Standardization of purification, detection, and reporting results of ctDNA analyses

- A guideline from the ctDNA Research Center

DCCctDNA ResearchCenter

January 2025

Standardization of purification, detection, and reporting results of ctDNA analyses

Published January 2025 by

The DCCC ctDNA Research Center

Table of Content

Working group

Ane Yde Schmidt (Dept. of Genomic Medicine, Rigshospitalet)

Cecilie Mondrup Jacobsen (Dept. of Biochemistry and Immunology, Vejle Hospital)

Estrid Høgdall (Dept. of Pathology, Herlev and Gentofte Hospital)

Laura Iisager (Dept. of Molecular Medicine (MOMA), Aarhus University Hospital, Aarhus University)

Lise Barlebo Ahlborn (Dept. of Genomic Medicine, Rigshospitalet)

Luisa Matos Do Canto (Dept. of Biochemistry and Immunology, Vejle Hospital)

Mads Heilskov Rasmussen (Dept. of Molecular Medicine (MOMA), Aarhus University Hospital, Aarhus University)

Maria Hønholt Jørgensen (Dept. of Molecular Medicine (MOMA), Aarhus University Hospital, Aarhus University)

Niels Pallisgaard (Dept. of Pathology, Næstved Hospital)

Rikke Fredslund Andersen (Dept. of Biochemistry and Immunology, Vejle Hospital)

Simone Karlsson Terp (Dept. of Molecular Diagnostics, Aalborg University Hospital)

Stig Egil Bojesen (Dept. of Clinical Biochemistry, Herlev and Gentofte Hospital)

Søren Egstrand (Dept. of Clinical Biochemistry, Herlev and Gentofte Hospital)

1 Background

The current work constitutes the first Danish national recommendations to laboratories in implementing and conducting cell-free tumor DNA (ctDNA) detection. The working group was appointed by the Danish National Center for Circulating Tumor DNA Guided Cancer Treatment. The paper will cover the pre-analytical factors to consider, the analytical quality control of ctDNA detection, and give recommendations to reporting the results to the physicians. This paper will not review the clinical aspects in appropriately timing of blood-sampling, e.g., in relation to medical or surgical treatment, or the current evidence for use of ctDNA detection as liquid biopsies. For these important clinical considerations, we refer to the recently published ESMO recommendations on the use of circulating tumor DNA assays for patients with cancer[1].

There is no international consensus on the requirements for the pre-analytical and analytical phases of ctDNA detection. The current recommendations are therefore a compilation of international publications and consensus among the members of the working group. The purpose is to facilitate high quality and reliability of ctDNA detection. A high degree of standardization is an important prerequisite for a successful transfer of ctDNA detection techniques from the research setting into broader clinical use.

1.1 Definitions

BSC, Bisulfite conversion

CF, Cytosine-free

cfDNA, Cell-free DNA

CHIP, Clonal hematopoiesis of indeterminate potential

CI, Confidence interval

CpG, 5'-Cytosine-phosphate-Guanidine-3'

CNV, Copy number variation

ctDNA, Cell-free tumor DNA

$CV_{\%}$, Coefficient of variance in %

dddPCR, Denaturation-enhanced ddPCR

ddPCR, Droplet digital PCR

dsDNA, double-stranded DNA

EDTA, Ethylenediaminetetraacetic acid

ESMO, European Society for Medical Oncology

EQA, External quality assessment

GATK, Genome Analysis ToolKit

GE, Genome Equivalents

GIAB, Genome-In-A-Bottle

INDEL, Insertion-deletion

NGS, Next-Generation Sequencing

LoB, Limit of blank

LoD, Limit of detection

LoQ, Limit of quantification

NTC, No-template control

PBC, Peripheral Blood Contamination

PCR, Polymerase chain reaction

RfB, Referenzinstitut für Bioanalytik

SD, Standard Deviation

SNV, Single nucleotide variant

ssDNA, single-stranded DNA

SV, Structual variation

TE, Total error

VAF, Variant allele frequency (in %)

2 Handling of blood samples prior to DNA extraction

Søren Egstrand, Stig Egil Bojesen, and Estrid Høgdall

For the initial handling of blood samples for ctDNA purification, we recommend following the current guidelines from the Bio- and Genome Bank Denmark (RBGB)[2]. The following section will be based on these recommendations. Blood should be collected in standard EDTA blood collection tubes or special cfDNA stabilization blood collection tubes. After initial centrifugation and isolation of plasma, we recommend collecting and freezing the buffy coat to allow for later analysis of clonal hematopoiesis of indeterminate potential (CHIP).

2.1 EDTA tubes

For blood collected in EDTA tubes, the samples are immediately transferred to the laboratory and centrifuged within 1½ hours to enable final handling within 3 hours after the blood draw.

First centrifugation: Set the centrifuge to slow deceleration corresponding to 45 sec. to avoid the separated phases from re-mixing. Centrifuge EDTA tubes at 2000g for 10 min at 4°C (recommended) or at room temperature. After the centrifugation has started, the process must not be interrupted.

Second centrifugation: Collect the separated plasma from the centrifuged EDTA tubes without disturbing the buffy coat by keeping the pipette 0.5 cm above the buffy coat layer. Transfer plasma into a new centrifuge tube and repeat centrifugation at the settings given above.

Transfer the plasma into a clean tube, keeping the pipette above the bottom 0.5 cm of the plasma to keep the pellet undisturbed. Disturbing the pellet can contaminate the sample, thereby giving false values in the subsequent analyses.

This procedure entails approximately 4 ml of plasma per 9 ml of whole blood for extraction of cfDNA.

Other procedures and centrifugation schemes to isolate the plasma may be used, but laboratories deviating from the protocol above must validate the procedure, especially regarding contamination with high molecular weight DNA.

NOTE: The protocol above is aimed at cfDNA extraction. If cell-free RNA is to be extracted, a higher centrifugation speed (10,000g) would be required in the second step to remove platelets.

2.2 cfDNA stabilization blood collection tubes

For blood collection in cfDNA stabilization blood collection tubes, the time to processing can be extended to several days to enable shipping of samples. Please refer to the manufacturer's instructions and scientific literature testing the specific tube[3]. We recommend using the validated centrifugation protocol for the specific tube or using the protocol outlined in the previous section.

3 Quality control of extracted DNA before ctDNA analysis

Rikke Fredslund Andersen, Luisa Matos Do Canto, and Cecilie Mondrup Jacobsen

In order to detect cfDNA in samples, highly sensitive techniques are used, which necessitates an extensive quality control regimen to ensure assay accuracy, precision, and reproducibility[4-6]. Sequential analyses of samples drawn from the same patient with the intent of monitoring treatment results over time further increase the need for rigorous quality control. Of particular concern is the lysis of leukocytes, as the release of high molecular weight DNA will hinder the detection of cfDNA due to dilution.

Several things should always be considered during quality control: the efficiency of the cfDNA extraction, the cfDNA concentration post-extraction, cfDNA fragment length, contamination with high molecular weight DNA, PCR inhibition by carryovers (e.g., ethanol, salt, Proteinase K), and the amount of single-stranded DNA (ssDNA) compared to double-stranded DNA (dsDNA).
In a routine clinical setting, in which the sample material is sparse, the amount of sample used for pre-analytical quality assessment should be limited. The pre-analytical workflow should be assessed extensively in establishing the analysis and monitored thereafter.

3.1 cfDNA extraction

Due to the finite amount of ctDNA in samples, the extraction method is of huge importance. Many commercial kits are available for both automated, semi-automated, and manual extraction. Before a new commercial kit is implemented, its specifications should always be validated in the lab: the cfDNA purification efficiency, fragment length bias, reproducibility, capacity, and sample turnover are important factors to consider. Since trade-offs are necessary, the kit choice should reflect the purpose of the analysis.

Isolation of cfDNA requires dedicated extraction kits designed for circulating nucleic acids that can recover a broad range of fragment sizes. There are several commercial kits designed for extracting cfDNA (with or without automation). Most commercial kits are based on the binding of DNA to silica gel membranes, magnetic particles, or organic chemicals, and a high level of variability among these methods regarding recovery efficiency, size discrimination, and reproducibility is observed. At present, the literature indicates that the manual QIAamp® Circulating Nucleic Acid kit from Qiagen (Cat. No. 55114) and the QIAsymphony DSP Circulating DNA Kit from Qiagen (Cat. No. 937556) give the best yield. However, the choice of kit should depend on the demands of the local lab. Further studies of available kits are recommended so that the choice of kit can be based on direct comparison of kits. For a review of the most commonly used kits, see Ungerer *et al*., 2020[7].

3.2 Efficiency of cfDNA extraction

A specific concentration of an exogenous spike-in control (e.g. soybean CPP1 DNA fragments, which is an in vitro generated 191 bp DNA fragment[8], or CEREBIS, which is a synthetic DNA construct[9]) can be added to each plasma sample before extraction to determine the DNA extraction efficiency. The spike-in should have a similar fragment length to cfDNA to ensure similar behavior in terms of denaturation, non-specific binding, and precipitation[10]. The extraction efficiency is the percent recovery of fragments following cfDNA extraction.

Extraction efficiency
% spike-in fragments recovered after extraction

3.3 cfDNA quantification

Accurate quantification of cfDNA yield can be performed using fluorometry (e.g., Qubit™, Invitrogen, Life Technologies) or PCR-based methods, which are considered the gold standard of DNA quantification. While fluorometry-based techniques assess the total amount of genomic material, PCR-based assays evaluate specific regions in a gene/genomic position. Qubit is less costly and time-consuming, and it can measure dsDNA (Qubit™ dsDNA HS/BR Assay Kit) or both ss- and ds-DNA (Qubit™ ssDNA HS/BR Assay Kit) with a good correlation with qPCR quantification[11].

Spectrophotometry-based methods (e.g., NanoDrop, ThermoFisher) are not recommended for cfDNA quantification.

Genome Equivalents (GE) in the cfDNA should be quantified using assays targeting regions that rarely show copy number alterations and are not within genes with pseudogenes. Frequently cleaved DNA regions, regarding nucleosome distribution, should also be considered when choosing a target region/gene. Examples of assays include targeting reference regions on chromosomes 3, 7, and 16[12, 13], or reference genes (e.g. *beta-2-microglobulin*, *EMC7*)[8, 14]. The analysis of more than one target should be considered when DNA from highly aneuploid tumors is being evaluated[15]. The total GE concentration can be described as copies/mL of plasma.

Quantification of cfDNA is important in order to comply with the protocols recommended minimum/maximum input mass, to maintain cfDNA-to-library efficiency and consequently the sensitivity of the method. For a sample with high amounts of cfDNA, increased sensitivity can be obtained by splitting into two or more library reactions. Quantification of library output (i.e. the PCR amplified library) can be used to estimate the cfDNA-to-library efficiency (effectively the output-input ratio) and be used to identify and possibly repeat samples that have failed the library preparation step.

3.4 Evaluating ssDNA and dsDNA

Depending on the extraction kit, a proportion of the cfDNA may be single-stranded after extraction. The presence of ssDNA can lead to incorrect quantification of total DNA by ddPCR and an overestimation of input DNA for sequencing library preparation where dsDNA is needed[16]. The use of a fluorometric assay (Bioanalyzer High Sensitivity, Agilent; Qubit dsDNA HS Assay Kit, Thermofisher) that specifically quantifies dsDNA can be used to avoid bias on quantification. To quantify DNA more precisely and consistently by ddPCR, the DNA can be denatured completely into ssDNA (e.g. incubate 2µL DNA at 95°C for one minute) before droplet generation (Denaturation-enhanced Droplet Digital PCR, dddPCR)[17].

The ratio of dsDNA and ssDNA can be measured by comparing ddPCR measurements of denatured and non-denatured DNA. The ssDNA/dsDNA ratio will be around two if the original sample contains mostly dsDNA and approach one if ssDNA is present. dddPCR has been shown to increase the number of data-positive droplets by 1.6 – 1.7-fold when applied to cfDNA[17].

3.5 cfDNA fragment length

Fragmentation patterns (DNA integrity index) of the purified cfDNA can be assessed using microfluidic electrophoresis (TapeStation and Femto Pulse Systems, Agilent Technologies), and more accurately quantified by analysis of two or more fragment sizes of a gene or genomic region (e.g., 250 bp and 65 bp fragments of *EMC7* gene). *EMC7* is a single copy gene located on chr. 15 and has never been reported to be involved in cancer, loss of heterozygosity (LOH), or amplifications[8]. Detection of increased concentration of long fragments can indicate potential contamination with high molecular weight DNA from lysed leukocytes.

DNA integrity index
ratio of long to short PCR amplicons

3.6 Contamination with high molecular weight DNA

High molecular weight DNA contamination of purified cfDNA samples can be more specifically assessed by estimating B-lymphocyte DNA content using an assay targeting the VDJ rearranged IGH locus-specific for B cells (Peripheral Blood Contamination, PBC)[10].

3.7 PCR inhibition

The presence of carryovers (ethanol, salt, Proteinase K, etc.) from the DNA purification process in the cfDNA eluate can inhibit downstream analyses leading to incorrect or inconclusive results[18]. Residual ethanol and salt can have a negative effect on droplet generation (in ddPCR) and inhibit PCR. Residual Proteinase K can also affect ddPCR and NGS reactions leading to decreased sensitivity or false-negative results[19]. Therefore, the use of standardized controls is recommended. Quantification of a spiked-in control (e.g. CPP1) with known

concentration is an option to test PCR efficiency and verify the presence of carryover inhibitors; another option is to use a spectrophotometer (NanoDrop, ThermoFisher). The presence of inhibitors can be tested by ddPCR by incubating samples and mastermix before PCR amplification. If inhibitors are present, clusters will shift position in a ddPCR plot and move closer together.

3.8 Evaluation of samples

Suggested cutoffs for each quality control assay are shown in Table 1. Positive samples are non-valid for quantification if one parameter is Red or two are Yellow but can be reported as 'positive – not quantifiable'. Negative samples should be reported as 'inconclusive' if one parameter is Red or two are Yellow.

3.9 Additional quality control for methylation analysis of cfDNA

For the analysis of DNA methylation markers, the methylation levels of a particular CpG or region are measured. Most pipelines include bisulfite conversion (BSC) of cfDNA followed by quantification of methylated or unmethylated CpG residues of target regions and control gene. DNA loss following BSC happens due to:

- fragmentation/degradation that prevents primer/probe annealing,
- the purification process and, therefore, the absence of the target fragment in the final elution,
- inefficient BSC, preventing quantification with a BSC-dependent PCR assay.

Conversion recovery can be evaluated by quantification of cfDNA before and after BSC[20]. The assessment of the deamination of unmethylated cytosine to uracil requires either different quantification assays before and after conversion or cytosine-free quantification assays that are capable of amplifying both converted and unconverted templates. Examples of assays are provided below.

Cytosine-free (CF) assay: An assay that amplifies a cytosine-free region of the genome, thereby enabling the use of the same assay for the quantification of both native and bisulfite-converted DNA. E.g., the CF assay targets a region on chromosome 1 that rarely shows copy number aberration in cancer. After bisulfite conversion, the CF assay is used for DNA quantification and recovery assessments[21, 22]. Since the assay does not distinguish between converted and native DNA, it is not necessarily a precise measure of converted DNA. It measures DNA present before and after conversion but not whether it has been converted.

BSC recovery
quantity after bisulfite conversion / quantity before

Cytosine-containing regions: Two different assays should be designed to detect a target region containing non-CpG cytosines before and after bisulfite conversion. After conversion, the assay should be capable of amplifying sequences only if bisulfite-converted. However, this approach compares quantification by two different assays which may not be completely accurate. The ratio between the assay used as the control gene after BSC (e.g. *ALB*) and the pre-BSC cfDNA surrogate marker (e.g. *EMC7 65*) can be used[8]. Although no cut-offs for BSC are defined, a minimum ratio of 0.2 is recommended.

Table 1. Quality control (QC) assays for samples undergoing circulating cell-free DNA analysis. Parameters for each QC are divided into green, yellow, and red. A sample cannot be quantified if one of the parameters is red or if two are yellow.

	Green	Yellow	Red
Purification efficiency CPP1	**>50% average level**	**25%< average level <50%**	**<25% average level**
Fragmentation *EMC7* 250/65	**<0.4 (ratio)**	**0.4< ratio <0.7**	**>0.7 ratio**
gDNA contamination PBC	**<0.5% PBC/cfDNA**	**0.5%< PBC/cfDNA <2%**	**>2% PBC/cfDNA**

4 Assay validation

Søren Egstrand, Stig Egil Bojesen, Ane Yde Schmidt, Lise Barlebo Ahlborn, and Mads Heilskov Rasmussen

4.1 Assay validation for ddPCR

For each ddPCR assay, the Limit of Blank (LoB), Limit of Detection (LoD), Coefficient of variance ($CV_{\%}$), linearity, and preferably the Limit of Quantification (LoQ) should be determined.

4.1.1 Limit of Blank (LoB)

LoB is the analytical signal limit below which 95% of blank samples are represented. We recommend using plasma samples from healthy young volunteers as blank samples. Assays differ in the frequency of false positive droplets (FP) and determining the LoB from blank samples will depend on whether this frequency follows a Gaussian-, non-Gaussian- or poisson-distribution.

Assuming a Gaussian distribution, LoB can be estimated by measuring an appropriate number of replicates (N≥20) of a blank sample to determine the resulting $mean_{blank}$ and SD_{blank}. LoB can then be calculated as:

$$LoB = mean_{blank} + 1.645 \cdot SD_{blank}$$

For non-Gaussian distributions, the number of replicates is increased (N≥60), results are ranked, and the LoB calculated as

$$LoB = Value[95^{th}\ percentile]$$

For poisson distributions with only a few false positive droplets, we can determine the number of positive droplets below which the probability corresponds to our predefined significance level at 95%:

$$P = \frac{\left(\frac{FP}{N}\right)^{x} \cdot e^{-\left(\frac{FP}{N}\right)}}{x!}$$

where *x* is the number of positive droplets in a measurement given the known rate of false positives of the assay: number of false positive *FP* droplets divided by the *N* number of measurements of blank samples.

Example of a Poisson distribution

We have measured 80 blank samples of which 4 exhibited one single positive droplet. We can now calculate the probability of finding 0 positive droplets in a new sample:

$$P = \frac{\left(\frac{4}{80}\right)^{0} \cdot e^{-\left(\frac{4}{80}\right)}}{0!} = 0{,}951 = 95{,}1\%$$

In this case 0 droplets will represent the LoB at which 95% of blank samples are represented.

LoB can be affected by the concentration of input DNA which can be an issue particularly for assays detecting transition changes: C>T and G>A. This is likely due to polymerase error during the PCR step[23]. For assays affected by input DNA quantity, we recommend determining LoB at several levels covering the relevant clinical spectrum or to use developed algorithms to account for the increase in false positive signal at increasing wildtype DNA levels[23].

4.1.2 Limit of Detection (LoD)

LoD is the lowest analytical signal limit to be reliably distinguished from the LoB. Assuming a Gaussian distribution, the conventional approach is to base LoD on the SD_{blank}:

$$LoD = LoB + 3 \cdot SD_{blank}$$

This approach is not based on positive samples and may be too conservative. Alternatively, LoD may be expressed as the limit above which 95% of positive low-concentration samples are called positive. Measuring an appropriate number of replicates (N>60) of low-concentration samples to determine the $SD_{low\ conc}$ and assuming a Gaussian distribution:

$$LoD = LoB + 1.645 \cdot SD_{low\ conc}$$

For non-Gaussian distributions, using low-concentration samples, results are ranked, and the LoD is calculated as

$$LoD = LoB + \left(\mu_{low\ conc} - Value[5^{th}\ percentile]\right)$$

Where $\mu_{low\ conc}$ is the average of low-concentration samples. If the assay exhibits a poisson distribution we can calculate the number

of positive droplets corresponding to a predefined separation from the blank samples, e.g. a probability of 0,1% of obtaining the result if the sample is blank. Using the same example from above:

Example: We measured 80 blank samples of which 4 exhibited a single positive droplet. We can now calculate the probability of finding ≥ 1 positive droplet in a blank sample which is equal to 1 - probability of finding 0 droplets:

$$P = 1 - \frac{\left(\frac{4}{80}\right)^0 \cdot e^{-\left(\frac{4}{80}\right)}}{0!} = 4{,}9\%$$

Likewise, we can calculate the probability of finding ≥ 2 positive droplets:

$$P = 1 - \frac{\left(\frac{4}{80}\right)^0 \cdot e^{-\left(\frac{4}{80}\right)}}{0!} - \frac{\left(\frac{4}{80}\right)^1 \cdot e^{-\left(\frac{4}{80}\right)}}{1!} = 1{,}2\%$$

And the probability of finding ≥ 3 positive droplets in a blank sample:

$$P = 1 - \frac{\left(\frac{4}{80}\right)^0 \cdot e^{-\left(\frac{4}{80}\right)}}{0!} - \frac{\left(\frac{4}{80}\right)^1 \cdot e^{-\left(\frac{4}{80}\right)}}{1!} - \frac{\left(\frac{4}{80}\right)^2 \cdot e^{-\left(\frac{4}{80}\right)}}{2!}$$

$$P = 0{,}002\%$$

In this case 3 droplets will represent the LoD at which <0,1% of blank samples are represented.

4.1.3 Coefficient of variance (CV%)

$CV_{\%}$ should be established using at least 60 replicates over a time period, preferably using different batches of reagents to best represent assay variation. We suggest using a sample at the level of clinical relevance, such as LoD.

$$CV_{\%} = \frac{SD}{mean} \cdot 100\%$$

4.1.4 Limit of Quantification (LoQ)

LoQ is the analytical signal limit above which predefined goals for total error (TE) are met. An acceptable total error of measurement is set at <50%, based on 40-60 replicates[24, 25]. A tentative LoQ can be set at LoD and gradually increased if an unacceptable total error is found. Total error is calculated as

$$TE_{\%} = Bias_{\%} + (1.645 \cdot CV_{\%})$$

To estimate bias, a "True" value is needed. As currently no reference method with higher sensitivity is available, ddPCR represents the gold standard for ctDNA quantification. We therefore suggest that a true value be determined by peer group comparisons. Alternatively, LoQ can be defined as the lowest level at which a predefined requirement for an acceptable $CV_{\%}$ is met (e.g. $CV_{\%}$ <25%)[26].

4.1.5 Linearity

Linearity can be determined using 2-3 replicates at 7 levels from the LoQ up to 100% variant allele frequency (VAF).

4.2 Assay validation for NGS

For NGS-assay validation, it is recommended to do a titration of samples to find the (upper and) lower amount of library input (ng of cfDNA) and how the input affects the detection of variants with low VAF. If a lab is starting NGS on cfDNA for the first time, dilution of tumor-DNA with germline-DNA can be used for the titration experiments (NB. it is important to fragment non-cfDNA samples to a size around 150 bp). Alternatively, commercially available samples can be used (for a description of reference materials, see section 5.1 *General considerations for reference control material*).

If the lab has established a ctDNA mutational analysis, samples with known variants can be used to test the new NGS assay. It is also recommended to characterize the assay using multiple negative samples without mutations in order to estimate the noise level of the assay, either across the whole assay, or better, on a per base (or region) – level.

If no ctDNA with known variants are available, it can be beneficial to sequence both a tumor and cfDNA sample from the same patients. This will help guide the initial data analysis.

It is highly recommended to include cfDNA samples of the same type as those intended to be analyzed by the assay (incl. collection tube, plasma isolation, extraction method, patient group).

5 Process Control

Maria Hønholt Jørgensen, Laura Iisager, Mads Heilskov Rasmussen, Luisa Matos Do Canto, Cecilie Mondrup Jacobsen, Rikke Fredslund Andersen, and Niels Pallisgaard

5.1 Reference control material

According to ISO guide 30:2015, a reference material is defined as *"material, sufficiently homogeneous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in a measurement process"* and reference materials can be used for calibration of apparatus, evaluation of measurement procedures and for internal or external quality control of measurements and laboratories. Reference materials can be spike-in controls, which are added to patient samples to assess between-sample technical errors or to serve as a scaling factor for normalization between samples. Spike-in controls need to be distinguishable from the molecules under investigation to avoid contaminating downstream analysis. Reference materials can also be external controls, which are not added to the patient samples, but undergo the same experimental procedures as the patient samples do. External controls can be derived from cell line DNA, be based on synthetic DNA fragment spike-in, or on well characterized clinical samples with known mutations. Such controls can assist in detecting experimental failures, in the calibration of methods, and the assessment of properties such as LoD for specific measurement methods.[27]

5.1.1 Applications of reference control materials for ctDNA analysis

Standardization of ctDNA analysis is needed for clinical integration. ctDNA workflows and processes should be normalized across and within facilities to achieve global standards for ctDNA detection. Thus, to ensure an accurate and robust detection of ctDNA, we recommend the use of control materials in every ctDNA laboratory protocol.

Reference ctDNA control materials can be integrated as an internal laboratory run control that assess everyday variations or batch-to-batch discrepancies. The inclusion of control materials in each laboratory run will allow for assessment and monitoring of quality parameters and assay performance, in terms of analytical sensitivity, specificity, and robustness. It will support deliberate changes (installation of new apparatus, changes in protocols, etc.), set up of new ctDNA detection laboratories, and assessment of user competency.

In general, the use of reference materials will support the development of national or even international standards for ctDNA detection, which is crucial for translating ctDNA results into clinical practice.

5.1.2 Available reference control materials

Several commercial reference materials appropriate for ctDNA analysis are on the market already, including from vendors like SensID, LGC, Horizon Discovery, ThermoFisher Scientific, Twist Bioscience and Promega. These reference materials differ in terms of manufacturing process, the variety of mutations included as well as the mutation allele frequency covered. For instance, companies like LGC, SensID, and ThermoFisher Scientific have created ctDNA-like material consisting of 10 - 40 selected variants (SNVs, INDELs, SVs, or CNVs) that are spiked into a background of fragmented genomic DNA from the B-Lymphocyte GM24385 Genome-In-A-Bottle (GIAB) cell line. Other companies, including Horizon Discovery and TWIST bioscience, have incorporated several hundreds of variants into their materials: Horizon Discovery has created mixtures of cell line DNA each carrying mutations introduced by CRISPR. TWIST Bioscience has created controls from synthetically short mutated sequences added to human-derived cell-free DNA from healthy donors. Commercial reference control materials are relatively costly and thus expensive to implement in daily laboratory routine flows. In addition, although some companies offer customized reference material (like Twist Bioscience), the flexibility in choosing mutations and mutation allele frequency, is in general not high.

The ctDNA center has developed its own ctDNA reference material, referred to as RefMat. With the aim of achieving common standards for

ctDNA detection across facilities, RefMat is freely available for laboratories affiliated with the center. The ctDNA center's RefMat is stable, unlimited, and well-characterized control DNA developed for the common ctDNA detection methods NGS and ddPCR. The standardized protocol for developing RefMat allows for easy and flexible scaling of future RefMats in accordance with particular research projects or ctDNA detection laboratory workflows.

The ctDNA center's RefMat consists of recombinant plasmids containing artificial mutated human DNA sequences of around 1000 bp, which are enzymatically fragmented to a size distribution resembling cfDNA, with a mode around 167 bp (15% variation). The artificial ctDNA-like fragments are added to a background of DNA from the human GM12878 healthy human B-lymphocyte GIAB cell line[28] that is also enzymatically fragmented to a size distribution resembling cfDNA, with a mode around 167 bp. Alternative background matrices can include DNA from peripheral blood mononuclear cells, artificial plasma and donor cfDNA. RefMat currently includes up to 102 SNV, INDEL, and SV variants which can be available in any desired VAF down to 0.05%. The selected variants cover the most recurrent cancer mutations, common drug targets, as well as technically challenging alterations, and are designed to include variants frequently analyzed by ctDNA laboratories in Denmark (decided at a workshop in 2023 for ctDNA center participants). RefMat is flexible regarding the variants incorporated in the particular RefMat sample and the VAF that each is presented in. The plasmid spike-in DNA and background cell line DNA are in principle unlimited due to storage in bacteria glycerol stocks and live cell freeze cryo-vials, respectively. Furthermore, the material is considered unchangeable with very low level of drifting mutations, as well as well-characterized due to GM12878 being part of the GIAB consortium.

5.1.3 Selection of the appropriate reference control

It is important to select an appropriate control for your particular analysis. The research question in focus, and the ctDNA workflow steps to standardize, are some of the important elements to take into account when choosing an appropriate control. For example, in order to compare pre-analytical procedures, such as handling of blood samples or cfDNA extraction, the included reference control could either be in a comparable blood plasma matrix and used as an external control, or of a non-human origin that can be added to the patient samples themselves and used as internal control. Upon the use of reference controls as laboratory process or batch controls, the material should optimally be very stable and ideally unlimited, or at least in great excess, so that it can be measured persistently over time.

It is important to have a uniform use of controls in order to standardize analyses. Upon interlaboratory comparison trials or benchmarking studies, the use of standardized common reference materials may also be crucial, since it can serve as a common reference point for comparisons.

5.2 Process Control for ddPCR

To ensure accurate quantification of ctDNA when performing ddPCR, several process parameters for both the ctDNA extraction, bisulfite conversion, and the amplification and read-out process should be considered. Similarly, the correct choice of control samples and successful droplet generation is important to report the results confidently.

5.2.1 Control samples for ddPCR analysis

Control samples for all analytical steps should be included (Figure 1). If results from several ddPCR plates needs to be compared, controls should be included on all plates. We recommend to include:

- A **no-template control (NTC)** is included to ensure that no external DNA contamination has taken place.
- A **negative control** is included to monitor the specificity of the ddPCR assays by asserting that negative samples are not reported as positive. Genomic DNA samples from healthy donors can be used to function as a biological comparable negative control. Commercial negative controls are also available.

- A **positive control** is included to ensure that the ddPCR assay works as expected and to guide the gating of the read-out when analyzing the results. A **genomic DNA control** can be used as positive control for the quality control assays. The positive control for ctDNA analysis should reflect the detection method; hyper/hypo-methylated for methylation analysis, mutated for mutation analysis etc. When performing methylation analyses, the positive control (if not already converted) also functions as a control of the bisulfite conversion.

- A **plasma control** can be included in the extraction step in the workflow to verify that the analytical workflow has been performed successfully. If possible, plasma samples can be acquired and pooled from leftover routine analyses performed at the hospital. If the control is to be used as a negative control as well, plasma from haematological and oncological patients should be excluded to avoid positive signals in the control. Methylation- and mutational status should always be examined before a control is taken into use.

When new primer-probe mixes are prepared, they should be tested on control samples before being used to analyze patient samples. Results from the analysis of control samples are registered to allow for the monitoring of quality control parameters over time. It should be noted when a new control is taken into use.

5.2.2 Droplet generation

The total number of droplets is an important process control parameter that must always be evaluated as it affects the dynamic range of the analysis. As ddPCR quantification is based on partitioning statistics and the Poisson distribution, a higher number of areas of

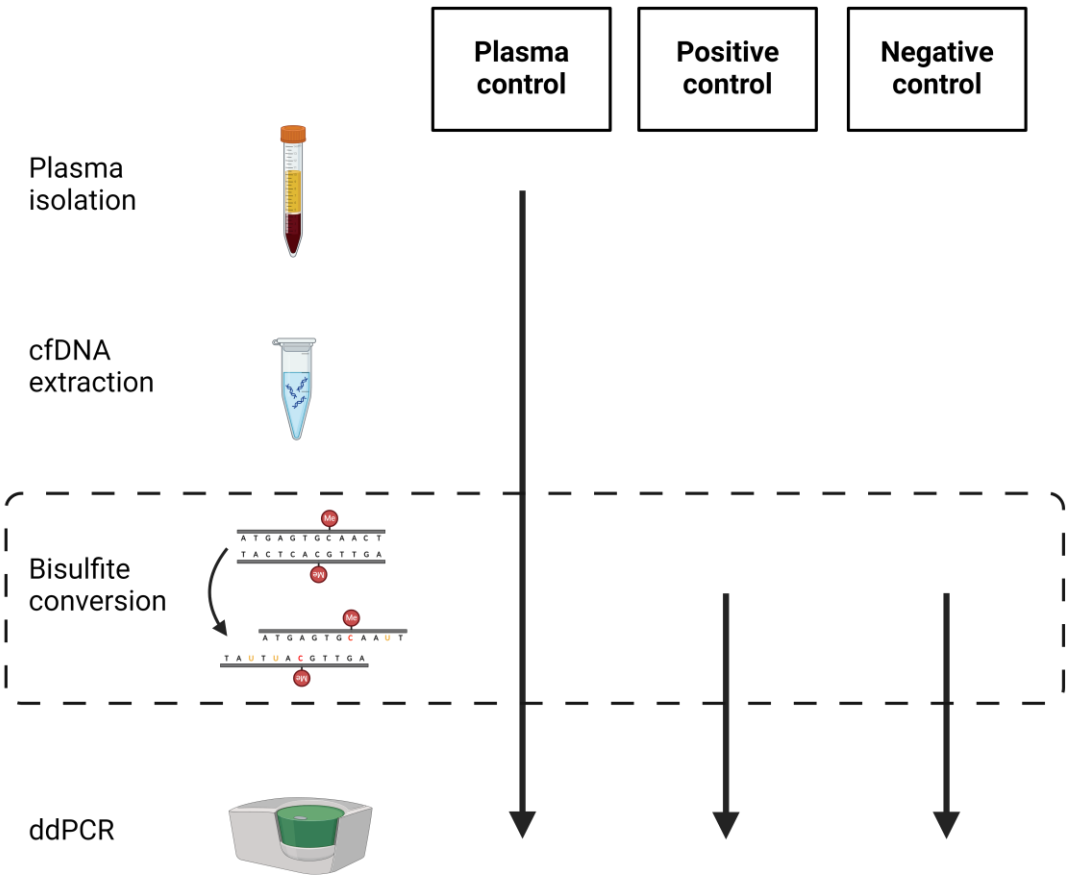

Figure 1. Overview of control samples for ctDNA analysis with ddPCR. Arrows indicate the analytical step where the controls are included. Methylation-specific ddPCR with bisulfite conversion is depicted in dashed lines. For other analyses that do not require bisulfite conversion (e.g. mutations or translocations), the positive and negative controls are included in the final step. Non-template control (NTC) is not included in the figure.

opportunity (number of partitions) and a low probability of success (nucleic acid targets per partition) must be present for accurate quantification[29]. Bio-Rad recommends a minimum droplet count of 10000 droplets for their QX200 and QX600 platforms[30]. If too few droplets are detected a quantitative result cannot be reported to the clinicians.

Evaluation of the clusters in the data analysis software is performed every time. The amplitude of droplets from patient samples and positive controls is compared to ensure similar placement of clusters and guide the gating. Similarly, the positive controls can indicate a drop in fluorescence, which can occur if reagents are past their expiration date.

Replicates of samples and controls ensure more robust results. Furthermore, results are ensured if an analysis fails in a well or if it contains too few droplets.

5.3 Process control for NGS

Since NGS workflows involve multiple steps each of which can introduce variability and errors, implementation of a comprehensive quality assurance framework for NGS assays - from sample preparation to data analysis - is important to ensure robust and reliable results[31]. Such process controls can be broken down into:

· Pre-Sequencing Process Controls, which are implemented before the sequencing step and are designed to ensure that the samples are adequately prepared and processed for sequencing, and to detect and exclude failed samples before sequencing.

· Post-Sequencing Process Controls, which includes indicators to evaluate the quality and reliability of the sequencing data and analysis results.

5.3.1 Pre-Sequencing Process Controls

The **quality of input DNA** needs to be assessed before sequencing to ensure that the DNA resembles cfDNA and is of sufficient high quality as described above in section 3 *Quality control of extracted DNA before ctDNA analysis.* Notably, contamination by carry-over EDTA and other enzymatic inhibitors can strongly affect the library reaction. Another factor that will negatively influence most commonly used library protocols is the denaturation of double stranded cfDNA into single-stranded DNA.

Quantification of the library yield, which can be done using Qubit or by Tapestation is also recommended as a pre-sequencing process control. The **Library Enrichment** is a proxy for how much input DNA has been converted into library and is applicable for protocols that include a PCR enrichment step after ligating adapters with DNA.

Library Enrichment
DNA input mass / library output mass

The *DNA input mass* is the total mass of cfDNA that was used in the library reaction, and *library output mass* is the total mass of DNA after PCR enrichment of the library. It is not important how the masses (concentrations) are determined (by ddPCR, Qubit or TapeStation), as long as the mass sufficiently represents the DNA (cfDNA or library) and that the same method is used to measure the concentration of input and output DNA. Reduced library enrichment in individual samples or sample batches can indicate the presence of contaminants, poor quality cfDNA or an irregularity in the laboratory workflow.

It is recommended to include a **batch control** as a pre-sequencing process control. Using a constant control sample on every library preparation batch can identify batches with inferior output and help locate the possible source for failed samples. The batch control can be identical aliquots of cell line DNA, or a reference control sample, whose expected library output is known (from experience).

5.3.2 Post-Sequencing Process Controls

The **Mean Sequencing Depth** reflects the sensitivity of the analysis and indicates how much of the input cfDNA has been interrogated by the sequencing method. This metric can be made using standard tools such as CollectHsMetrics from the Genome Analysis ToolKit (GATK). In case one is doing targeted sequencing, this tool also provides another important post-sequencing quality parameter, namely the **On-Target Rate**, which is information about the fraction of reads that are associated

with the targeted region, i.e. an indicator on the efficiency of the procedure used for enriching particular genomic loci.

The **Sequencing Conversion Rate** represents the efficiency of converting input cfDNA into analyzable sequence data and is particularly relevant for ultra-deep panel sequencing aiming at doing saturated sequence, i.e. sequencing all cfDNA converted to library in order to maximize sensitivity. The Sequencing Conversion Rate can be calculated as the unique (i.e. after removal or consensus-collapsing of PCR duplicates) Mean Sequencing Depth obtained divided with the total number of genome equivalents that was used in the library.

Batch Control Samples should be included in each sequencing batch in order to monitor batch-to-batch variability and ensure consistency across different experimental runs. As a batch control one can use an aliquot of library from the pre-sequencing batch control mentioned above.

Identity SNPs (IDSNPs) should be analysed to document consistency and agreement between samples from the same individual and throughout the analysis workflow in order to ensure accurate and reliable results. This can be done by extracting informative single nucleotide polymorphisms (SNPs) directly from data to assess sample identity and purity. With custom panels, it is possible to identify SNPs with high heterozygosity in the population and add them to the capture design. In a variation of this, in order to avoid sequencing the IDSNP regions to the same depth as the target genes, one can design a separate IDSNP panel that can be used in diluted amounts together with the target gene panel. In principle, 20 well-chosen SNPs with a high heterogeneity index are sufficient to distinguish samples. In case of large capture designs (e.g. Whole Exome Sequencing) or whole genome sequencing, a constant set of informative SNP loci can be chosen and used as IDSNPs.

Evaluating the **level of background noise** in sequencing data is crucial for distinguishing true signals from artifacts. Tools such as CollectSequencingArtifactMetrics from GATK readily provides this information from a SAM/BAM file.

It is recommended to monitor the **rate of duplicated reads** in sequencing data in order to assess sequencing quality and library complexity. This information can be added to SAM/BAM files using MarkDuplicates from GATK.

6 External quality assessment

Laura Iisager, Niels Pallisgaard, Mads Heilskov Rasmussen, and Lise Barlebo Ahlborn

In contrast to internal controls, monitoring imprecision of an analysis, external quality assessments (EQA) aim to monitor analysis bias compared to a reference standard. ctDNA reference samples can be used for identifying best laboratory practice in interlaboratory comparison trials and can serve as a common reference point for assay documentation. Quality assurance trials, using control materials, will allow for the identification of workflow weaknesses and validation of interlaboratory concordance, as well as the assessment of analytical processes yielding robust and sensitive results. In this regard, ctDNA control samples would be able to compare pre-analytical processing procedures (e.g. purification), ctDNA detection methods, and bioinformatic pipelines, thus benchmarking ctDNA methods throughout the entire workflow. It is strongly recommended for laboratories to complement their internal controls with periodical participation in external quality assessment procedures to maintain quality standards and ensure continuity of analyses within and between institutions.

EQA should be done on ddPCR assays that are used regularly by >80% of participation laboratories as a proficiency testing, where unknown samples for testing are sent to laboratories and the results from all laboratories are analyzed centrally and reported back to the laboratories. For ddPCR assays that are rarely used, but used by >2 laboratories, an inter-laboratory exchange of samples between laboratories should be performed.

6.1 Commercially available EQA programs

To the knowledge of the working group, only one commercial ctDNA EQA program is currently available from Referenzinstitut für Bioanalytik (RfB), offering biannual surveys to European laboratories. RfB offers surveys to test the detection of specific mutations of KRAS, BRAF, EGFR, and NRAS in 3 ml EDTA plasma samples.

6.2 EQA programs within the ctDNA Research Center

In the ctDNA Research Center, QA trials are conducted across laboratories in Denmark, and all Danish laboratories conducting ctDNA analyses are welcome to participate in these. At the time of writing, two EQA trials have been conducted testing the cfDNA purification process, and two EQA trials have been conducted testing ddPCR and NGS detection of specific mutations using the control material developed in the Center as described in section 5 *Process Control*.

7 Report format

Simone Karlsson Terp, Ane Yde Schmidt, Lise Barlebo Ahlborn, Mads Heilskov Rasmussen, Søren Egstrand, Stig Egil Bojesen, and Niels Pallisgaard,

7.1 Report format for ddPCR

ddPCR results can be reported in the ordinary Laboratory Information System and should state assay-specific information: the gene name, the reference genome used, the genomic reference sequence (NCBI Reference Sequence number e.g. NG_00XXX.X), the nucleotide numbering and DNA level descriptions (e.g. g.12345A>G for substitution, g.12345_12349_del/dup for deletion/duplication, or g.12345_12346AAA for insertions). For methylation analyses the assay position in a reference genome can be reported (e.g., chr1:123456-123556).

When reporting results from ddPCR measurements, it must be decided whether to communicate only a single entity to ease interpretation, i.e. only *mutant copies per mL* or only *VAF(%)*, or to present all results to enable a more comprehensive interpretation when the background *wildtype copies per mL* differ substantially between repeated samples. As we expect the clinicians receiving and interpreting these results to be experienced specialists within clinical oncology, we advise to disclose all entities for full comparability between repeated samples.

The LoD and other limitations of the specific assay should be included in the reporting of the results. Results should be reported as follows:

- Results below LoB should be reported as "*Not detected*".
- Results between LoB and LoD should be reported as "*Inconclusive*".
- Results above LoD should be reported as "*Detected*" with results additionally above LoQ reported in both *mutant copies per mL*, *wildtype copies per mL,* and as *VAF(%)*.

Results will be reported as *"Failed"* if requirements for QC and process control are not met (please refer to sections 3 and 5). In some cases, it may be advisable to provide a result even if the requirements are not fully met, e.g. if a sample is irreplaceable. In these cases, a result should be accompanied by a caveat describing the suboptimal QC parameters and highlighting the higher uncertainty of the given result. Figure 2 shows an example of a report for a ddPCR ctDNA analysis.

Presuming low concentration of the mutant copies, as expected for the majority of clinical samples, the variance introduced by the distribution of mutant copies during the partitioning will represent the majority of variance with negligible remaining experimental and biological variation[32]. Comparing the Poisson confidence intervals of repeated measurements might allow significant differences to be identified. Therefore, to increase comparability of repeated measurements it is advised to also report the Poisson confidence intervals calculated when conducting the ddPCR measurements[33]. If possible, comments on results could be reported with figures or other graphics to support the comments and/or the results.

7.2 Report format for targeted NGS for mutation calling

The report should clearly state patient name, CPR, diagnosis, requesting doctor, sample ID. Always state the methods (incl. version number) used, incl. sequencing platform, capture kit, analysis software, minimum coverage, and VAF cut-off (depends on sequencing platform) etc. The report should reflect the clinical question, e.g., if only BRCA1/2 is requested, only these genes are included in the report, and this should be clear from the report. Always state the reference genome used and which mutation caller is used and also analysis software if used. For reported mutations always include at least gene isoform (e.g. NM number), c./p. nomenclature according to HGVS, coverage, and VAF. Always confirm the reported variants in the raw sequencing reads to exclude sequencing artifacts to be reported. Relevant/oncogenic variants should be included in the report depending on the clinical scope of the analysis while in general polymorphisms and benign variants should NOT be included (>1% gnomAD).

Variant classification: include which information the variant classification is based upon e.g., ACMG or literature, databases, *in silico* prediction, population frequency, etc.
If no germline sample is subtracted from the tumor sample, be aware of germline variants (VAF in the range 30-70% and ClinVar references and reported germline in patients). If it is unknown/unclear if a variant is germline or not, this should be stated in the report. Clonal hematopoiesis: these variants can be subtracted by running a normal blood sample. But at least state that CHIP variants cannot be separated from tumor variants.

ctDNA analysis for specific variant: BRCA1, c.5096G>A

Laboratory: Dept. of X, X University Hospital

Requested by: Dept. of Oncology, X University Hospital

Date received: 06/04/2024

Date reported: 03/05/2024

Authorized by: Jørgen Jensen, molecular biologist

Jens Jørgensen, molecular biologist

Patient:
CPR: XXXXXX-XXXX
Name: NN

Results/Conclusion:
Positive: BRCA1, c.5096G>A variant identified with a VAF of 10%

Internal reference:
Analysis id: NPU58702
Order id: ctDNA-BRCA1-XX
LIMS id: REKV000XXXX

Sample material:
EDTA-plasma
Tube id: 12345641 (1212155DD221) received 06/04/2024
Analyte id: XXXX-123456789

Clinical information/indication:
OBS resistance to treatment

Method of analysis:
Droplet digital PCR has been performed using primers and Tagman probes specific for the variant BRCA1: LRG_292t1:c.5096G>A p.(Arg1699Gln). Reference genome: hg38. [BIC: 5215G>A]. Limit of detection (LoD) for the analysis is a VAF of 0,1% and Limit of Blank (LoB) for the analysis is X. Data has been analyzed in QX Manager (Version 2.1).

Variant(s) testet:
BRCA1: LRG_292t1:c.5096G>A p.(Arg1699Gln). [BIC: 5215G>A]

Figure 2. Example on a format for reporting results of ctDNA analysis using ddPCR

8 References

1. Pascual J, Attard G, Bidard FC*, et al.* ESMO recommendations on the use of circulating tumour DNA assays for patients with cancer: a report from the ESMO Precision Medicine Working Group. *Annals of Oncology* 2022; **33:** 750-768.

2. RBGB: Instructions for handling blood for ctDNA analysis, version1, 29-06-2022: https://www.rbgb.dk/wp-content/uploads/2024/04/Instructions-for-handling-blood-for-ctDNA-analysis.pdf. In

3. 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:** 3104-3109.

4. Alcaide M, Cheung M, Hillman J*, et al.* Evaluating the quantity, quality and size distribution of cell-free DNA by multiplex droplet digital PCR. *Sci Rep* 2020; **10:** 12564.

5. Meddeb R, Pisareva E, Thierry AR. Guidelines for the Preanalytical Conditions for Analyzing Circulating Cell-Free DNA. *Clin Chem* 2019; **65:** 623-633.

6. Yuwono NL, Boyd MAA, Henry CE*, et al.* Circulating cell-free DNA undergoes significant decline in yield after prolonged storage time in both plasma and purified form. *Clin Chem Lab Med* 2022; **60:** 1287-1298.

7. Ungerer V, Bronkhorst AJ, Holdenrieder S. Preanalytical variables that affect the outcome of cell-free DNA measurements. *Crit Rev Clin Lab Sci* 2020; **57:** 484-507.

8. Lefèvre AC, Pallisgaard N, Kronborg C*, et al.* The Clinical Value of Measuring Circulating HPV DNA during Chemo-Radiotherapy in Squamous Cell Carcinoma of the Anus. *Cancers (Basel)* 2021; **13**.

9. Goh SK, Cox DRA, Wong BKL*, et al.* A Synthetic DNA Construct to Evaluate the Recovery Efficiency of Cell-Free DNA Extraction and Bisulfite Modification. *Clin Chem* 2021; **67:** 1201-1209.

10. Pallisgaard N, Spindler KL, Andersen RF*, et al.* Controls to validate plasma samples for cell free DNA quantification. *Clin Chim Acta* 2015; **446:** 141-146.

11. Ponti G, Maccaferri M, Manfredini M*, et al.* The value of fluorimetry (Qubit) and spectrophotometry (NanoDrop) in the quantification of cell-free DNA (cfDNA) in malignant melanoma and prostate cancer patients. *Clin Chim Acta* 2018; **479:** 14-19.

12. Andersen CL, Lamy P, Thorsen K*, et al.* Frequent genomic loss at chr16p13.2 is associated with poor prognosis in colorectal cancer. *Int J Cancer* 2011; **129:** 1848-1858.

13. Christensen E, Nordentoft I, Vang S*, et al.* Optimized targeted sequencing of cell-free plasma DNA from bladder cancer patients. *Sci Rep* 2018; **8:** 1917.

14. Demuth C, Spindler KG, Johansen JS*, et al.* Measuring KRAS Mutations in Circulating Tumor DNA by Droplet Digital PCR and Next-Generation Sequencing. *Transl Oncol* 2018; **11:** 1220-1224.

15. Markus H, Contente-Cuomo T, Farooq M*, et al.* Evaluation of pre-analytical factors affecting plasma DNA analysis. *Sci Rep* 2018; **8:** 7375.

16. Simbolo M, Gottardi M, Corbo V*, et al.* DNA qualification workflow for next generation sequencing of histopathological samples. *PLoS One* 2013; **8:** e62692.

17. Fitarelli-Kiehl M, Yu F, Ashtaputre R*, et al.* Denaturation-Enhanced Droplet Digital PCR for Liquid Biopsies. *Clin Chem* 2018; **64:** 1762-1771.

18. Schrader C, Schielke A, Ellerbroek L*, et al.* PCR inhibitors - occurrence, properties and removal. *J Appl Microbiol* 2012; **113:** 1014-1026.

19. Tozaki T, Ohnuma A, Kikuchi M*, et al.* Robustness of digital PCR and real-time PCR against inhibitors in transgene detection for gene doping control in equestrian sports. *Drug Test Anal* 2021; **13:** 1768-1775.

20. Worm Ørntoft MB, Jensen S, Hansen TB*, et al.* Comparative analysis of 12 different kits for bisulfite conversion of circulating cell-free DNA. *Epigenetics* 2017; **12:** 626-636.

21. Bjerre MT, Nørgaard M, Larsen OH*, et al.* Epigenetic Analysis of Circulating Tumor DNA in Localized and Metastatic Prostate Cancer: Evaluation of Clinical Biomarker Potential. *Cells* 2020; **9**.

22. Jensen S, Øgaard N, Ørntoft MW*, et al.* Novel DNA methylation biomarkers show high sensitivity and specificity for blood-based detection of colorectal cancer-a clinical biomarker discovery and validation study. *Clin Epigenetics* 2019; **11:** 158.

23. Henriksen TV, Drue SO, Frydendahl A*, et al.* Error Characterization and Statistical Modeling Improves Circulating Tumor DNA Detection by Droplet Digital PCR. *Clin Chem* 2022; **68:** 657-667.

24. Hallermayr A, Benet-Pagès A, Steinke-Lange V*, et al.* Liquid Biopsy Hotspot Variant Assays: Analytical Validation for Application in Residual Disease Detection and Treatment Monitoring. *Clin Chem* 2021; **67:** 1483-1491.

25. Armbruster DA, Pry T. Limit of blank, limit of detection and limit of quantitation. *Clin Biochem Rev* 2008; **29 Suppl 1:** S49-52.

26. Milosevic D, Mills JR, Campion MB*, et al.* Applying Standard Clinical Chemistry Assay Validation to Droplet Digital PCR Quantitative Liquid Biopsy Testing. *Clin Chem* 2018; **64:** 1732-1742.

27. Geeurickx E, Hendrix A. Targets, pitfalls and reference materials for liquid biopsy tests in cancer diagnostics. *Mol Aspects Med* 2020; **72:** 100828.

28. Coriell-Institute. http://ccr.coriell.org/Sections/Search/Search.aspx?PgId=165&q=GM12878.

29. Basu AS. Digital Assays Part I: Partitioning Statistics and Digital PCR. *SLAS Technol* 2017; **22:** 369-386.

30. Bio-Rad. Droplet Digital™ PCR Applications Guide, Bulletin 6407.

31. Hallermayr A, Keßler T, Fujera M*, et al.* Impact of cfDNA Reference Materials on Clinical Performance of Liquid Biopsy NGS Assays. *Cancers (Basel)* 2023; **15**.

32. Lievens A, Jacchia S, Kagkli D*, et al.* Measuring Digital PCR Quality: Performance Parameters and Their Optimization. *PLoS One* 2016; **11:** e0153317.

33. Spindler KG, Jakobsen A. Circulating tumor DNA: Response Evaluation Criteria in Solid Tumors - can we RECIST? Focus on colorectal cancer. *Ther Adv Med Oncol* 2023; **15:** 17588359231171580.