Innovative Technology to Automate and Improve the Detection Sensitivity of Ultra-low Frequency Mutations Directly from Blood Liquid Biopsies

Killpack J, Galova K, Christova R, Palmer E, Barratt G, Greenwood C, Fraccari R, Acquah JR, Reed S, Wisniewski M

DNAe London, United Kingdom and Carlsbad, California, USA

CONTACT: Jarrett Killpack
jarrett.killpack@dnae.com

INTRODUCTION

Blood liquid biopsy contains circulating cell-free DNA (cfDNA), a portion of which may be shed from tumors. This tumor-derived cfDNA, or circulating tumor DNA (ctDNA), can be analysed to detect cancers and identify their mutation profiles. Sensitive and specific cancerous mutation detection is critical to guide selection of the correct treatment regimens and lead to improved patient outcomes[1].

DNAe is developing the first rapid, sample-to-result, next-generation sequencing (NGS)-based platform, the LiDia-SEQ™ platform, which enables clinically relevant NGS ctDNA analysis and reporting within a work shift for use in routine clinical care. The whole workflow is fully automated.

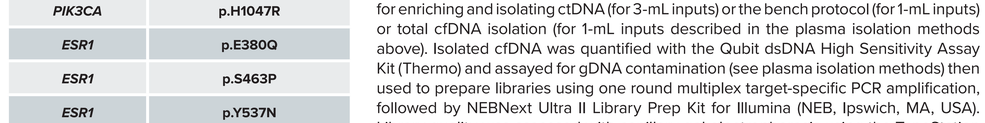

Figure 1. Photo of the LiDia-SEQ™ prototype instrument (left) and a schematic of its fully automated NGS workflow from whole blood or plasma input to clinical report generation (right). For research use only. Not for use in diagnostic procedures.

DNAe has developed a novel sample preparation method for isolating plasma from whole blood samples without the need for centrifugation[2]. Additionally, DNAe has developed a proprietary pre-amplification method for rapidly isolating enriched ctDNA directly from background non-tumor cfDNA in plasma, named Specific Variant Capture (SVC, patent application submitted 09-SEP-2025). This isolated and enriched cfDNA can be processed by the LiDia-SEQ™ platform to deliver a clinical report detailing the actionable mutation profile of ctDNA.

MATERIALS & METHODS

PLASMA ISOLATION

MATERIALS 10-mL fresh healthy whole blood (BioIVT, London, UK) collected in PAXgene ccfDNA tubes (BD, Franklin Lakes, NJ, USA) was spiked with 220 copies of a synthetic 165-base pair (bp) mock cfDNA geneBlock fragment (IDT, Coralville, IA, USA) then immediately processed.

METHODS Whole blood samples were processed with either of two methods of plasma isolation for comparison: 1) the DNAe automated method or 2) the standard double-centrifuged manual method (centrifuge 1.0 RCF at 4 °C for 10 minutes, pipette supernatant and decant to new tube, centrifuge 2.0 RCF at 4 °C for 10 minutes, pipette supernatant and decant to new tube). Total cfDNA was then isolated from the plasma with the cfDNA MagMAX kit (Thermo, Waltham, MA, USA) according to manufacturer instructions. Three DNA targets were tested with qPCR using Platinum SuperFi PCR master mix (Thermo): 1) a synthetic 165-bp mock cfDNA geneBlock target to assess spike recovery; 2) a 165-bp KRAS target to assess background levels of cfDNA; and 3) an 815-bp GAPDH target to assess the amount of genomic DNA (gDNA) from lysed nucleated blood cells.

Table 1. SVC Test Panel – 9 single nucleotide variant targets were selected from a larger panel of important breast cancer markers

Gene Target	Amino Acid Variant
PIK3CA	p.E545K
PIK3CA	p.E545G
PIK3CA	p.Q546R
PIK3CA	p.H1047R
ESR1	p.E380Q
ESR1	p.S463P
ESR1	p.Y537N
ESR1	p.Y357C
ESR1	p.Y537S

MUTANT ENRICHMENT WITH SVC

MATERIALS 80 ng cfDNA multiplex reference material (SensID, Rostock, Germany) containing 0.25% variant allelic frequency (VAF, gene loci listed in Table 1) was spiked either into 10-mL fresh healthy human whole blood (BioIVT) collected in PAXgene ccfDNA tubes (BD), or 1-mL synthetic plasma (SensID) before immediate processing.

METHODS Gene targets were selected as a representative test panel for important breast cancer mutations (Table 1). Plasma was isolated from spiked whole blood samples as described in the plasma isolation methods above. 4-mL of isolated plasma was aliquoted into 1- or 3-mL volumes or 1-mL spiked synthetic plasma was processed with SVC using the automated LiDia-SEQ™ prototype instrument with single-use cartridges for enriching and isolating ctDNA (for 3-mL inputs) or the bench protocol (for 1-mL inputs) or total cfDNA isolation (for 1-mL inputs described in the plasma isolation methods above). Isolated cfDNA was quantified with the Qubit dsDNA High Sensitivity Assay Kit (Thermo) and assayed for gDNA contamination (see plasma isolation methods) then used to prepare libraries using one round multiplex target-specific PCR amplification, followed by NEBNext Ultra II Library Prep Kit for Illumina (NEB, Ipswich, MA, USA). Library quality was assessed with capillary gel electrophoresis using the TapeStation 4200 system (Agilent Technologies, Santa Clara, CA, USA). Libraries were then either directly assayed for enrichment with absolute allele-specific probe-based quantitation with the Naica 3-color droplet digital PCR system (Stilla, Paris, France) or sequenced on the MiniSeq sequencing system (Illumina, San Diego, CA, USA) for comparison.

RESULTS

AUTOMATED PLASMA ISOLATION

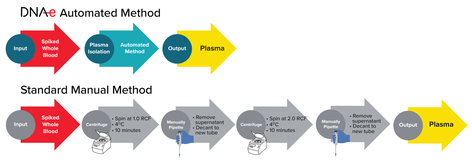

Figure 2. Two plasma isolation workflows: DNAe's automated centrifugation-free workflow (top) and the standard manual workflow with centrifugation (bottom).

We compared performance of DNAe's automated plasma isolation method to the standard manual method (Figure 2). Plasma isolated from both methods was qualitatively equivalent—plasma was straw-like in color, showing little to no hemolysis (Figure 3). Quantitative analysis also showed equivalence between the methods for recovery of background cfDNA, spiked synthetic mock cfDNA, and gDNA (Figure 4). The DNAe automated method yielded 75% plasma volume in approximately 15 minutes or 90% in 30 minutes compared to the standard manual method which could only achieve a maximum of 75% yield in 30 minutes due to the protocol requirement of leaving approximately 1-mL plasma after the first centrifugation step to avoid aspirating the buffy coat (Table 2).

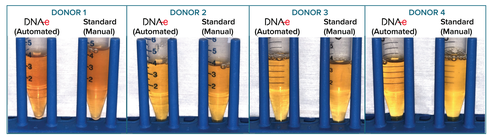

Figure 3. Photos of isolated plasma from four healthy donors using two methods: 1) DNAe's automated method or 2) the standard manual method; plasma isolated from both methods appears qualitatively equivalent with little to no visible hemolysis.

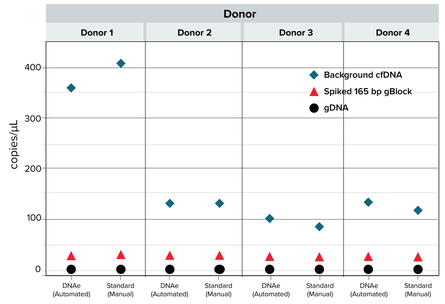

Figure 4. Comparison of DNA recovery from spiked whole blood of four healthy donors with the two plasma isolation methods: three qPCR assays measuring background cfDNA, recovery of spiked synthetic mock cfDNA, and gDNA from lysed nucleated cells.

Table 2. Performance comparison of the two plasma isolation methods.

Plasma Separation Method		DNAe (Automated)	Standard (Manual)
Automatable		✓	✗
LiDia-SEQ Compatible		✓	✗
Yield	In 15 minutes	75%	✗
	In 30 minutes	90%	75%
Quality	No hemolysis	✓	✓
	No gDNA contamination	✓	✓
cfDNA Recovery	Background cDNA	equivalent	equivalent
	Spiked mock cDNA	equivalent	equivalent

SVC FROM WHOLE BLOOD (4-PLEX)

Figure 5. The DNAe in-development automated NGS workflow (teal arrows) with orthogonal sequencing (gray arrow): from whole blood to bioinformatics report.

The automated plasma isolation method was then joined with the downstream workflow testing four *PIK3CA* targets for enrichment (Figure 5). Background cfDNA from plasma of a no-spike control from the same donors was quantified to be 5.1 ng/mL on average adjusting the input to 25.1 ng/mL with a VAF of 0.19% before enrichment. Enrichment levels across the non-optimized 4-plex targets ranged from 60- to 115-fold with the instrument protocol and from 38- to 144-fold with the bench protocol (Figure 6). Only two of the four targets performed similarly on both protocols (p.E545K and p.H1047R) while the others (p.E545G and p.Q546R) had differing performance with larger standard error in the 1-mL bench runs, likely due to stochastic effects from low DNA inputs in those samples (i.e., 25.1 ng at 0.19% VAF, or 13.8 mutant copies absolute).

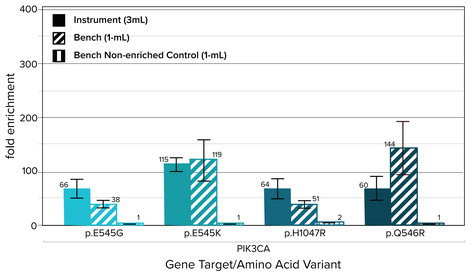

Figure 6. DNAe's NGS workflow results showing detection and enrichment levels of four important breast cancer targets from spiked whole blood compared to non-enriched controls at clinically relevant inputs. Starting cfDNA input before enrichment was 25.1 ng/mL with 0.19% VAF.

EXPANDING SVC MULTIPLEX (9-PLEX)

Figure 7. The DNAe in-development automated NGS workflow (teal arrows) with orthogonal sequencing (gray arrow): from plasma to bioinformatics report.

The SVC multiplex was then expanded to include the same four *PIK3CA* mutations and an additional five *ESR1* mutations (non-optimized, Table 1) and joined with the downstream workflow (Figure 7). Enrichment levels across the non-optimized 9-plex targets ranged from 55- to 371-fold with repeatable performance across replicates (Figure 8).

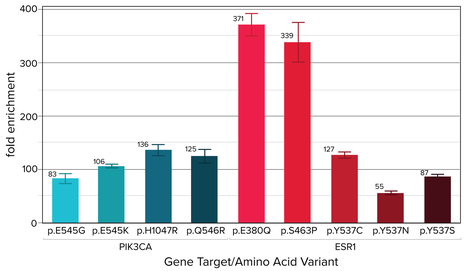

Figure 8. DNAe's NGS workflow results showing detection and enrichment levels of 9 important breast cancer targets from spiked synthetic plasma. Starting cfDNA input before enrichment was 80 ng/mL with 0.25% VAF.

CONCLUSION & DISCUSSION

DNAe is developing a rapid, fully automated sample-to-result NGS platform to enable reporting of clinically actionable sequencing results in routine clinical care for applications such as infectious diseases and oncology diagnostics — making NGS accessible in decentralized settings where it is currently not possible. As part of the oncology diagnostics workflow, DNAe has developed two novel automated sample preparation methods: 1) isolation of high-quality cell-free plasma without the need for centrifugation and 2) isolation and enrichment of ctDNA from cfDNA directly from blood plasma. These innovative technologies have been integrated with a downstream NGS workflow, demonstrating end-to-end capability of processing whole blood or plasma liquid biopsy samples and delivering sequencing results with very high detection sensitivities of ultra-low frequency mutations from clinically relevant inputs. However, mutant enrichment changes the oncology NGS assay detection paradigm from a quantitative measurement (i.e., VAF) to a binary response and thus would only be applicable in certain clinical contexts where very high sensitivities are required to detect ultra-low frequency mutations (e.g., early detection, minimal residual disease, resistance monitoring, etc.). DNAe plans to optimize and expand the SVC multiplex capabilities and, ultimately, to commercialize multiple NGS diagnostic assays for multiple oncology applications among others in separate clinical domains (e.g., infectious diseases, pharmacogenomics, transplant matching, etc.).

REFERENCES:
1. Foser S, Maiese K, Digumarthy SR, Puig-Butille JA, Rebhan C. Looking to the Future of Early Detection in Cancer: Liquid Biopsies, Imaging, and Artificial Intelligence. Clin Chem. 2024;70(1):27-32. doi:10.1093/clinchem/hvad196
2. Marturano A, Fatoyinbo HO, Saha A, Killpack J, inventors; DNAe Diagnostics Limited, assignee. Improvements in or relating to fluid sample preparation. WIPO Patent Application No. WO2022079447A1. October 15, 2021.