

Development and Analytical Validation of a Two-Step Circulating Tumor DNA Workflow for Monitoring Camizestrant Response in ER+ Breast Cancer PDX Models

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Abstract

Circulating tumor DNA (ctDNA) enables minimally invasive tumor burden monitoring and is a mainstay of clinical response assessment, yet standardized workflows for patient-derived xenograft (PDX) models remain constrained by low murine plasma volumes. To bridge preclinical pharmacodynamic monitoring with clinical ctDNA readouts, we developed and validated a two-step workflow combining droplet digital PCR (ddPCR) quantification of human-specific LINE-1 retrotransposon elements with reflexed targeted next-generation sequencing (NGS). Applied to an *ESR1*-mutant ER+ breast cancer PDX model (CTC174), LINE-1 levels correlated with tumor volume and growth rate. Treatment with the next-generation oral SERD *camizestrant* produced a > 97% reduction in plasma ctDNA versus vehicle. Samples exceeding the LINE-1 threshold were reflexed to targeted NGS, which detected *ESR1* and *PIK3CA* mutations in control but not camizestrant-treated plasma. This workflow enables rational sample prioritization, concurrent variant surveillance, and exploratory profiling from minimal plasma, providing a preclinical analogue of clinical liquid biopsy monitoring.

Introduction

Liquid biopsy approaches have become established in routine clinical practice as minimally invasive methods for the sensitive detection and quantification of tumor burden, with circulating tumor DNA (ctDNA), the tumor-derived fraction of cell-free DNA (cfDNA), representing the most widely adopted analyte underpinning these strategies. By enabling the molecular characterization of tumors from a simple blood draw, ctDNA-based liquid biopsies hold considerable potential to guide therapeutic stratification, facilitate longitudinal monitoring of treatment response, and support adaptive clinical decision-making within precision oncology frameworks (Crowley et al., 2013; Wan et al., 2017; Ignatiadis et al., 2021). Accordingly, ctDNA has emerged as a particularly promising biomarker for non-invasive disease surveillance across multiple solid tumor types.

Patient-derived xenograft (PDX) models represent an essential platform in translational oncology research offering a preclinical system that faithfully recapitulates human tumor biology by preserving the genomic, epigenomic, transcriptomic, and histopathological architecture of the parental malignancy (Hidalgo et al., 2014). Given the increasing clinical reliance on ctDNA-guided treatment decisions, there is a compelling rationale to develop analogous molecular monitoring methodologies within PDX frameworks to enable enhanced preclinical-to-clinical back-translation and greater concordance with contemporary clinical endpoints. In PDX models, all detected human cfDNA in the murine host is considered tumor-derived, as it originates exclusively from the engrafted human tumor. The relationship between ctDNA levels and tumor burden has been demonstrated across multiple clinical and preclinical settings. Diehl et al. (2008) showed a strong correlation between ctDNA levels and tumor volume in colorectal cancer patients, Bettgowda et al. (2014) demonstrated ctDNA detection in over 75% of patients with advanced cancers, and Izumchenko et al. (2017) confirmed that ctDNA levels in PDX models closely mirror tumor growth and regression patterns, providing real-time assessment of disease progression and treatment efficacy.

Long Interspersed Nuclear Element 1 (LINE-1) is a retrotransposon present at approximately 500,000 copies in the human genome. Critically, human LINE-1 sequences diverge substantially from their mouse counterparts at the sequence level, enabling the design of species-specific qPCR primers that amplify only human-derived DNA. This property makes LINE-1 an ideal endogenous reference for quantifying human ctDNA in mouse plasma without relying on tumor-specific mutations making it applicable across cell line and PDX models of any cancer type. Droplet digital PCR (ddPCR) is well-suited for quantifying these elements, offering absolute quantification without standard curves, detection of as few as 3–4 target copies per reaction, and a dynamic range spanning five orders of magnitude (Hindson et al., 2011). Importantly, ddPCR can generate robust measurements from the limited plasma volumes (20–100 µl) obtainable from serial murine blood draws, enabling longitudinal monitoring without compromising animal welfare (Rago et al., 2007; Siravegna et al., 2017).

Despite these individual technological advances, no integrated workflow has been described that combines rapid, low-input ddPCR-based tumor burden triage with reflexed next-generation sequencing (NGS) for molecular profiling in PDX models using minimal plasma volumes. Here, we describe the development and analytical validation of such a two-step liquid biopsy workflow. In this approach, ddPCR quantification of human-specific LINE-1 elements serves as a rapid triage step to assess ctDNA burden from as little as 20 µl of murine plasma. Samples exceeding a defined tumor fraction threshold are then reflexed to targeted hybrid-capture NGS and low-pass whole-genome sequencing (WGS) for somatic variant detection and exploratory fragmentomic characterization. We conducted a proof-of-principle in PDX CTC174, a D538G ESR1 mutant ER+ advanced breast cancer model treated with the next-generation oral selective estrogen receptor degrader (SERD) camizestrant. We describe a unified analytical framework capable of concurrent tumor fraction estimation, pharmacodynamic assessment, variant-level mutation surveillance, and fragmentation analysis, consolidated within a single resource-efficient pipeline engineered to operate within the stringent sample input limitations of preclinical xenograft models.

Methods

PDX Model Establishment and Efficacy Study

All animal experiments were conducted in accordance with UK Home Office legislation, the Animal Scientific Procedures Act 1986, and the AstraZeneca Global Bioethics policy. All experimental work underwent review by an Animal Welfare and Ethical Review Board, followed by Home Office approval. Experiments followed the principles of good statistical practice and the PREPARE and ARRIVE guidelines. AstraZeneca is a signatory to the Concordat on Openness on Animal Research in the UK.

Tumors were established from the hormone-independent ER+ breast cancer PDX model CTC174 (Ladd et al. 2016), with fragments inserted into the mammary fat pad of female NOD SCID gamma (NSG) mice. For the efficacy study, animals were randomized into treatment groups once tumor volume reached 150–300 mm³. Tumors were measured twice weekly by caliper (length × width), where length was the

longest diameter across the tumor and width the corresponding perpendicular. Tumor volume (TV) was calculated as $TV \text{ (mm}^3\text{)} = (\pi \times \max(\text{length, width}) \times \min(\text{length, width})^2) / 6,000$.

Camizestrant was formulated in tetraethylene glycol (TEG; 40% of final dosing volume) and 12.5% Captisol® in water (60% of final volume). Vehicle control consisted of 40% TEG and 60% Captisol at 12.5%. Camizestrant monotherapy at 10 mg/kg was administered once daily (QD) via oral gavage (PO) at a volume of 0.05–0.1 mL per 10 g body weight (n = 4–6 per group). Vehicle was dosed on the same schedule and volume.

Growth rate was estimated by fitting each tumor's growth curve to an exponential model: $\log_{10}(\text{tumor volume}) = a + b \times \text{time} + \text{error}$, where *a* represents log initial volume and *b* represents growth rate, assuming normally distributed error terms.

Blood Collection and Plasma Processing

Blood samples were collected at the end of the efficacy study via cardiac puncture under terminal anesthesia. Approximately 200–500 µl of whole blood was collected into K₂-EDTA-coated microtubes. Plasma was separated by centrifugation at 13,000 × g for 4 minutes at 4°C. Separated plasma was aliquoted and stored at –70°C until cfDNA extraction.

Cell-Free DNA Extraction

Cell-free DNA was extracted using the Mag-Bind cfDNA Kit (Omega BioTek # M3298-02). Samples were extracted and modified from manufacturers recommendations by miniaturizing the 2 mL starting volume by 10 times but using 5 µl of Mag-Bind particles CH. cfDNA was eluted in 50 µl of elution buffer. cfDNA yield was not independently quantified prior to ddPCR, as the LINE-1 ddPCR assay itself served as the primary quantification step for human-derived DNA content.

Droplet Digital PCR (ddPCR) – LINE-1 Quantification

Droplet digital PCR was performed using the Bio-Rad QX200 Droplet Digital PCR system to quantify human-specific LINE-1 retrotransposon sequences in murine plasma cfDNA. The assay exploits the high copy number (~500,000 copies per human genome) and complete absence of LINE-1 in the mouse genome to provide a sensitive and specific measure of human tumor-derived ctDNA.

Assay design

Primers and a hydrolysis probe were designed to target a conserved region within the LINE-1 open reading frame 2 (ORF2), selected for maximal representation across LINE-1 subfamilies and minimal homology to murine repetitive elements. The probe was labeled with FAM at the 5' end and conjugated to a Black Hole Quencher (BHQ-1) at the 3' end. Primer and probe sequences were validated in silico

against both the human reference genome (hg38) and mouse reference genome (mm10) to confirm species specificity.

Reaction setup

Each 20 µl ddPCR reaction contained 10 µL of 2× ddPCR Supermix for Probes (no dUTP; Bio-Rad), 900 nM forward primer, 900 nM reverse primer, 250 nM hydrolysis probe, and 5 µl of cfDNA template (adjusted based on available extracted volume), with nuclease-free water to final volume. Reactions were prepared in a PCR-clean environment to minimize contamination.

Droplet generation

Assembled reactions and droplet generation oil (Bio-Rad) were loaded into DG8 cartridges and processed using the QX200 Droplet Generator, producing approximately 20,000 nanoliter-sized water-in-oil droplets per well. Each droplet serves as an independent PCR micro-reactor containing zero, one, or a few target DNA molecules.

Thermal cycling: Droplets were transferred to a 96-well PCR plate, heat-sealed with pierceable foil, and amplified on a C1000 Touch Thermal Cycler (Bio-Rad) using the following protocol: 95°C for 10 minutes (enzyme activation); 40 cycles of 94°C for 30 seconds (denaturation) and 60°C for 1 minute (annealing/extension); 98°C for 10 minutes (droplet stabilization); hold at 4°C. A ramp rate of 2°C/second was used throughout to ensure uniform heating of droplets.

Droplet reading and analysis

Following amplification, plates were read on the QX200 Droplet Reader. Fluorescence amplitude data were analyzed using QuantaSoft software (Bio-Rad, version 1.7 or later). A manual fluorescence amplitude threshold was applied consistently across all experiments to classify droplets as positive (containing amplified LINE-1 target) or negative (no amplification). Wells with fewer than 10,000 accepted droplets were excluded from analysis. A LINE-1 concentration of ≥ 10 copies/µl in the final eluate was defined as the minimum threshold for reflexing to NGS library preparation. This threshold was empirically derived from the observed relationship between LINE-1 concentration and on-target sequencing coverage. Samples below this threshold produced insufficient coverage to support reliable variant calling.

Targeted Sequencing and Whole-Genome Sequencing

Samples that exceeded the LINE-1 ddPCR threshold for adequate ctDNA content were reflexed to next-generation sequencing. Whole-genome libraries (WGL) were constructed using the Kapa Biosystems HyperPrep Kit with 1–10 ng of cfDNA input (where available) and 8–12 cycles of PCR amplification. For targeted sequencing, libraries were hybridized to the AZ700 panel (Supplemental Fig. 1), a custom

hybrid-capture panel covering the exonic regions of 697 cancer-relevant genes including *ESR1* and *PIK3CA*.

Sequencing was performed on the Illumina NovaSeq X Plus platform using 2×150 bp paired-end reads. Targeted panel sequencing aimed for a mean on-target coverage of $\geq 100\times$, though achieved coverage ranged from 47.8X to 239.5X across samples due to variability in cfDNA input. Low-pass WGS was performed at approximately 0.5–1X genome-wide coverage for fragmentomic and copy number analyses.

Bioinformatic analysis

Sequencing reads were aligned to a combined human (hg38) and mouse (mm10) reference genome. Disambiguation of human versus mouse-derived reads was performed using the DRAGEN Bio-IT Platform (Illumina), which assigns reads to the genome with the higher alignment score. Variant calling on targeted cfDNA data was performed using the DRAGEN somatic pipeline with a minimum variant allele frequency (VAF) threshold of 1% for reporting. Whole-genome data were processed for copy number alterations using ichorCNA (Adalsteinsson et al., 2017). Fragment size distributions and end-motif analyses were derived from paired-end WGS reads aligned to the human genome.

Statistical analysis

Differences between the two independent groups were analyzed using an unpaired t-test in GraphPad Prism. Simple linear regression was conducted using GraphPad Prism. Statistical significance was evaluated by testing whether the slope differed significantly from zero. The reported p-value was derived from the t-statistic for the fitted slope coefficient.

Results

Overview of the Two-Step Workflow

Figure 1 outlines the integrated testing scheme deployed in this study. Plasma separated from whole blood collected from PDX-bearing mice underwent cfDNA extraction, followed by LINE-1 ddPCR as the first-step triage assay. This step quantifies human ctDNA and determines whether sufficient tumor material is present to warrant downstream sequencing. For samples where LINE-1 detection confirmed adequate ctDNA, libraries were constructed and subjected to both targeted hybrid-capture sequencing (AZ700 panel) and low-pass WGS for variant detection and exploratory fragmentomic analysis, respectively.

Step 1: LINE-1 ddPCR Quantification of Tumor Burden and Treatment Response

In mice bearing ER+ breast cancer CTC174 patient-derived tumors, camizestrant (10 mg/kg QD) treatment produced marked tumor growth inhibition, with reduced growth rate for the camizestrant treated arm, over 28 days relative to vehicle-treated controls (Fig. 2a, 2b). We then assessed whether plasma ctDNA levels, as measured by the LINE-1 ddPCR assay, reflected this therapeutic response.

At the Day 28 terminal timepoint, vehicle-treated mice harbored approximately 390 copies/μl of human LINE-1 ctDNA in mouse plasma cfDNA, consistent with substantial tumor burden (Fig. 2c). In contrast, camizestrant-treated mice showed a significant reduction to approximately 10 copies/μl, representing a > 97% decrease in ctDNA. LINE-1 concentrations were positively correlated with both tumor volume and tumor growth rate across all samples (Fig. 2d), confirming that the ddPCR assay provides a reliable surrogate measure of disease burden in this model. These findings establish the quantitative basis for the LINE-1 reflex threshold, confirming that ddPCR-derived ctDNA burden is a meaningful and reliable criterion for gating entry to Step 2 of the workflow.

Step 2a: Targeted NGS for Somatic Variant Tracking

Having established that LINE-1 levels reliably reflect tumor burden and discriminate treatment response, we next assessed whether samples passing the ddPCR triage threshold yielded sufficient material for downstream NGS-based molecular profiling.

We observed a strong correlation between LINE-1 concentration and sequencing quality metrics, including on-target coverage (Fig. 3a; Labrousse et al., 2024), confirming that samples exceeding the empirically defined ≥ 10 copies/μl reflex threshold consistently achieved coverage adequate for somatic variant detection. Those below the threshold were excluded from library preparation to maximize resource efficiency.

The ER+ advanced breast cancer CTC174 PDX model harbors two established driver mutations: D538G in *ESR1* and N345K in *PIK3CA* (Ladd et al., 2016). Targeted sequencing using the AZ700 panel consistently detected both mutations in tumor tissue and in plasma cfDNA from vehicle-treated mice at Day 28 (Supplemental Fig. 2). Coverage across samples ranged from 47.8X to 239.5X, which limits sensitivity for detecting very low tumor burdens but was adequate for variant detection in samples with sufficient LINE-1 signal.

In the camizestrant-treated arm, neither the D538G *ESR1* nor the N345K *PIK3CA* mutation was detected above the assay's reporting threshold in plasma cfDNA at Day 28, in contrast to their consistent detection in vehicle-treated samples (Fig. 3b). This finding is consistent with substantial suppression of tumor-derived DNA following camizestrant treatment. However, it is important to note that non-detection at the achieved sequencing depths does not exclude the persistence of residual disease below the assay's analytical sensitivity; at the coverage levels obtained, variants present at VAFs below approximately 1–2% would not be reliably detected. Together, these results confirm that the two-step workflow successfully propagates a meaningful molecular signal from the initial ddPCR triage through to

variant-level resolution, demonstrating end-to-end analytical coherence within a single minimal-volume plasma pipeline.

Step 2b: Exploratory Fragmentomic Characterization

As a proof-of-concept extension of the primary workflow, we performed exploratory fragmentomic profiling, including fragment size distribution and trinucleotide end-motif analysis, from low-pass WGS data of vehicle-treated samples. Consistent with published observations in clinical specimens (Cristiano et al., 2019; Serpas et al., 2019), vehicle-treated PDX cfDNA showed enrichment of shorter fragments and altered end-motif composition relative to healthy human donor cfDNA (Figs. 4 and 5), observations that are technically feasible within the workflow but require validation in larger cohorts before any monitoring utility can be assessed.

Discussion

This study provides proof-of-principle for an integrated two-step ctDNA workflow in a defined preclinical setting, demonstrating that LINE-1 ddPCR triage combined with reflexed NGS enables rational sample prioritisation, reduces library preparation costs by excluding low-input samples, and supports concurrent tumor fraction estimation, variant surveillance, and exploratory fragmentomic analysis from a single minimal-volume plasma sample. The strong correlation between LINE-1 concentration and downstream sequencing quality detected validates this triage approach. Gorges et al. (2012) were among the earliest to validate the principle that repeat-element qPCR could serve as a pharmacodynamic biomarker in xenograft drug efficacy studies. Our transition from qPCR to ddPCR for LINE-1 quantification extends this established approach by providing absolute quantification without standard curves, improved precision at low template concentrations, and tolerance of PCR inhibitors, giving advantages particularly relevant to the small plasma volumes available from murine blood draws. Critically, by conditioning the NGS step on LINE-1 concentration, our workflow combines the broad applicability and low cost of repeat-element quantification with the molecular resolution of sequencing, enabling simultaneous assessment of tumor burden, variant tracking, and fragmentomic features from a single plasma sample.

In the proof-of-concept study, camizestrant treatment produced marked inhibition of tumor growth in the ER+ breast cancer PDX model CTC174, as previously described (Mawson et al., 2023). Our results show a > 97% decrease in detectable LINE-1 ctDNA following 28 days of camizestrant treatment, with positive correlations between LINE-1 concentration, tumor volume, and growth rate (cm^3/day). These findings extend prior qPCR-based work with circulating human LINE-1 and Alu elements (Mishra et al., 2021; Gorges et al., 2012) to a ddPCR platform, providing absolute quantification from minimal plasma input and reinforcing LINE-1 as a broadly applicable tumor burden biomarker across independent cancer types and therapeutic mechanisms.

Notably, Mishra et al. (2021) also examined LINE-1 dynamics following ionizing radiation (6 Gy single dose) and observed transient measurement discrepancies between ctDNA and imaging readouts in the

immediate post-treatment period, consistent with an acute burst of apoptotic and necrotic cfDNA release following cytotoxic insult. This phenomenon, documented mechanistically by Rostami et al. (2020) and Marques et al. (2020), is an important consideration for the interpretation of our camizestrant treatment data. The endpoint design of our study likely captures the net effect of sustained tumor suppression rather than acute treatment-induced cfDNA spikes, which typically resolve within 24–48 hours of therapy initiation. Future longitudinal sampling designs incorporating early post-treatment timepoints will be important to characterize cfDNA release kinetics following targeted treatment response like camizestrant administration and to define optimal blood collection windows for pharmacodynamic assessment.

The detection of D538G *ESR1* and N345K *PIK3CA* driver mutations in vehicle-treated plasma of PDX CTC174 and their absence below the reporting threshold following camizestrant treatment provides a compelling pharmacodynamic signal consistent with effective target engagement and tumor suppression. This demonstrates that the workflow can track clinically relevant mutations in a preclinical liquid biopsy context. We emphasize that variant non-detection is contingent on assay sensitivity, sequencing depth, and input DNA quantity. At the coverage levels achieved in this study (47.8X–239.5X), variants present at VAFs below approximately 1–2% would not be reliably detected. The absence of detectable *ESR1* and *PIK3CA* mutations in the camizestrant arm therefore reflects substantial reduction in tumor-derived DNA rather than definitive biological eradication of all tumor cells. Future studies employing error-corrected sequencing or molecular barcoding approaches may enable more definitive assessment of minimal residual disease in this context.

Beyond confirming the presence or clearance of expected variants, NGS-based analyses at differing tumor levels should yield similar VAFs because the proportion of mutant to wild-type molecules is preserved from the originating tumor. To quantify tumor burden independently of variant-specific assays, we propose using the ratio of sequencing reads aligned to the human (hg38) versus mouse (mm10) genomes, which normalizes for differences in sample input. Our data support this approach and show concordance with LINE-1-based quantification, providing an orthogonal method for tumor fraction assessment within the same sequencing data (Supplemental Fig. 3). This dual-quantification strategy complements prior approaches such as copy-number-derived xTF metrics (Sauer et al., 2022) and human-specific single-copy gene targets (Kamat et al., 2006), and adds an additional layer of analytical confidence without requiring extra assays or sample aliquots.

Exploratory fragmentomic features – including a shift toward shorter fragment sizes and altered end-motif composition in vehicle-treated samples are consistent with published observations in clinical cancer specimens (Cristiano et al., 2019; Serpas et al., 2019) and demonstrate that while this analysis layer is technically accessible within the workflow, their translational relevance requires further validation.

Future studies across more diverse PDX models, tumor types, and treatment contexts will be needed to establish generalisability, refine the LINE-1 threshold for NGS reflexing, and improve analytical

robustness. With further validation, standardised ctDNA workflows of this kind could enhance the predictive value of preclinical oncology studies, support earlier detection of resistance, and help accelerate therapeutic development.

Declarations

Funding Declaration

This work was funded by AstraZeneca through internal sponsorship. No additional funding was received.

Author Contribution

P.L., S.R., H.R., and D.S. wrote the main manuscript P.L., S.R., H.R., H.F., D.Shera and D.Stetson prepared figures All authors reviewed and provided feedback for the manuscript

Data Availability

All data supporting the findings of this study are available within the paper and its Supplementary Information.

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Figures

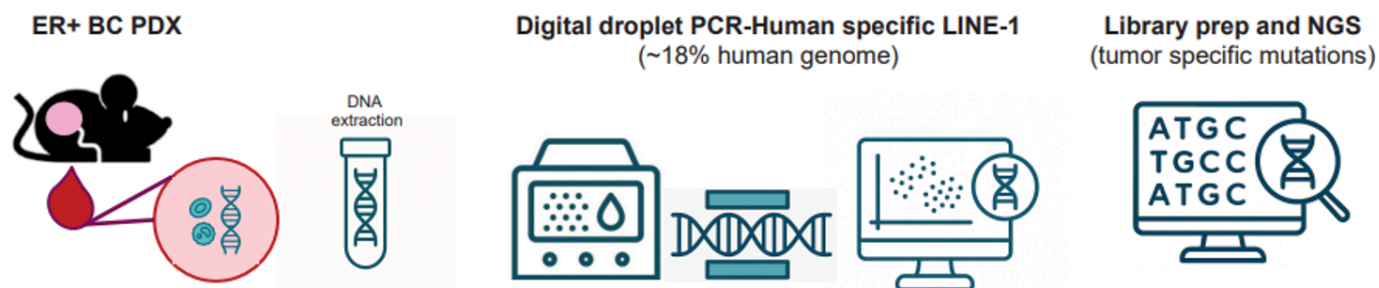


Figure 1

Outline of the two-step testing scheme. Plasma is separated from whole blood collected from PDX mice, followed by cfDNA isolation. The cfDNA is first tested with the LINE-1 ddPCR assay to quantify tumor-derived human DNA. For samples exceeding the tumor fraction threshold, libraries are prepared and subjected to hybrid-capture NGS (AZ700 panel) and low-pass WGS for somatic variant detection and fragmentomic characterization.

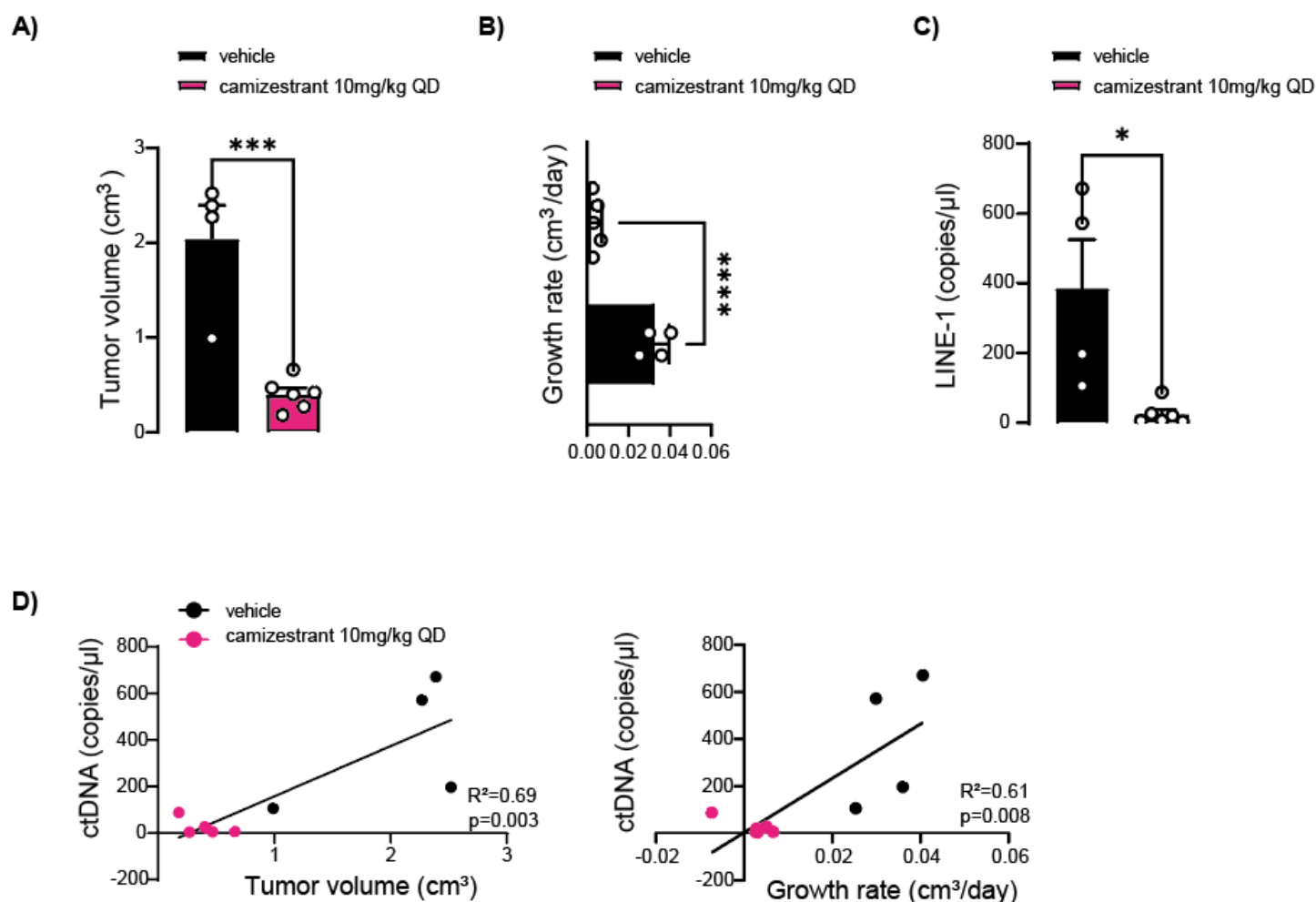


Figure 2

a) Tumor volume at the end of study day 28 for group treated with camizestrant (10 mg/kg QD) over 28 days relative to vehicle (n=4–6 per group). b) Growth rate for the 28-day study (QD oral dosing, n=4–6 per group); mean growth rate $\pm$ SD (cm³/day) estimated by exponential model fitting. c) LINE-1 quantification in murine plasma at Day 28 (end of study, vehicle n=4 camizestrant n=6). Mean $\pm$ SEM; significant decrease in LINE-1 in camizestrant-treated samples. d) Correlation between tumor volume and tumor growth rate versus ctDNA concentration as measured by the LINE-1 assay. P values were calculated using unequal variance t-test, *p<0.05; ***p<0.001; ****p<0.0001, correlation between LINE-1 levels and Growth rate, with p value displayed in plot.

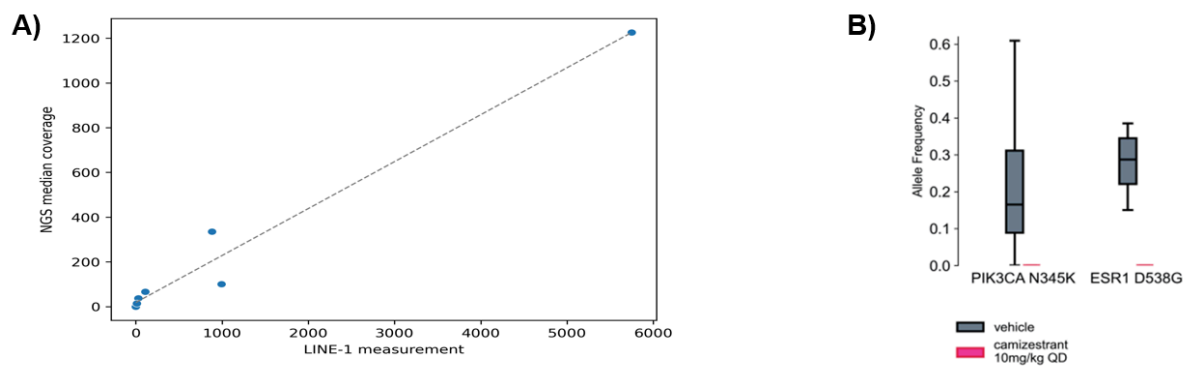


Figure 3

a) Correlation between LINE-1 concentration and sequencing coverage, supporting the use of ddPCR as a triage tool for NGS. b) Detection of D538G *ESR1* and N345K *PIK3CA* in vehicle-treated plasma cfDNA and their absence below the reporting threshold in camizestrant-treated samples at Day 28.

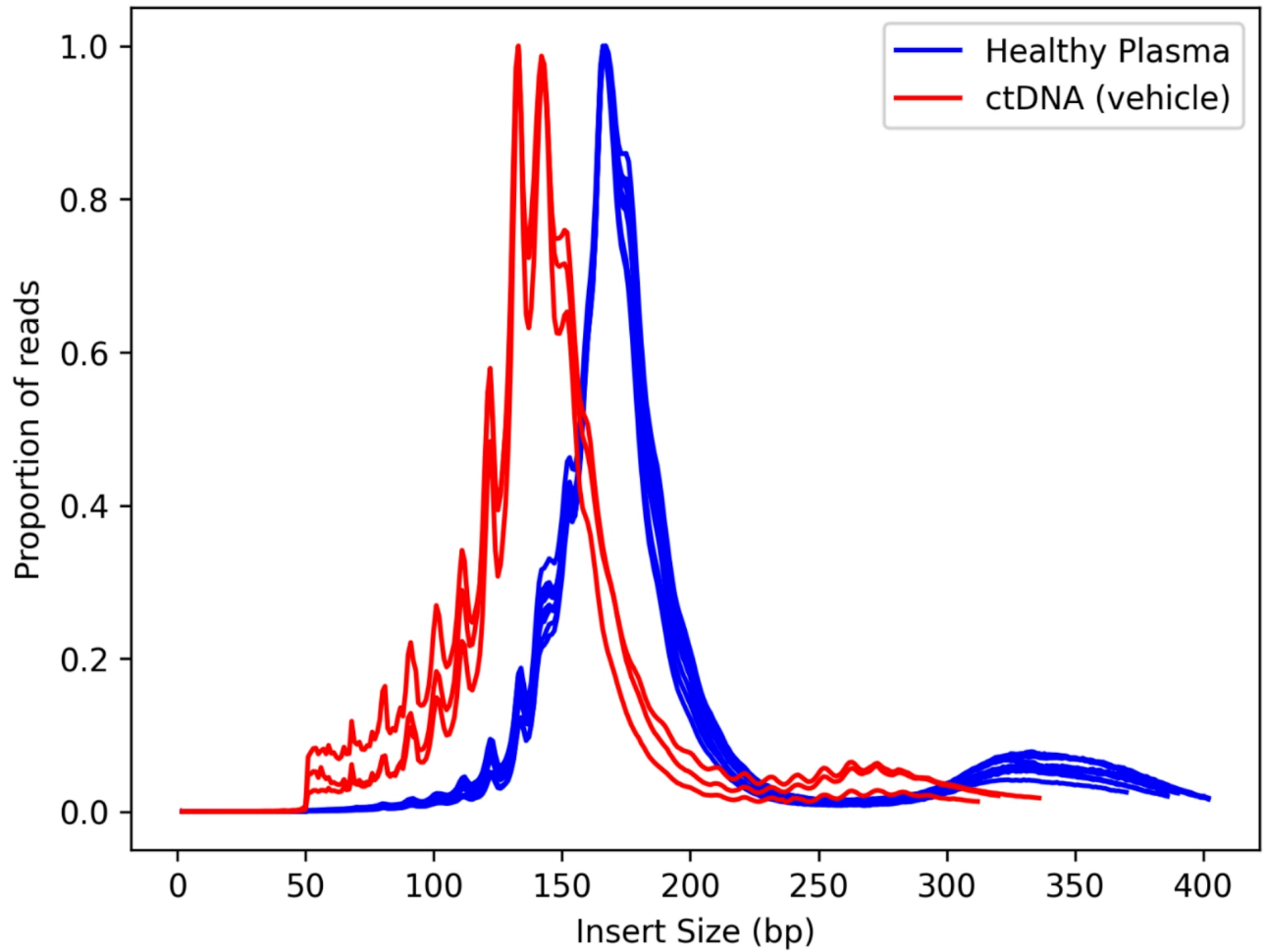


Figure 4

Fragment size distribution of cfDNA from vehicle-treated PDX samples versus healthy human donor reference. The vehicle arm shows a leftward shift indicating enrichment of shorter fragments relative to the healthy donor.

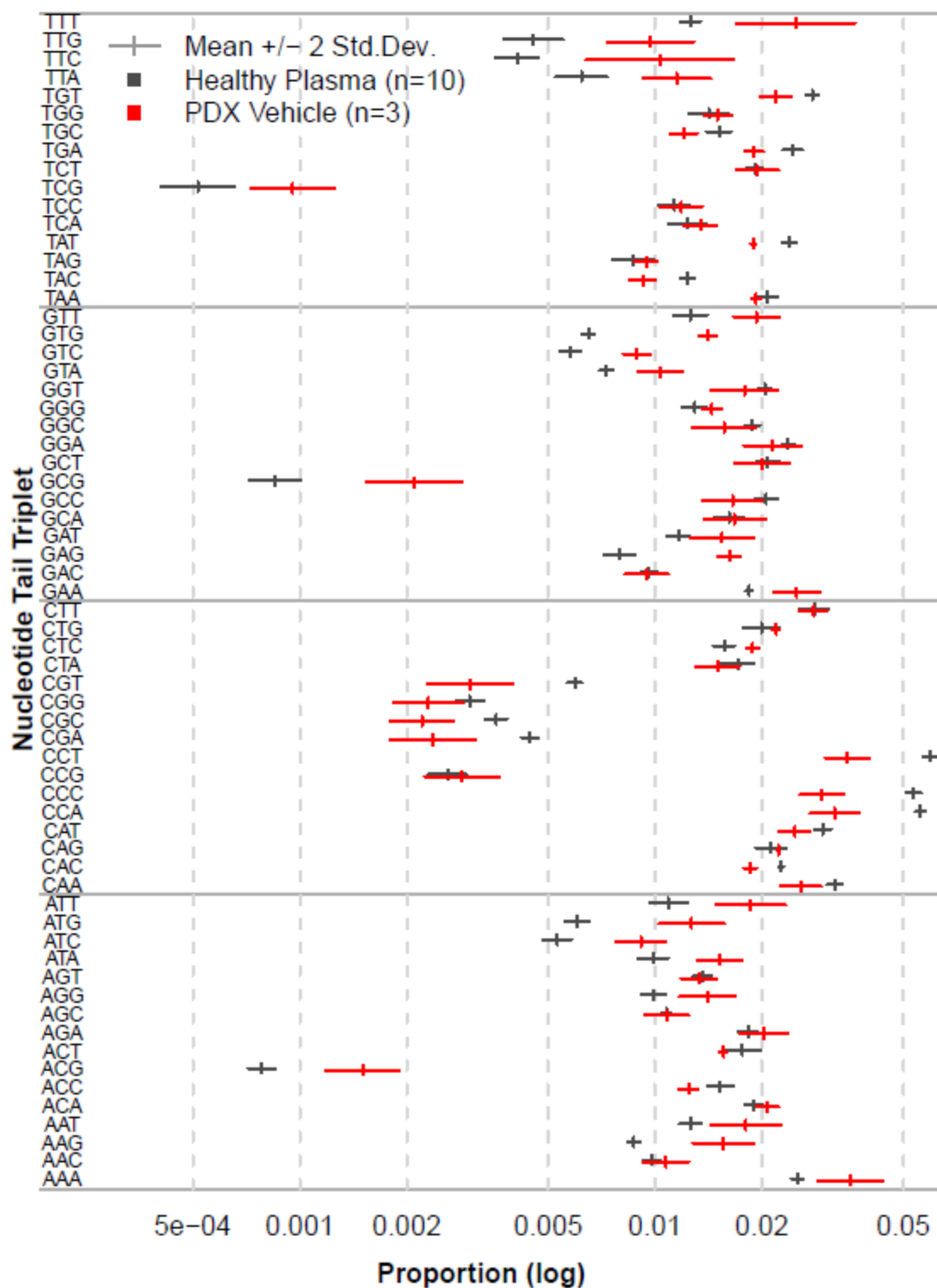


Figure 5

Aggregate trinucleotide end-motif data from healthy human donor plasma and ctDNA derived from the vehicle PDX group. Data shows relative proportion (log₁₀) of all trinucleotide configurations. Line length is determined by mean $\pm$ 2 standard deviations.

Supplementary Files

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