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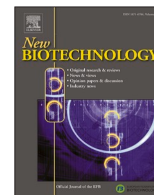

Interlaboratory evaluation of quality control methods for circulating cell-free DNA extraction

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ABSTRACT

Analysis of circulating cell-free DNA (ccfDNA) isolated from liquid biopsies is rapidly being implemented into clinical practice. However, diagnostic accuracy is significantly impacted by sample quality and standardised approaches for assessing the quality of ccfDNA are not yet established. In this study we evaluated the application of nucleic acid "spike-in" control materials to aid quality control (QC) and standardisation of cfDNA isolation for use in *in vitro* diagnostic assays. We describe an approach for the design and characterisation of in-process QC materials, illustrating it with a spike-in material containing an exogenous *Arabidopsis* sequence and DNA fragments approximating to ccfDNA and genomic DNA lengths. Protocols for inclusion of the spike-in material in plasma ccfDNA extraction and quantification of its recovery by digital PCR (dPCR) were assessed for their suitability for process QC in an inter-laboratory study between five expert laboratories, using a range of blood collection devices and ccfDNA extraction methods. The results successfully demonstrated that spiking plasmid-derived material into plasma did not deleteriously interfere with endogenous ccfDNA recovery. The approach performed consistently across a range of commonly-used extraction protocols and was able to highlight differences in efficiency and variability between the methods, with the dPCR quantification assay performing with good repeatability (generally CV <5%). We conclude that initial findings demonstrate that this approach appears "fit for purpose" and spike-in recovery can be combined with other extraction QC metrics for monitoring the performance of a process over time, or in the context of external quality assessment.

Introduction

Molecular *in vitro* diagnostics underpin many of the advances being made in precision medicine, however, the field suffers from a lack of standardisation with many pre-analytical and analytical processes currently lacking comparability between laboratories and methods [1]. Pre-analytical processes are associated with a high proportion of diagnostic errors [2] and are important to standardize for diagnostics and biobanking, especially for new classes of companion diagnostics based on liquid biopsy [3]. Nucleic acid (NA) extraction is a key part of a liquid biopsy-based diagnostic workflow and can potentially impact the accuracy and sensitivity of downstream analysis by inefficiency in NA recovery or lack of affinity for the targeted NA species. Biomarkers such as circulating tumor DNA are present at very low levels with a short fragment size of ≤ 167 bp, compared to longer 'contaminating' genomic DNA (gDNA) released by leukocytes [4,5]. Several studies have reported large variations in efficiency, yield, presence of co-purified inhibitors and bias associated with fragment size for different extraction methods [6–8], however accepted approaches and metrics for pre-analytical QC have not yet been established for routine laboratory use.

Abbreviations: ADH, alcohol dehydrogenase; ccfDNA, circulating cell-free DNA; CNA, QIAamp Circulating Nucleic Acid kit; Cq, quantification cycle; dPCR, digital PCR; DR, proprietary development/research method; EQA, external quality assurance; gDNA, genomic DNA; GE, genome equivalents; IVDR, In Vitro Diagnostic Regulation (EU) 2017/746; ME, QIAamp MinElute ccfDNA kit; NA, nucleic acid; PAX, PAXgene Blood ccfDNA blood tube; QC, quality control; RMP, reference measurement procedure; RSC, Maxwell® RSC ccfDNA Plasma kit; STK, Cell-Free DNA BCT blood tube (Streck); SYM, QIAsymphony DSP Circulating DNA kit.

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The EU FP7 and Horizon 2020 projects SPIDIA and SPIDIA4P aimed to standarise and improve pre-analytical procedures for *in vitro* diagnostics, with the latter project focussing on workflows applied to precision medicine [9]. The projects also support the implementation of the EU *In Vitro* Diagnostic Regulation (IVDR) which requires that manufacturers demonstrate the performance of tests including the "determination of appropriate criteria for specimen collection and handling" [10]. As well as developing CEN and ISO documentary standards specifying pre-analytical processes for a range of analytes [11], SPIDIA/SPIDIA4P also established external quality assessment schemes (EQAs) for liquid biopsy. The approach of using an "in-process" quality control (QC) material could be a potential performance indicator in EQAs for ccfDNA, where significant variability between laboratories in pre-analytical procedures has been observed [12].

The current study, conducted under SPIDIA4P, utilises the approach of spiking a fragmented plasmid material, containing a non-human/mammalian sequence, into sample plasma prior to ccfDNA extraction. This approach has previously shown promise for measuring ccfDNA extraction efficiency, linearity of extraction yield and bias associated with fragment size [6]. In the work described here, the candidate QC approach was further developed and its fitness for purpose assessed through an inter-laboratory study involving five expert laboratories. The sizes of the spike-in DNA fragment sizes were modified to 189 bp and 1009 bp, relevant to the discrimination of 'true' ccfDNA generated through apoptosis and mostly of short length (major peak ~180 bp) from contaminating gDNA originating from white blood cells, which has been described in studies as being of longer length (≥ 1 kb) [13]. A duplex dPCR assay was utilised for value assignment of the material and tested for ease of use as a QC procedure in the interlaboratory study, where the QC material was spiked into plasma prepared from alternative blood collection tubes and processed with a number of commonly used ccfDNA extraction methods. In addition, results from application of the spike-in material were compared with assays measuring endogenous ccfDNA by qPCR, fluorimetry and electrophoresis.

Materials and methods

Production and characterisation of the ADH spike-in material

Assay development

A duplex dPCR assay for the QX200 (Bio-Rad) dPCR system (Hercules, CA, USA) was developed using two assays, Adh-beta and Adh-delta, which detect target sequences in the *Arabidopsis thaliana* alcohol dehydrogenase (ADH) gene that are present on the 189 bp and 1009 bp ADH material fragments respectively Table M1-M2 (Supplementary File 1); SPIDIA4P Task 2.3 Interlaboratory Study Protocol dPCR (Supplementary File 2A). The assays were tested in both uniplex and duplex to confirm that the duplex format did not lead to a reduction in copy number concentration results. A protocol for performing a duplex Adh-beta / Adh-delta qPCR assay was prepared, SPIDIA4P Task 2.3 Interlaboratory Study Protocol dPCR (Supplementary File 2B) based on a previous publication [14].

Production of the material

The ADH material plasmid pSP64/ADH (stock prepared by Eurofins, Ebersberg, Germany) was digested with *Bpm*I (Part no. R0565S) (10 U) and *Nsp*I (Part no. R0602S) (20 U) using CutSmart Buffer (Part No. R0602) (NEB, Ipswich, MA, USA) in final volume of 50 µL containing 3.7 µg plasmid DNA for 60 min at 37 °C followed by enzyme inactivation (20 min at 80 °C). The digested plasmid was diluted in 1 ng/µL yeast tRNA (Part No. AM7119, Thermo Fisher Scientific (Carlsbad, CA, USA)) to an approximate concentration of 10^6 copies/µL. Accurate quantification of the digested ADH material was performed by dPCR prior to the preparation of the study material. Three hundreds units of ADH Control Material containing ~ 2.5 × 10^4 copies/µL in a background of 1 ng/µL yeast tRNA (total volume per unit 300 µL) were produced and stored at − 20 °C. The material was evaluated for homogeneity and stability and value assigned as described below.

Homogeneity

The homogeneity of the ADH material (Suppl. Fig. S1) was evaluated according to ISO Guide 35 [15] by measuring 10 units of the material by duplex dPCR (n = 8 replicate assays per unit) using a randomised plate layout. Homogeneity was assessed by 1-way ANOVA using Graphpad Prism v9.4 (Boston, MA, USA) with variation between units (s_b) calculated by comparing total variation (MS_b) to the residual variation corresponding to dPCR measurement repeatability (s_r). A significant difference between units was found for analysis of both Adh-beta and Adh-delta ($p < 0.001$). Modest relative variation between unit (s_b) of 4% (Adh-beta) and 3% (Adh-delta) was observed. No significant difference in the copy number concentration of the Adh-beta and Adh-delta containing plasmid fragments was observed.

Value assignment

The assigned value of the ADH material was calculated as the mean copy number concentration of the homogeneity study data (2.1 ×10^4 copies/µL). The measurement uncertainty of the assigned value was calculated by combining the variances due to inhomogeneity (average between unit SD of 865 copies copies/µL) and repeatability (average repeatability SD of 512 copies/µL) divided by the average number of measurements per unit. The standard combined uncertainty (883 copies/µL) was converted to an expanded uncertainty (0.2 ×10^4 copies/µL, 95% confidence) using a coverage factor (k) of 2.26 based on the degrees of freedom (9) associated with the number of units evaluated (n = 10) (95% confidence range of assigned value 1.9–2.3 ×10^4 copies/µL).

Stability

The stability of the material was evaluated by measuring independent tubes of the material 5 months and 7 months after production (Suppl. Fig. S2). Measurements of the Adh-beta and Adh-delta containing fragments were within the assigned value uncertainty range (1.9–2.3 ×10^4 copies/µL), indicating that the ADH material was stable for the duration of the inter-laboratory study.

Interlaboratory assessment

Design

In order to assess the factors which could affect the performance of the ADH material as a spike-in control for extraction of ccfDNA from plasma, laboratories sourced six individual plasma samples each (Fig. 1). In addition, laboratories were able to select the type of blood collection tube and ccfDNA extraction method to be used in line with routine practice. Each plasma sample was processed with or without spiking the ADH material in order to test whether the additional nucleic acids (plasmid and carrier yeast tRNA) influenced the recovery of endogenous cfDNA. The reproducibility of ADH duplex analytical methods (dPCR and qPCR) for measuring recovery of the ADH material was also tested by dividing each sample eluate into two aliquots, with half being analysed by the participant and half being analysed by the lead laboratory. To support evaluation of the metrics associated with the ADH material with other commonly used sample QC approaches for measuring the endogenous ccfDNA, laboratories were invited to perform additional analysis (such as fluorimetry or qPCR measuring a human genomic reference) with their half of the sample eluate which could be compared to the ALUJ qPCR performed at the lead laboratory [16] (Table M1, M4 (Supplementary File 1)).

Study protocols

Protocols were developed based on previous publications [6,17] for spiking the ADH control into plasma (20 µL/extraction). dPCR and qPCR protocols were also provided with a suggested plate layout

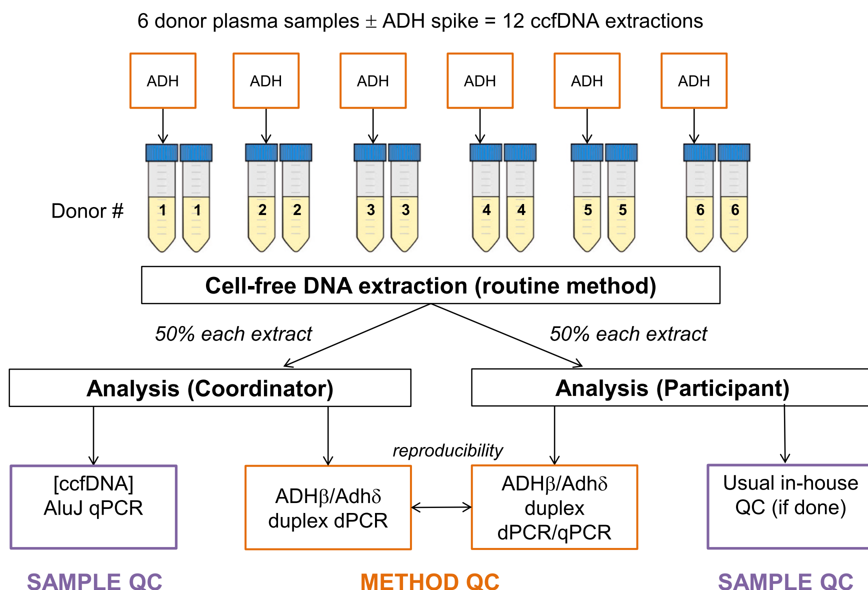

Fig. 1. Inter-laboratory study design. Each participating laboratory performed 12 ccfDNA extractions from 6 donor blood samples, with paired extractions with/without spiked with the ADH control material ('ADH'). Half of the sample eluate was retained by the participant and analyzed by the participant using the Adh quantification assay ('Adhβ/Adhδ') in dPCR or qPCR format and half of the eluate sent to the coordinating laboratory for replicate dPCR measurements. In some cases, participants also performed alternative QC methods measuring the endogeous ccfDNA. The coordinator also measured the concentration of ccfDNA by AluJ qPCR.

(Supplementary File 2). Participants were asked to freeze their ccfDNA eluates following extraction in order that the samples analysed by the participant would have undergone the same number of freeze-thaw cycles (1) as those analysed at the lead laboratory (Fig. 1). The participants were asked to use the remainder of the same tube of ADH spike-in material as a control (n = 6) within the dPCR or qPCR analysis as a control for calculating the extraction efficiency and checking the performance of the dPCR assay at the participant's laboratory. dPCR users were provided with a Quantasoft template file which enabled data exported from Quantasoft software to be imported into the Study Protocol excel file. qPCR users were provided with a Data Analysis sheet which calculated the ADH copy number per reaction and %recovery of the ADH based on the assigned value of the material and mean Cq value of the control reactions respectively. A deltaCq calculation was applied using the published PCR efficiencies of the Adh-beta and Adh-delta assays [6]. The dPCR and qPCR Study Protocols also included a Reporting form to capture details of the extraction method, dPCR equipment and consumables and report fluorescence amplification plots for positive control, samples and negative controls.

Participation

Five expert laboratories participated in the study using three types of blood collection tubes and five cfDNA extraction approaches (Table 1). Additional method information is available in Supplementary File 1.

Coordinator's analysis of interlaboratory samples

The coordinating laboratory received 50% of each extract from the participating laboratories (Fig. 1). The ADH copy number concentration and recovery of the short (189 bp) and long (1009 bp) plasmid fragments was measured in each extract using the ADH-beta and -delta assays. Endogenous cfDNA concentration was measured using a qPCR assay to AluJ sequences [16]. Primer/probe sequences, thermal cycling conditions and qPCR standard curve information are provided in 'Coordinating Laboratory dPCR and qPCR Methods' section in

Table 1
Participation in Inter-laboratory study.

Laboratory	Workflow Number*	Blood tube	Extraction method (Abbreviation)	ADH analysis method (s)	Additional analysis
Lab 1	1	PAXgene Blood ccfDNA (QIAGEN, Hilden, Germany) (**PAX**)	QIAamp Circulating Nucleic Acid kit (QIAGEN) (**CNA**)	dPCR	Cell-free DNA ScreenTape /Tapestation (Agilent Technologies, Santa Clara, CA, USA)
	2	Cell-Free DNA BCT (Streck, La Vista, NE, USA) (**STK**)	CNA		
Lab 2	3	EDTA	CNA	dPCR	N/A
	4	EDTA	Maxwell® RSC ccfDNA Plasma kit (Promega, Madison, WI, USA) (**RSC**)		
Lab 3	5	EDTA	QIAamp MinElute ccfDNA (QIAGEN) (**ME**)	qPCR	QuantiPlex Pro qPCR (91/353 bp amplicons) (QIAGEN)
	6	PAXgene Blood ccfDNA (PAX)	ME		
	7	EDTA	Proprietary development/research method (**DR**)		
Lab 4	8	STK	QIAsymphony DSP Circulating DNA kit (QIAGEN) (**SYM**)	dPCR and qPCR	Fluorimetry: Qubit HS DNA (Thermo Fisher Scientific)
Lab 5	4	EDTA	RSC	dPCR	Fluorimetry: Quantus DNA One (Promega) Alu qPCR (83/244 bp amplicons[35])

* Workflow is numbered based on combination of blood collection and ccfDNA extraction method; both Laboratories 2 and 5 applied the same workflow (4).

Supplementary Methods.

Statistical analysis

Linear regression analysis of ccfDNA concentration values (measured with the AluJ qPCR assay) for spiked vs. non-spiked plasma samples was performed with robust linear regression (due to the non-normal data distribution and presence of outliers) using the 'rlm' function in R version 4.2.1 [18]. The remaining statistical analysis was performed using Graphpad Prism v9.4. Participants' and coordinator's measurment of ADH spike-in 189 and 1009 bp fragments were analysed by 2-way ANOVA with repeated measures (paired ccfDNA extracts) with Sidak's post-hoc test to compare each participant laboratory's with the coordinator's values.

ADH spike-in recovery (extraction efficiency) and ADH spike-in 189/1009 bp ratio results showed inequal variance between groups and were therefore analysed by Brown-Forsythe ANOVA test. Dunnett's T3 multiple comparison test was used to compare each laboratory/workflow with the established EDTA-CNA workflow (Laboratory 2 workflow 1, Table 1) as a point of reference.

Alternative analytical methods for quantification of ccfDNA in samples from Laboratory 3 (AluJ qPCR and Quantiplex Pro 91 bp amplicon qPCR) were analysed by mixed effects model with matching between plasma samples (across workflows) and between extracts (between analytical methods), with Sidak's post-hoc test to compare between AluJ and Quantiplex Pro for each of the three workflows at Laboratory 3. Alternative analytical methods for quantification of ccfDNA (AluJ qPCR and Qubit fluorimetry) in samples from Laboratory 4 were analysed by paired *t*-test. Alternative analytical methods for quantification of ccfDNA in samples from Laboratory 5 (AluJ qPCR, Alu 83 bp qPCR and Quantus fluorimetry) were analysed by one-way

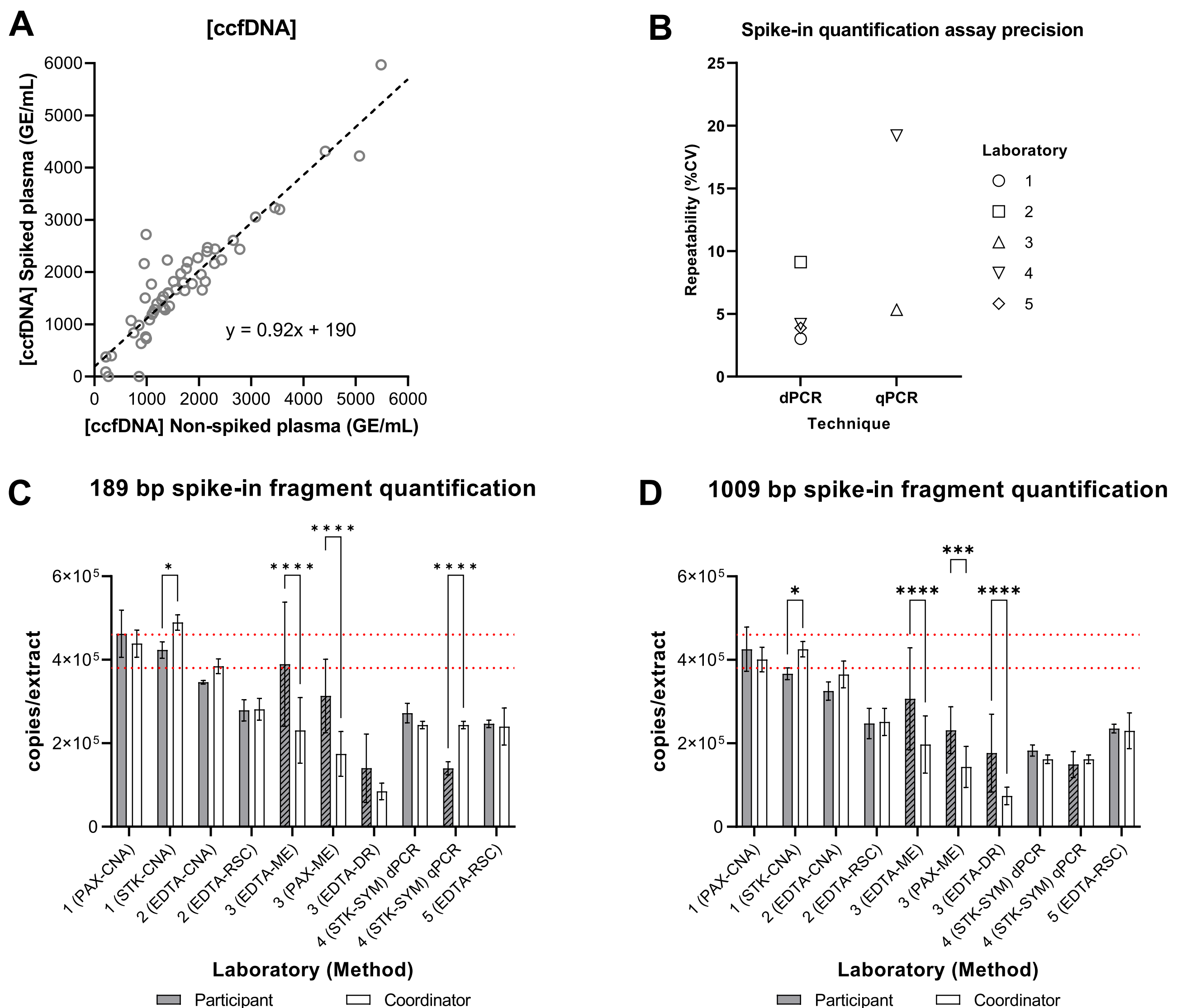

Fig. 2. Performance of spike-in control material and quantification assays. (A) Impact of spiking ADH material on ccfDNA purification from plasma. Concentration of ccfDNA (expressed as haploid Genome Equivalents (GE)/mL) was measured by AluJ qPCR and results for the same plasma sample plotted for extraction with/without spiking the ADH material (all laboratories, $n = 108$ samples). Robust linear regression was performed (best-fit line and equation shown on graph). (B) Intralaboratory precision of Adhβ assay for quantification of short (189 bp) ADH fragment in the non-spiked ADH material. %CV is shown for each participating laboratory (number of replicate dPCR/qPCR reactions: Laboratories 1–2, $n = 12$; Laboratories 3–4, $n = 6$ and Laboratory 5, n = 4 due to two wells being excluded with a dPCR droplet count <10,000). (C-D) Inter-laboratory reproducibility of short (C) and long (D) ADH fragment measurements (mean ± SD ($n = 6$ extractions with the exception of Laboratory 3 workflows PAX-ME and EDTA-DR where results for $n = 5$ shown for both participant and coordinator due to insufficient eluate for analysis at participant laboratory). Solid grey bars show dPCR measurments; hashed grey bars show qPCR measurements. Red dashed line show expanded uncertainty (95% confidence) of ADH control material reference value. Significant differences between the participant and coordinating laboratory are indicated with asterisks: * $p < 0.05$, * ** $p < 0.001$, * ** * $p < 0.0001$.

ANOVA with repeated measures (sample extract), with posthoc analysis using Dunnett's comparison test. Alternative analytical methods for % short ccfDNA (Tapestation, Quantiplex Pro qPCR and Alu amplicon qPCR) were only compared within laboratory as all laboratories processed different plasma samples. No significant differences between % short ccfDNA were found between workflows within laboratory (laboratory 1 and 3).

Results

Performance of spike-in material and dPCR/qPCR quantification assays

All participanting laboratories were able to follow the protocols provided by the coordinating laboratory and perform ccfDNA extractions spiked with the ADH material and measure ADH short (189 bp) and long (1009 bp) plasmid fragments using the Adh-beta and Adh-delta assays respectively by dPCR (4 laboratories) or qPCR (2 laboratories; Laboratory 4 performed both). The performance of the ADH spike-in material and associated duplex dPCR and qPCR assays for quantification of each ADH fragment (189 bp and 1009 bp) was assessed (Fig. 2). In aliquots of each extract sent by the participants to the coordinating laboratory ($n = 108$), the impact of spiking the ADH material was assessed by comparing endogenous ccfDNA concentrations in paired extractions from the same plasma sample with- and without- spiking the material (Suppl. Fig. S3A). There was good correlation between extraction yields with and without ADH (slope of 0.92 (95% confidence interval: 0.84–0.99)). The ratio of the [ccfDNA] with and without the ADH material was close to 1.0 for the majority of laboratory workflows (Suppl. Fig. S3), with a median ratio of 1.06 (25th to 75th percentile, 0.94–1.16) from all paired extractions, indicating that for the methods tested, addition of the ADH material did not influence the process of ccfDNA purification.

The analytical precision of the ADH dPCR and qPCR assays were evaluated based on control (non-spiked) ADH material analysed in each experiment by participants (Fig. 2B). dPCR repeatability (%CV) was $< 5\%$ in three of the four participating laboratories using this approach

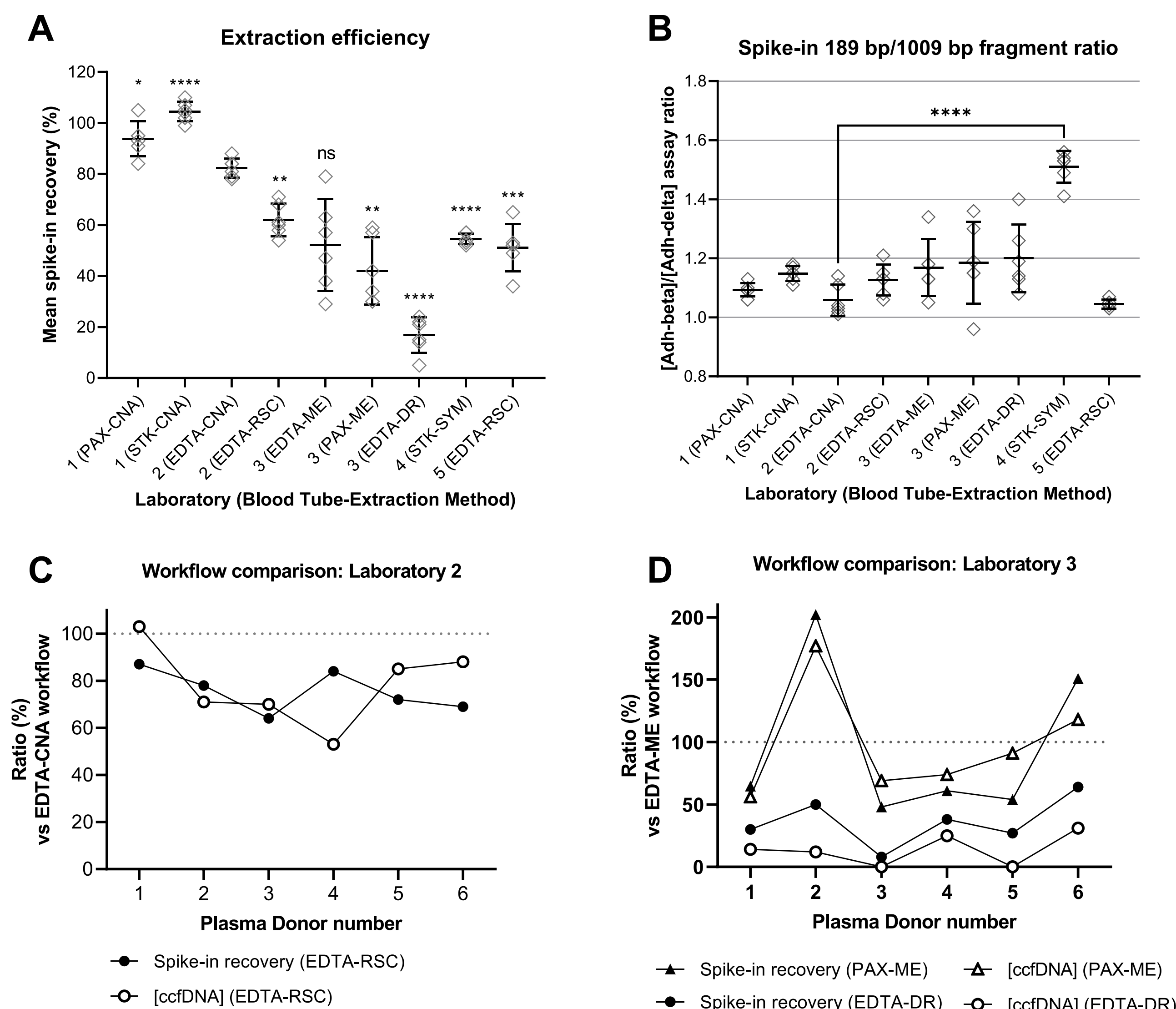

Fig. 3. Extraction QC metrics based on spike-in recovery. (A) Extraction efficiency for each laboratory and workflow shown as % ADH spike-in recovery (ratio of ADH (189 bp) concentration in sample eluate to the reference value in parallel measurements of the same unit of non-spiked ADH control). The significance of differences in extraction efficiencies compared to EDTA-CNA (Laboratory 2) are shown with asterisks. (B) Ratio of short to long ADH plasmid fragments for each laboratory and workflow. Mean ± SD shown ($n = 6$ extractions). The significance of differences in short/long fragment ratio compared to EDTA-CNA (Laboratory 2) are shown with asterisks. (C,D) Comparison of % spike-in recovery and [ccfDNA] measured by AluJ qPCR for alternative pre-analytical workflows: (C) Laboratory 2: metrics for EDTA-RSC workflow shown relative to EDTA-CNA workflow. (D) Laboratory 3: metrics for PAX-ME and EDTA-DR workflows shown relative to EDTA-ME workflow for the same donor. Datapoints reflect single measurements. * $p < 0.05$, * * $p < 0.01$, * ** $p < 0.001$, * ** * $p < 0.0001$, ns, not signficant.

and < 10% in the other laboratory, compared to qPCR repeatabilities of 5% in one laboratory and 19% in another. The interlaboratory reproducibility of ADH quantification was assessed by comparison of the participants' and coordinator's measurements of short and long ADH fragments (Fig. 2C-D). dPCR values generally agreed well between the participant and coordinating laboratories; only small difference between spike-in concentrations measured at Laboratory 2 vs. coordinator for the STK-CNA (workflow 2) samples was noted. In the case of participants using qPCR analysis, laboratory 3′s qPCR values tended to be higher than the corresponding coodinator's dPCR measurements. In contrast, laboratory 4′s qPCR measurements of the 189 bp ADH fragment, but not the 1009 bp ADH fragment, were ~50% lower than those of the dPCR values. As the qPCR measurements were less reproducible than the dPCR-based values, dPCR values from the coordinator's analysis were used for evaluation of the ADH spike-in as a cfDNA extraction QC material.

Spike-in extraction metrics and comparison with endogenous cfDNA yield

The extraction efficiency for each laboratory and each workflow was calculated based on the recovery of the ADH material as a percentage of the value of the control (analysed in the same dPCR experiment as the extracts) (Fig. 3A). The average extraction efficiency of the CNA kit was > 80% for the three sets of extractions using this method. As a frequently used and established workflow, the use of EDTA blood collection tubes with the CNA extraction kit was selected as a reference for comparison with alternative workflows (Fig. 3A). Automated workflows showed similar extraction efficiencies: RSC kit (62% and 51% at laboratories 2 and 5 respectively) and SYM method (55% used at Laboratory 4). For the three workflows tested at Laboratory 3, the ME extractions tended to be more efficient (52% and 42% for EDTA and PAX blood collection tubes) than those with the DR kit (17%). As well as being an indicator of yield, the ADH spike-in recovery reflected extraction variability, with the workflows with lower yield based on %ADH recovery also more variable based on %CV between replicate extractions (Table 2). The ratio of the ADH 189–1009 bp plasmid fragment copy number concentrations was calculated to evaluate whether any workflow showed a bias towards recovery of short *vs.* long DNA fragments (Fig. 3**B**). The majority of workflows showed an average ADH short/long fragment ratio of between 1.05 and 1.2 however a higher ADH fragment ratio (1.5) was observed for the workflow at Laboratory 4 ($p < 0.0001$) indicating preferential isolation of shorter molecules. Three laboratories (1, 2 and 3) performed paired extractions from the same donor with multiple pre-analytical workflows. Intralaboratory differences in average %ADH recovery were observed between workflows at Laboratories 2 and 3 (Fig. 3A), therefore it was evaluated whether these were mirrored by the yield of endogenous ccfDNA in the sample extracts (Fig. 3C-D). The lower %ADH recovery in the RSC method extractions at Laboratory 2 was accompanied by a reduction in endogeous ccfDNA yield in the majority of extracts (Fig. 3C). Comparison of the PAX-ME and EDTA-DR workflows with the EDTA-ME workflow at Laboratory 3 also showed that %ADH recovery followed the same trend as yield of ccfDNA for individual cfDNA extracts (Fig. 3D). This suggests that the ADH recovery can be an indicator of sub-optimal performance of a ccfDNA extraction workflow.

Table 2
Extraction repeatability based on %ADH recovery. Intra-laboratory repeatability was estimated based on 6 extractions.

Laboratory	Blood tube	Extraction method	Repeatability (%CV) ADH recovery
1	PAX	CNA	7%
	STK	CNA	4%
2	EDTA	CNA	5%
	EDTA	RSC	10%
3	EDTA	ME	35%
	PAX	ME	31%
	EDTA	DR	42%
4	STK	SYM	4%
5	EDTA	RSC	18%

Alternative QC metrics for cfDNA extraction

Participating laboratories performed a range of sample QC approaches measuring both the quantity and fragment size profile of cfDNA extracts (Fig. 4). The coordinating laboratory also quantified all extracts using AluJ qPCR. The concentrations of ccfDNA measured by Quantiplex Pro qPCR at Laboratory 3 were in close agreement with AluJ qPCR (Fig. 4A), confirmed by correlation analysis (Suppl. Fig. S4A) (slope = 0.72; $R^2 = 0.87$). Laboratories 4 and 5 quantified sample extracts by fluorimetry (Qubit and Quantus fluorimeters respectively). DNA concentration values reported by the Qubit HS DNA fluorimetric assay tended to be approximately 2-fold higher than those measured by AluJ qPCR (Fig. 4B), whilst the Quantus assay measurements were approximately 2-fold lower than the AluJ qPCR measurements (Fig. 4C). Laboratory 5 also measured ccfDNA concentration using an *Alu* assay with an 83 bp amplicon: this gave results approximately double those of the coordinator's AluJ assay, which comprises a 94 bp amplicon (Fig. 4C; slope = 2.1; $R^2 = 0.85$ (Suppl. Fig. S4B)). Three approaches were used to analyse ccfDNA fragment size profile (Fig. 4D): cfDNA ScreenTape (Agilent Technologies) (Laboratory 1), which estimates "% cfDNA" based on the proportion of DNA < 700 bp; the Quantiplex Pro 91 bp/353 bp amplicon qPCR assays (Laboratory 3) and *Alu* 83 bp/244 bp amplicon assays (Laboratory 5), which provide metrics for % shorter DNA from the quantities measured by the respective shorter and longer amplicons. Whilst the analyses cannot be compared directly as they relate to extractions from different plasma samples, all assays suggest that the purified samples contain a majority of shorter DNA fragments. In keeping with the different size ranges included in ratios of short to total ccfDNA by the cfDNA ScreenTape (< 700 bp), Quantiplex Pro (< 353 bp) and Alu assay (<244 bp), the ScreenTape-based % short fragments was lower (60–88%) than the Quantiplex Pro- and Alu-based proportions (79–95% and 82–90% respectively). No significant differences in fragment size ratios were found between the two workflows at Laboratory 1 or between workflows at Laboratory 3.

Discussion

Recent studies and reviews have highlighted the importance of pre-analytical variables in the translation of liquid biopsy approaches into the clinic [8,19–21]. Spike-in controls have proved a useful approach in characterizing pre-analytical variables such as biofluid source (plasma/serum), blood tube stabilizing agents and extraction kit, as well as effects on variant allelic frequency, copy number variation or microRNA quantification [21–27] and recent International Federation of Clinical Chemistry and Laboratory Medicine cfDNA Working Group guidance "highly [recommends]. that an internal non-human DNA standard [is] used to control for DNA extraction efficiency" [19].

Our inter-laboratory assessment of a DNA spike-in-based QC approach successfully demonstrates that the addition of such non-human internal standards in a relatively low concentration of yeast tRNA carrier (20 ng per extraction) does not interfere with extraction or detection of endogenous ccfDNA using the common kits and blood tubes assessed in this study. The workflow applying the spike-in material can be readily incorporated into routine laboratory use when provided with a detailed protocol and a validated assay for quantification of the recovered spike-in molecules post-extraction. The duplex dPCR assay detecting the 189 bp and 1009 bp spike-in fragments enables users to measure the recovery of both fragments with minimal usage of sample and was found to show good precision within laboratory. The paired analysis of samples at both the participants' and coordinator's laboratories also demonstrated high interlaboratory reproducibility of the

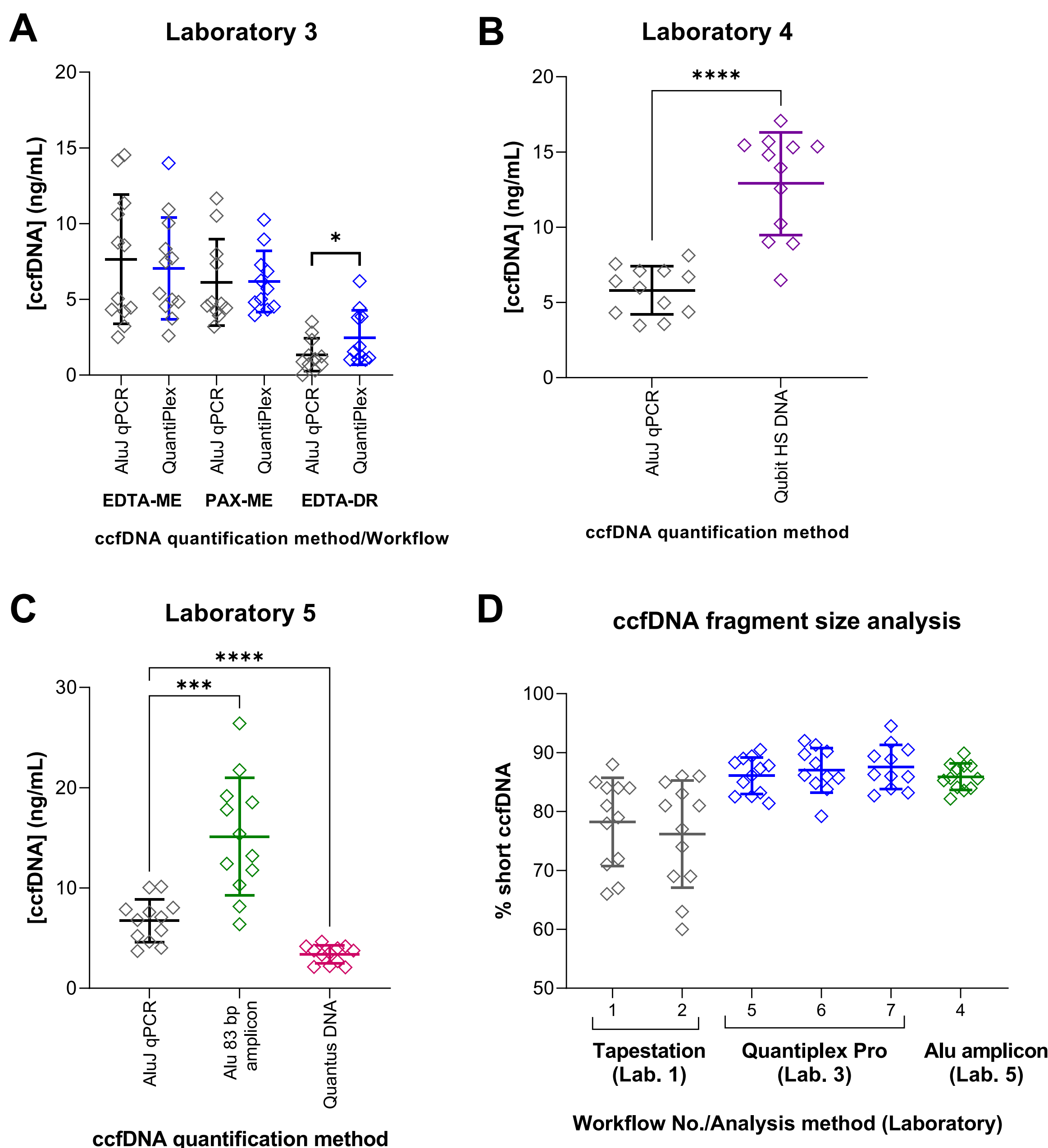

Fig. 4. Comparison of ccfDNA yield and fragment size metrics. (A-C) Comparison of AluJ qPCR (94 bp amplicon) (coordinator's laboratory) and alternative quantification methods for total ccfDNA (A) Quantiplex Pro (91 bp amplicon); (B) Qubit High Sensitivity DNA assay (Invitrogen); (C) Alu 83 bp amplicon [35], Quantus DNA One assay (Promega). (D) Fragment size metrics based on ratio of short to total DNA based on Cell-free DNA Screentape (% DNA < 700 bp/total DNA); qPCR assays calculated as 1-($Q_{long\ amplicon}/Q_{short\ amplicon}$) where $Q_{long\ amplicon}$ is the quantity of DNA measured by the 353 bp amplicon (Quantiplex Pro) or 244 bp (Alu amplicon assay) and $Q_{short\ amplicon}$ is the quantity measured by the 91 bp amplicon (Quantiplex Pro) or 83 bp Alu amplicon. For Workflow number, see Table 1. Datapoints reflect single measurements with line and error bars showing mean and SD (A-C), based on $n = 12$ extractions with except of Laboratory 3: PAX-ME and EDTA-DR $n = 11$ (missing data due to limited eluate). The significance of differences in estimates of ccfDNA yield of the same set of plasma samples (A-C) measured with alternative analytical methods are shown with asterisks $p < 0.05$, * ** $p < 0.001$, * ** * $p < 0.0001$.

quantification assay, consistent with other studies of dPCR method performance [28]. Goh et al. also applied dPCR for the quantification of a spike-in controlling for cfDNA extraction and bisulfite modification, and found that it was a highly accurate method [29]. Moreover, as dPCR is a candidate reference measurement procedure (RMP) for DNA copy number concentration traceable to the International System (SI) of units [30,31], the approach outlined in the current study for value-assignment of the QC material, offers the potential benefit of long-term metrological traceability. The traceability of calibrators and QC materials to higher order RMPs or reference materials is another performance criterion stipulated by the IVDR [10].

The results of the qPCR assay for ADH spike-in quantification were less consistent than those for the dPCR approach. Unlike the dPCR method, the qPCR requires normalization to a non-spiked control, which was performed using a PCR efficiency-corrected delta-Cq approach [32]. Differences in the amplification of the cfDNA extracts compared to the control may have lead to variability in the qPCR copy number concentration measurements (for example, some cfDNA extraction methods such as the CNA contain high levels of carrier RNA which may influence qPCR amplification profile). A standard curve-based quantification approach which controls for plate-to-plate variation in PCR efficiency or an ADH reference control in a background specific to the extraction kit

being monitored may overcome such problems.

The exogenous spike-in method was amenable to integration into workflows using plasma from commonly-used blood collection tubes, including plasma from both EDTA and preservative blood tubes (STK and PAXgene), and a range of ccfDNA extraction kits, including both manual column-based approaches (CNA, ME) and automated magnetic bead-based methods (RSC and QIAsymphony). The approach was able to highlight differences between the kits and provide QC metrics for cfDNA extraction. The differences seen between kits in terms of ADH fragment recovery mirrored the recovery of endogenous ccfDNA across kits when comparing workflows at two participating laboratories, indicating that the material is capable of behaving in a similar manner to endogenous cfDNA. QC metrics based on the recovery of one or both ADH fragments include % extraction efficiency based on recovery compared to input, and the ratio of short (189 bp) to long (1009 bp) fragments monitors the affinity of the extraction adsorption matrix for DNA molecules which approximate to cfDNA of ~ 1 nucleosome length and contaminating gDNA. The ~1:1 ratio of short/long fragments for the CNA kit in this study is consistent with previous evaluations of this kit [6,17]. The ME and RSC extraction methods used in this study also showed a similar ADH ratio, whilst fewer 1009 bp ADH spike-in molecules compared to the 189 bp spike-in were measured following QIAsymphony ccfDNA isolation. The ADH fragment size ratio would be expected to reflect technical performance of extraction methods and affinity for shorter *vs*. longer molecules. This is complementary to endogenous ccfDNA fragment size analysis but cannot fully replace it as additional information reflecting pre-analytical steps such as storage and centrifugation steps, as well as intrinsic biological factors, is provided by analysis of the size distribution of endogenous ccfDNA fragments. As well as electrophoretic analysis, amplicon size-based qPCR (such as the QuantiPlex Pro and Alu 83/244 bp assays tested here) and dPCR methods are useful DNA fragment size monitoring approaches [8,33–37]. New studies indicate that "true" ccfDNA can also be of longer length beyond 250 bp [38].

The ability to spike in a relatively high copy number of the internal control (4.2×10^5 copies/extraction) compared to the endogenous ccfDNA genomic copy number (typically 10^3 GE/mL) enables extraction performance to be monitored more precisely than endogenous ccfDNA measurements alone. Based on the spike-in control, the majority of workflows showed an extraction efficiency > 50% with a repeatability (%CV) < 20%, which is comparable with other studies using spiked cell-line DNA [39]. DNA spike-in controls have also been utilised to evaluate cfDNA extraction in alternative liquid biopsy matrices, such as urine [40], cerebrospinal fluid [41] and bile [42], however the stabilization and processing of cfDNA from these biofluids is less standardized, therefore the interaction between the biological or stabilisation matrix and the non-protein bound spike-in molecules (for example, due to pH) should be considered in establishing QC procedures in these areas.

The spike-in recovery and extraction repeatability results provided by the current study may be utilised in developing performance specifications for ccfDNA procedures [43] and could form an initial stage in the development of control charts for laboratory internal QC combining the exogenous spike-in recovery metrics with results from other QC materials containing cancer variant sequences [8,44]. The current inter-laboratory feasibility study only measured the recovery of spike-in material in single experiment at each of the participating laboratories. Between-day and between-operator comparisons are required to establish robust intermediate precision measures and to fully explore the potential of the materials for highlighting sub-optimal performance of a method. Introduction of intentional perturbations in to the process to mimic sub-optimal conditions may help to identify the extent to which the materials can highlight issues.

Conclusion

This study had the overall aim to characterise and test "in process" quality control materials to monitor efficiency and reproducibility of extraction methods, and support comparability and standardisation of nucleic acid extractions. The study outlines a process for generation of spike-in control materials of ccfDNA-relevant fragment size and characterisation using dPCR, which, through the availability of published spike-in sequences and assays, may form the basis of SI-traceable approaches for monitoring cfDNA isolation. Protocols for spiking into plasma and dPCR measurement by laboratory end-users were shown to be robust and demonstrate "fitness for purpose" across multiple laboratories, ccfDNA extraction kits and blood collection tubes. This cfDNA extraction QC approach can form the basis for the development of control charts which combine exogenous spike-in metrics with other extraction QC metrics for highlighting sub-optimal peformance of a method or gradual "drift" in the performance of a process over time, or could also be provided with EQA samples as an additional metric of laboratory performance.

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CRediT authorship contribution statement

AD and CF conceived the study; AD, GJ and CF developed extraction and dPCR methodology; OK, PP and SB provided plasma samples. GJ, AFG, OK, JT, SG and SB performed ccfDNA extraction and/or qPCR/dPCR experiments. AD performed data analysis and visualization. AD and PP supervised the work on the project. AD, OK and CF contributed to project administration. CF acquired funding. AD and CF drafted the manuscript. AD, OK, PP, SB and CF reviewed and edited the manuscript. All authors have read and approved the final manuscript.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Alison Devonshire reports a relationship with Roche that includes: paid expert testimony.

Data Availability

Data is available in Supplementary File 4. Raw data available upon request. Declaration of Competing Interest

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2023.09.005.

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