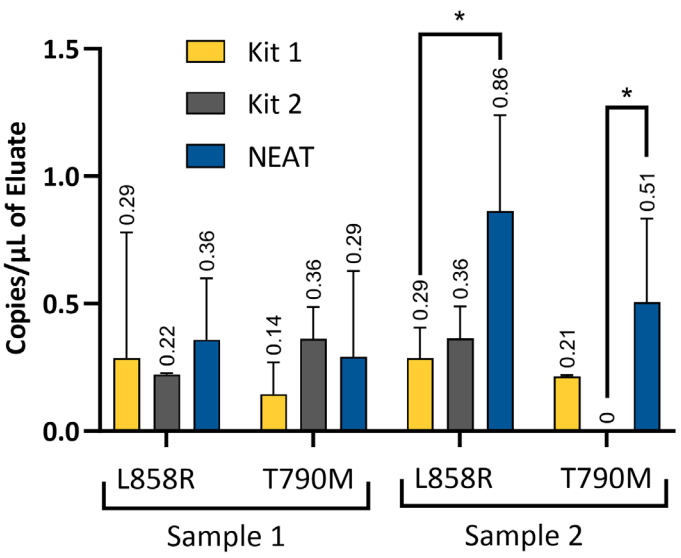


Figure 3: Bar graph copies/µL of eluate for extraction of cfDNA from 4 mL of plasma. K2EDTA plasma was collected from two healthy donors and spiked with SeraCare Seraseq cfDNA Standards at 0.125% mutant allele frequency. The spiked plasma samples were processed using three different cfDNA extraction methods. Kit 1 was a manual column-based extraction method. Kit 2 and NEAT Liquid Biopsy Kit samples were processed using the KingFisher Apex. Samples were run in triplicate. The eluted DNA was analyzed on the QIAcuity digital PCR system to determine mutant allele frequency. Statistical analysis graphed, *p<0.05.

Conclusions

The NEAT Liquid Biopsy Kit integrates the Nanotrap technology platform into a simple and easy-to-use workflow. This innovative approach demonstrates superior performance in capturing and concentrating cfDNA from plasma samples while reducing gDNA contamination, which improves detection of mutant alleles for multiple oncology markers when utilizing molecular assays.

Moreover, the NEAT Liquid Biopsy Kit eliminates the need for a second centrifugation step after plasma preparation from whole blood collected in K2EDTA tubes. This streamlines the pre-processing of plasma before cfDNA extraction and reduces the hands-on time required. The NEAT Liquid Biopsy Kit can be used to process 1 mL, 2 mL, and 4 mL K2EDTA plasma samples. The entire workflow is automated on the KingFisher Apex and KingFisher Flex Systems, providing a convenient and high-throughput solution for extraction processes.

References

1. Yan YY, Guo QR, Wang FH, et al. Cell-Free DNA: Hope and Potential Application in Cancer. Front Cell Dev Biol. 2021;9:639233. Published 2021 Feb 22. doi:10.3389/fcell.2021.639233

2. Buszka K, Ntzifa A, Owecka B, et al. Liquid Biopsy Analysis as a Tool for TKI-Based Treatment in Non-Small Cell Lung Cancer. Cells. 2022;11(18):2871. Published 2022 Sep 14. doi:10.3390/cells11182871

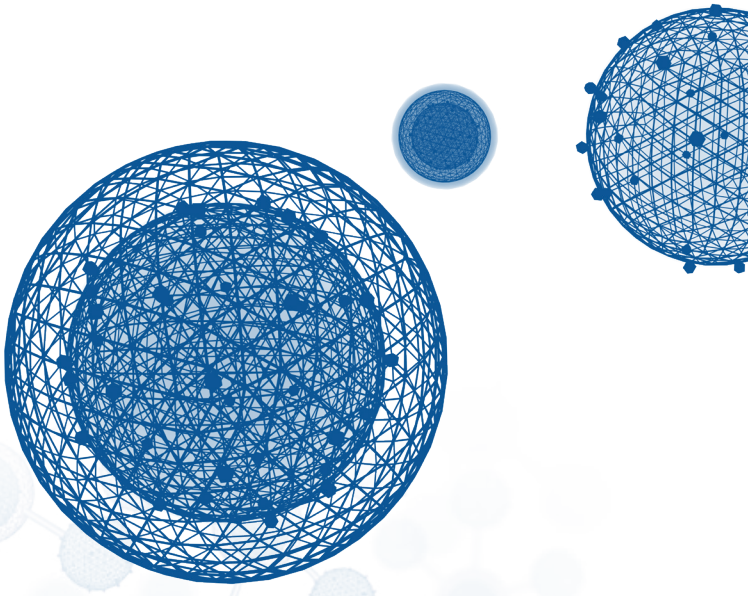
3. Liu S, Wang J. Current and Future Perspectives of Cell-Free DNA in Liquid Biopsy. Curr Issues Mol Biol. 2022;44(6):2695-2709. Published 2022 Jun 10. doi:10.3390/cimb44060184

4. Herberts C, Wyatt AW. Technical and biological constraints on ctDNA-based genotyping. Trends Cancer. 2021;7(11):995-1009. doi:10.1016/j.trecan.2021.06.001

5. Greytak SR, Engel KB, Parpart-Li S, et al. Harmonizing Cell-Free DNA Collection and Processing Practices through Evidence-Based Guidance. Clin Cancer Res. 2020;26(13):3104-3109. doi:10.1158/1078-0432.CCR-19- 3015

6. Mouliere F, Robert B, Arnau Peyrotte E, et al. High fragmentation characterizes tumour-derived circulating DNA. PLoS One. 2011;6(9):e23418. doi:10.1371/journal.pone.0023418.

7. Zaborowska, Ł. "Sensitivity and Specificity Calculator". Available at: https://www.omnicalculator.com/statistics/sensitivity-and-specificity. Accessed: 14 September 2023.



Contact Us

Phone: +1.800.615.0418x1
Web: www.ceresnano.com
Email: info@ceresnano.com

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