# Critical Factors in the Analytical Work Flow of Circulating Tumor DNA-Based Molecular Profiling

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**Review**

# Critical Factors in the Analytical Work Flow of Circulating Tumor DNA-Based Molecular Profiling

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**BACKGROUND:** Liquid biopsy testing, especially molecular tumor profiling of circulating tumor DNA (ctDNA) in cell-free plasma, has received increasing interest in recent years as it serves as a reliable alternative for the detection of tumor-specific aberrations to guide treatment decision-making in oncology. Many (commercially available) applications have been developed, however, broad divergences in (pre)analytical work flows and lack of universally applied guidelines impede routine clinical implementation. In this review, critical factors in the blood-based ctDNA liquid biopsy work flow are evaluated.

**CONTENT:** In the preanalytical phase, several aspects (e.g., blood collection tubes [BCTs], plasma processing, and extraction method) affect the quantity and quality of the circulating cell-free DNA (ccfDNA) applicable for subsequent molecular analyses and should meet certain standards to be applied in diagnostic work flows. Analytical considerations, such as analytical input and choice of assay, might vary based on the clinical application (i.e., screening, primary diagnosis, minimal residual disease [MRD], response monitoring, and resistance identification). In addition to practical procedures, variant interpretation and reporting ctDNA results should be harmonized. Collaborative efforts in (inter)national consortia and societies are essential for the establishment of standard operating procedures (SOPs) in attempts to standardize the plasma-based ctDNA analysis work flow.

**SUMMARY:** Development of universally applicable guidelines regarding the critical factors in liquid biopsy testing are necessary to pave the way to clinical implementation for routine diagnostics.

## Introduction

Blood environs cells and cellular components originating from virtually every organ can serve as analytes in diagnostics. The characterization of circulating nucleic acids can be applied to prenatal testing, organ transplant monitoring, and molecular profiling of cancer (1). In oncology, tumor-derived nucleic acids can be extracted from circulating tumor cells (CTCs), extracellular vesicles (EVs), tumor-educated platelets (TEPs) or from the cell-free plasma fraction of blood. CTCs are generally present at very low levels and only in few patients for most types of cancer (2, 3). Tumor cell-derived exosomes—a subset of EVs—cargo molecular components which could serve as biomarkers in liquid biopsy, however, clinical applications remain restrained by the lack of isolation and analysis methods of sufficient quality (4, 5). Although RNA expression-based profiling and tumor classification has shown promising results (6), tumor-derived RNA levels in TEPs are too low to reliably measure clinically relevant aberrations (7). Therefore, there is a growing interest in oncological diagnostics to efficiently extract and characterize circulating nucleic acids, in particular the circulating cell-free DNA (ccfDNA) in plasma (2, 8–12). Through apoptosis, necrosis, and active secretion, cells release DNA into the bloodstream and, in the case of cancer, a fraction of the total ccfDNA derives from the tumor cells—referred to as circulating tumor DNA (ctDNA)—which harbors similar molecular aberrations as the cells it originated from (13). Although ccfDNA and ctDNA are collected through minimally invasive phlebotomy, their unique biological features impede proper molecular analysis. First, ccfDNA is enzymatically degraded around the nucleosome-protected fragments averaging around 166 base pairs (bp) in the bloodstream (14). Degradation patterns are slightly different for ctDNA compared to general ccfDNA, thereby enabling separation between the two using fragment size analysis (15). Second, the analytical sensitivity required to detect the ctDNA could not be reached in a subset of cancer patients related to stage of disease, tumor size, and tumor type amongst other confounders (8, 14). Third, specific tumor types (e.g., brain, kidney, and thyroid) have, in general, lower levels of ctDNA compared to other cancers (e.g., breast, colorectal, and lung) (16). Finally, ccfDNA—encompassing ctDNA—is cleared by liver and spleen and excreted renally, eventually resulting in a short half-life (approximately

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30 min) (14, 17–20). Hence, although ctDNA represents an actual image of the tumor molecular status, its instability complicates subsequent analyses. Nevertheless, when detectable, ctDNA could therefore be used to perform similar molecular diagnostics as presently executed routinely on tumor tissue. The ability to detect ctDNA at an early stage provides valuable information in various clinical settings (21). In the absence of tumor tissue specimens, ctDNA analysis of, for example, somatic (driver) mutations and methylation patterns, could be regarded as a reliable alternative to perform molecular tumor profiling for screening and primary diagnosis (16, 22). Additionally, studies on longitudinal ctDNA monitoring in various cancer entities have shown the ability to identify responders to immune checkpoint inhibitor (ICI) treatment with a considerable lead time to imaging (2, 23). Furthermore, although generally accepted criteria must be composed and validated, previous studies revealed that ctDNA-based early response prediction outperforms imaging as a surrogate marker for overall survival (24). Therefore, it is believed that ctDNA monitoring enables the analysis of consecutive time points while reducing exposure of patients to insensitive computed tomography (CT)-scanning or invasive collection of multiple tumor tissue biopsies. Longitudinal testing for ctDNA allows minimal residual disease (MRD) detection, on-treatment response monitoring, and identification of acquired resistance mutations (8, 25). Despite these developments, ctDNA analysis has not been implemented universally in routine clinical practice for predictive testing or disease monitoring applications as clinical utility has yet to be demonstrated.

To date, the major issue delaying clinical implementation is the lack of harmonization in (pre)analytical work flows (11, 26–28). For each proposed clinical application of ctDNA testing, divergent strategies to perform molecular tumor profiling are applied, due to the absence of universal standard operating procedures (SOPs), and therefore generate insufficient evidence of clinical validity and utility. Despite the analytical considerations required for clinical implementation having been previously recommended (2, 22), the variety of commercially available products (e.g., blood collection tubes [BCTs], ccfDNA extraction kits, and ctDNA detection assays) keeps expanding. Many studies reported previously on the effect of such factors on the quantity and quality of ccfDNA and ctDNA, which have been reviewed extensively (26, 28, 29). However, the attempts to establish (pre)analytical protocols and guidelines (e.g., European Society for Medical Oncology (ESMO), European Liquid Biopsy Society (ELBS), International Association for the Study of Lung Cancer (IASLC), International Society of Liquid Biopsy (ISLB), The Blood Profiling Atlas in Cancer (BLOODPAC)) have not specified quality standards that products must meet and thereby still allow divergences that result in critical discrepancies in analytical outcomes, hampering the clinical implementation of harmonized work flows (11, 27, 30). In addition, there is significant international variation in structure and funding of healthcare systems for regulatory approval and assay reimbursement of liquid biopsy testing, resulting in differences in available assays between countries (31).

Here, the critical factors in the blood-based ctDNA liquid biopsy work flow are evaluated from blood collection to variant interpretation and reporting. Although methylation among other biomarkers has shown great promise in liquid biopsy research (2, 32, 33), this review focuses specifically on considerations for somatic variant analyses as (pre)analytical protocols differ considerably. Since specific factors have been reviewed previously (8, 11, 12, 16, 19, 21, 27, 28), special emphasis has been placed on potential applications of liquid biopsies in oncological diagnostics, including screening and molecular target identification for diagnosis and treatment decision-making, and MRD and disease monitoring, including early detection of therapy resistance. In addition, recommendations on (inter)national collaborative efforts of proving clinical utility that would support routine implementation are discussed.

## Preanalytical Considerations

The preanalytical phase of liquid biopsy approaches is crucial to properly isolate the analytes of interest. Whole blood contains ctDNA, circulation tumor RNA (ctRNA), CTCs, TEPs, tumor-derived proteins and metabolites, and tumor-derived EVs, each with their own stability (2, 10, 14). The blood processing method determines whether the analytes can be extracted with sufficient quantity and quality. The preanalytical phase of plasma-derived ctDNA analysis encompasses blood collection, cell-free plasma processing, and ccfDNA extraction.

### BLOOD COLLECTION

*Blood collection procedure.* During blood collection, there are several factors that can affect the quality of the ccfDNA in plasma. Phlebotomy using butterfly needles significantly reduces hemolysis compared to conventional intravenous catheters (34). Cellular breakage contaminates the ccfDNA with high molecular weight (HMW) genomic DNA (gDNA), thereby increasing background DNA levels and impeding the detection of ccfDNA (28, 35). In addition, gDNA can contain nontumorous variants—in particular, variants related to clonal hematopoiesis of indeterminate potential (CHIP)—which complicates molecular interpretation (36, 37). To this end, hemolysis must be prevented as much as possible to facilitate sensitive ctDNA detection and ensure

accurate variant identification. Although the use of needles with larger gauges is associated with less hemolysis (28, 38), confirmation on whether needle gauge size affects ccfDNA integrity is lacking. Despite that biological factors, including psychosocial and physical stress, site of venipuncture, food consumption, and circadian rhythm, are proposed to influence ccfDNA properties, little evidence is available to support a direct relation with these factors and ccfDNA integrity (28, 39, 40). However, brief spikes in ccfDNA concentrations in plasma have been observed following rigorous exercise due to elevated cell degradation (17), which might impair ctDNA detectability more prominently than, for example, choice of needles. Since ccfDNA is rapidly cleared in blood, ccfDNA levels will normalize shortly after exertion. Limiting physical activity directly prior to phlebotomy is therefore recommended.

*Sample quantity.* The volume of blood collected during a blood draw is primarily based on preference and ethical constraints. Whereas researchers prefer to collect high volumes of blood to enable repetitive testing of samples, the physical conditions of and the burden on the patients should be considered. A single 10 mL BCT generally yields 4 to 5 mL of cell-free plasma. Despite the fact that many commercially available liquid biopsy tests rely on 2 mL (e.g., Idylla), 4 mL (e.g., Cobas, Oncomine, and Avenio), or 8 mL (e.g., FoundationOne® Liquid CDx, and Guardant 360® CDx) of plasma, no standardized blood collection volumes are reported in guidelines. Recent recommendations for the practical implementation of ctDNA mutation testing suggest the collection of $2 \times 10$ mL of blood (30). Applications that rely on high ccfDNA input to secure sufficient analytical sensitivity such as screening and MRD detection might require even higher plasma volumes (8).

*Blood collection tubes.* Improper preservation and processing of blood samples induces deterioration of cells, thereby releasing HMW gDNA into the cell-free plasma fraction and diluting the ctDNA even more. The DNA-containing cellular fraction of blood consists predominantly of peripheral blood mononuclear cells (PBMCs), which are prone to accumulation of somatic mutations resulting from replication errors in their rapidly dividing hematopoietic progenitors. Prevention of HMW DNA contamination of the ccfDNA is therefore crucial to enable reliable evaluation of tumor-derived mutations. BCTs without preservative agents (e.g., EDTA) require fast processing (within 2 to 4 h) of blood sampling to limit hemolysis. Thus, the use of BCTs that contain formaldehyde-free cell preservative agents (e.g., Streck or PAXgene) facilitate the processing procedure since they stabilize blood cells, eliminating the need for rapid processing. Of note, the blood should be mixed properly with the preservative agents for optimal prevention of cell degeneration. For ctDNA testing, heparin BCTs should be avoided because of interference with PCR applications (41). The use of different BCTs has been extensively studied and reviewed previously (26–28, 30, 42). These studies concluded that ccfDNA levels and quality were similar between preservation BCTs and EDTA when worked up within the recommended maximal processing times. The choice of BCTs is therefore often based on costs and the biobanking infrastructure. BCTs with preservative agents are approximately 50- to 100-fold more expensive than EDTA tubes, so if fast processing can be achieved, use of EDTA tubes is often preferred. However, facilitating biobanking whenever a sample is received is often a major financial burden on laboratories that outweighs the costs of the BCTs, except for high-throughput laboratories.

*Sample transport and handling.* Blood collection is a routine procedure performed in all healthcare centers. However, processing blood for ccfDNA extraction and ctDNA testing are in general (regionally) centralized in a dedicated department within (academic) hospitals or specialized institutions. Therefore, blood samples have to be transported within hospitals or from outpatient clinics to the central laboratories. In many cases, transportation exceeds the maximal storage time of BCTs without stabilizing agents and thus requires preservation BCTs. During transportation BCTs should be handled with care to prevent cellular degeneration due to mechanical stress and sample agitation. Recently, a study confirmed that transportation through pneumatic tube systems within an institution augments the release of cellular DNA in plasma collected in EDTA BTCs compared to delivery by courier (43). In contrast, DNA levels in Streck BCTs remained similar, implicating better resistance to mechanical stress for preservation BCTs. Vacuum BCTs were originally made of glass but are currently more often constructed of plastic materials. Compared to glass, plastic tubes minimize exposure to potentially biohazardous material following breakage due to greater shock resistance and toleration of higher centrifugation speeds (41). Therefore, glass BCTs require caution during transportation in shipping envelopes through protective blisters, absorbing materials, and safety bags.

*Storage of blood specimens.* Prolonged storage of BCTs without preservative agents before processing induces the degradation of ccfDNA and increases the deposition of gDNA in the plasma, thereby impairing the integrity of the sample and impeding the detection of ctDNA (44). Time to processing should therefore be kept as short as possible. Manufacturers of BCTs containing

preservative agents report ccfDNA stability of up to 14 days if stored appropriately, confirmed for up to 7 days (42). Nevertheless, prolonged storage of preservative BCTs (even though in a vacuum) induces hemolysis and evaporation and, therefore, a preferred storage time of less than 2 days at room temperature is recommended (11, 27, 30). Storage of BCTs at 4°C instead of room temperature did not show any improvement in sample quality (45).

## CELL-FREE PLASMA PROCESSING

*Centrifugation.* Single low-speed centrifugation renders remnant cells in plasma—which are prone to hemolysis—and therefore is insufficient to make plasma cell-free (46). Previous studies have shown that double centrifugation drastically reduced gDNA contamination, hence, the most commonly used protocols recommend performing a two-step centrifugation (28, 35, 47). In the first centrifugation step, blood cells (both PBMCs and erythrocytes) are separated from the plasma while minimizing cell degeneration. The upper cell-free plasma fraction should be removed carefully without agitating the buffy coat layer, which harbors leukocytes and CTCs (if present). As pipetting of the plasma inevitably disturbs the cellular layers, thereby contaminating the plasma with cells, a second centrifugation step is required to pellet and fully remove residual cells and remaining debris. Centrifugation temperatures do not affect the sample quality (42, 47). The initial low-speed centrifugation step is generally performed at 800*g* to1600*g* to prevent cellular degradation, while the second centrifugation step is often at high velocity (16 000*g* to 20 000*g*) (28, 42, 48). Comparative studies have revealed comparable ccfDNA concentrations using either one-step or two-step centrifugations protocols, however, cell-free plasma isolation using one-step protocols contained elevated levels of gDNA contamination (48, 49). The second centrifugation step can also be performed prior to ccfDNA extraction after durable storage and thawing of cell-free plasma and yield similar ctDNA recovery (50).

*Centrifuge braking.* Despite appropriate centrifugation conditions rendering the plasma cell-free, to optimally separate the cellular and plasma fractions, braking of the centrifuge should be avoided, in particular during the first centrifugation step, to limit cell resuspension. However, not applying a brake during centrifugation significantly prolongs the duration of processing. Therefore, most current biobanking protocols do not provide recommendations for brake settings and virtually all laboratories use light braking to facilitate efficient work flows and time management (47). Package inserts from BCT providers allow the use of light braking (https://www.streck.com/wp-content/uploads/sync/Stabilization/Cell-Free_DNA_BCT_RUO_CE/01_Instructions_(IFU)/01_Cell-Free_DNA_BCT_RUO_IFU.pdf). When a light brake in the first centrifugation step is applied, the second centrifugation step ensures the removal of any remnant cells. Heavy braking should be avoided at any time.

*Storage of cell-free plasma.* The cell-free plasma from different BCTs of the same blood draw must be pooled and homogenized to ensure that each stored aliquot has a similar composition. Long-time storage of cell-free plasma should be at −80°C since ccfDNA concentrations deteriorate at −20°C after a couple of months (51). In addition to temperature, the selection of tubes for long-time storage is important. DNA helices are strongly hydrophilic and can bind to polypropylene polymers in plastics resulting in conformational changes, complete denaturation, and strand separation, predominantly affecting short-sized DNA fragments (52). Therefore, cell-free plasma samples should be stored in low-bind virgin polypropylene tubes to enable efficient ccfDNA recovery.

## CCFDNA EXTRACTION

*Thawing conditions.* Thawing of stored plasma samples should be executed slowly on ice to avoid reactivation of nucleotide-degrading enzymes. After only a few freeze–thaw cycles, loss of ccfDNA integrity is observed due to degradation (50, 52). As freeze–thaw cycles must be minimized, it is recommended to store the plasma in small fractions (e.g., 1 mL) to ensure efficient usage for multiple analyses. If plasma needs to be transported for analysis, it is crucial that it remains frozen solid on dry ice since thawing during transport reduces the quality (53).

*Plasma and elution volumes.* The ccfDNA available for molecular analyses depends on the amount of plasma used during ccfDNA extraction. ccfDNA concentration from cancer patients varies from non-detectible values to 1000 ng/mL of plasma, averaging around 10 ng/mL (19, 28, 54). Therefore, 8 to 10 mL of plasma collected from two 10 mL BTCs contain sufficient ccfDNA to detect and quantify ctDNA for most analytical applications. However, it is of importance to consider what plasma and elution volumes to use during extraction as this ultimately determines the concentration and availability of the ccfDNA sample. Many technologies have limited input volumes and would therefore benefit from the use of high plasma volumes during extraction to generate concentrated eluates and increase the analytical sensitivity. Lowering the elution volume, however, reduces the ccfDNA extraction efficiency (55). On the other hand, partitioning plasma over several extractions and increasing elution volumes allows for repetitive

testing of ccfDNA from the same blood collection, but lowers the ccfDNA concentration. At a minimum, plasma and elution volumes should be consistent throughout preanalytical work flows to ensure accurate comparative quantification between patients and during serial testing.

*ccfDNA extraction chemistry.* There are many ccfDNA extraction kits that are commercially available from multiple companies. Previous comparison studies analyzed a few of these methodologies using reference material, (patient-derived) plasma, or serum, and frequently yielded contradictory results (28, 53). There are 3 types of chemistries utilized in ccfDNA extraction methods. Most kits use either silica membrane columns or magnetic beads to extract the DNA from bodily fluids (particularly cell-free plasma); some kits are based on polymer-mediated enrichment [reviewed elsewhere (49, 56)]. Silica membrane- and magnetic bead-based extraction methods, especially the QIAamp Circulating Nucleic Acids Kit (Qiagen) and the Maxwell RSC LV ccfDNA Kit (Promega), respectively, have been studied and compared extensively (26, 45, 53). In general, ccfDNA yield is the most evident measure of extraction efficiency. Many studies using either patient-derived plasma or artificial material show that silica membrane-based kits yield more ccfDNA than methods using magnetic beads [reviewed in (53)]. However, as ccfDNA consists of DNA originating from cells all over the body (57), preferential extraction of tumor-derived DNA compensates for a less efficient total ccfDNA extraction. Although no methodologies are specifically designed to extract solely short DNA fragments, previous studies advocate that magnetic bead-based extraction enriches for short-sized ccfDNA fragments, mainly due to less efficient recovery of HMW gDNA (58). Despite some confirmation using artificial reference samples (26), no differences in ccfDNA fragment size distributions were observed in real-life clinical patient-derived plasma samples (53), indicating that no preferential ctDNA extraction occurs. In line with this, detection of ctDNA levels correlated with the ccfDNA yield and showed higher mutant molecule levels in silica membrane-based kits (53). To date, a high total ccfDNA yield remains the most reliable measure to indicate suitability of a sample for accurate tumor mutational profiling.

*ccfDNA extraction work flow.* The work flow for ccfDNA extraction must be considered carefully, especially for larger, high-throughput laboratories. Virtually all silica membrane-based methodologies require manual labor, while many magnetic bead-based assays can be fully automated or have automation capabilities. Automated applications reduce the hands-on time of the extraction as well as operator-based variability, thereby increasing the concordance of intra- and interlaboratory results (26). Although this is a very important matter in terms of harmonization of the preanalytical phase, automated work flows generally yield significantly less ccfDNA than manual procedures (53). Optimization of (semi)automatic methodologies that do not reduce total ccfDNA recovery should be pursued. Moreover, automated work flows are only cost-efficient with a large throughput of samples (59).

#### PATIENT-MATCHED REFERENCE SAMPLES

For accurate ctDNA-based molecular tumor profiling, patient-matched reference samples are required to differentiate between tumor-derived and germline variants. DNA from PBMCs is frequently used as patient-specific reference material in the analysis of germline and clonal hematopoietic variants. PBMCs are abundant in blood and can be isolated from each type of BCT after the first centrifugation step without processing time restrictions. Either the buffy coat layer or the entire cellular fraction could be collected and stored for years to decades at −80°C (60, 61). Some protocols recommend lysing red blood cells with ammonium-chloride-potassium (ACK) lysing buffer prior to storage of the cellular fraction to purify the PBMCs and reduce the sample volume. After storage, DNA can be extracted using a general cell-based isolation protocol. Since the DNA from PBMCs is HMW, the DNA must be fragmented before ccfDNA-based analysis.

## Critical Factors in the Analytical Work Flow

For optimal utilization of the usually small amount of ccfDNA, several factors should be considered. Since plasma-derived ccfDNA is precious material, the ability to perform multiple tests on the same eluate is preferred and encouraged for reproducibility of results. Quantitative and qualitative factors influence the type of analysis that should be performed for each application. Therefore, optimizing analytical considerations is crucial for routine clinical implementation, and analysis may differ depending on the application.

#### CCFDNA QUANTIFICATION AND ANALYTICAL INPUT

For many liquid biopsy applications, a defined input is required and therefore quantification of the ccfDNA prior to ctDNA testing is needed. Molecular profiling techniques often have limited input volumes. Due to that limitation, not every sample has a high enough ccfDNA concentration to reach the analytical sensitivity of that method. Therefore, quantitative analysis ensures that solely samples with sufficient ccfDNA abundance are evaluated. Quantification of ccfDNA is performed using fluorescence-based assays (e.g., Qubit, Nanodrop, and

TapeStation) or PCR-based tools (e.g., quantitative PCR (qPCR), droplet digital PCR (ddPCR), and mass spectrometry). Fluorescence-based assays generally have short turnaround times and are easy to implement in a work flow. However, they have large margins of error compared to time-consuming PCR-based methods (26). Therefore, many laboratories apply a fluorescence-based approach to estimate the analytical input. On the other hand, PCR-based assays quantify only the amplifiable ccfDNA and thereby represent the fraction of material applicable for subsequent analyses (45, 62, 63). For screening, MRD follow-up, and resistance mutation identification, sufficient sensitivity to detect tumor-derived mutations is of utmost importance. Higher ccfDNA input elevates the chance of detecting the low-frequency ctDNA fragments required to determine the presence of cancer cells (8). To this end, analyzing most of the available material is recommended irrespective of the ccfDNA concentration. Guidelines should be composed to set thresholds of minimal ccfDNA inputs below which sufficient analytical sensitivity cannot be ensured. For primary diagnosis and on-treatment disease monitoring in advanced-stage disease, applying maximal amounts of ccfDNA is less essential since ctDNA derived from abundant tumor cells is often present in the plasma for molecular profiling. In fact, for reliable comparative quantitative monitoring, use of similar fractions of the extracted ccfDNA is important as changes in the actual number of mutant molecules harbor prognostic value. However, sufficient ccfDNA remains crucial to ensure the analytical sensitivity of the assay. Again, criteria should be defined to determine the minimal wild-type coverage required for accurate interpretation.

### CCFDNA QUALIFICATION

Several quantitative assays (e.g., TapeStation, size-based ddPCR, and mass spectrometry) are able to determine qualitative properties of the ccfDNA as well. The presence of HMW gDNA, probably due to inaccurate blood sample handling resulting in hemolysis, disturbs the analysis of extracted ccfDNA (64). Therefore, fragment size distribution qualification of ccfDNA indicates whether a sample is suitable for accurate tumor mutational profiling. Alternatively, recent studies apply shallow whole genome sequencing (sWGS) techniques followed by the evaluation of somatic copy number alterations (SCNAs) to determine the actual tumor fraction in the plasma (65–67). This tumor fraction could be used to determine the quality of a ccfDNA sample, to estimate the quantity of ctDNA, and to indicate whether it is appropriate for subsequent analysis. Presently, the clinical value of SCNA detection remains limited mainly due to poor sensitivity of sWGS. However, a combination of SCNA analysis and fragmentomics has recently been reported as a promising tool to increase detection sensitivity using low ccfDNA input (68). To date, sample qualification is considered optional since the added value has not been studied in detail yet and interpretation of results or guidelines on when to exclude samples for analysis are lacking.

### CTDNA ANALYSIS METHOD

ctDNA testing enables early detection of tumor markers harboring relevant clinical value. For each application of liquid biopsy testing (i.e., screening, primary diagnosis, MRD, response monitoring, and resistance identification), however, different requirements apply to select an appropriate analytical assay. There are many molecular tumor profiling techniques commercially available, designed to target either a single target (e.g., ddPCR), single or multiple gene panels (e.g., Cobas, Idylla, and UltraSEEK®), or elaborate NGS panels (e.g., Oncomine, Avenio, FoundationOne® Liquid CDx, and Guardant 360® CDx) (11, 16, 27, 30). The differences in performance between these platforms have been extensively studied [reviewed in (29, 69–71)]. In general, there are 2 liquid biopsy strategies with regards to molecular tumor profiling: tumor-informed testing and tumor-agnostic analysis. Here, considerations for selection of a certain strategy are addressed.

*Tumor-informed testing.* Personalized molecular profiles can be achieved by sequencing primary tumor tissue biopsy specimens. For several cancer types in the metastatic setting (e.g., lung, colorectal, breast, and melanoma) (16), tumor tissue next-generation sequencing (NGS) is currently routinely implemented using SOPs to properly diagnose patients and assist in treatment decision-making. Based on the targets identified in the tumor tissue, patient-specific (driver) mutations can be retrieved in plasma using commercially available assays or personalized panels. Several assays [e.g., RaDaR, Signatera, CAPP-Seq, Safe-SeqS, FoundationOne Tracker, and ArcherDX; reviewed in (72)] combine tumor tissue whole genome sequencing (WGS) with the development of personalized targeted panels which could be applied for longitudinal analyses. Previous studies have shown high concordances between tumor tissue NGS and plasma-derived ctDNA testing across various platforms. Tumor-informed analyses are particularly suitable for MRD detection and surveillance (72), and for monitoring of ctDNA dynamics (73) as, when a mutation is detected, they could be regarded as a surrogate for, respectively, recurrence or tumor load. For MRD and monitoring purposes, limiting testing to one or a couple of targets provides a cost-efficient alternative compared to broad sequencing strategies. However, tumor tissue NGS is only performed on a small sample of the total tumor mass and lacks information on tumor heterogeneity and clonal evolution of the disease. Furthermore,

development of on-treatment resistance mutations might be missed by limiting analysis to a minimal number of targets. Therefore, single- or limited-target monitoring of tumor-derived mutations seems suitable to resemble tumor load but requires additional elaborate analysis to identify predictive markers associated with disease progression.

*Tumor-agnostic analysis.* The detection of tumor-derived mutations could be performed in a tumor-agnostic manner. In theory, elaborate plasma-derived plasma molecular tumor profiling offers a suitable alternative to overcome the recurrent challenges of conventional tumor tissue-based diagnostics such as intra- and inter-tumor heterogeneity and tumor accessibility (74). Tumor-agnostic testing therefore is most appropriate for screening and primary tumor mutation profiling when tumor tissue is unavailable, and for identification of acquired resistance mutations at disease progression. Tumor-agnostic MRD and surveillance (75) and ctDNA monitoring (76) have been described as well, however, they require additional quality controls for specific variant calling. In general, ensuring that an identified mutation is truly derived from the tumor is essential. Uninformative variants originating from various sources throughout the body—especially those from cells of the hematopoietic lineage referred to as CHIP—have to be excluded from the data (36). Previous studies have identified one or multiple CHIP-related variants in 45% and 40% of non-small cell lung cancer (NSCLC) patients, respectively, stressing the impact of clonal hematopoiesis and the interpretation of ctDNA results for clinical decision-making (76). Parallel analysis of patient-matched reference samples, preferably PBMCs, aids in removal of uninformative variants. By limiting the analyzed targets to only clinically relevant mutations, the need for reference sample testing is reduced. However, one should be wary that CHIP-related variants occur in genes that affect treatment decisions as well (e.g., *KRAS* and *TP53*) (36, 76). Additionally, tumor-agnostic approaches analyzing molecular targets other than mutations are reported as well, including fragmentomes and methylation. In fragmentomics, exploitation of physiological differences between general ccfDNA and ctDNA, such as fragment length distributions and fragment end sequences, could be used to identify ctDNA in subsequent data interpretation. Tumor-specific methylation patterns are well known for many tumor types and occur early in the tumor [reviewed in (77, 78)]. PCR- or sequencing-based methylation profiling can discriminate cancer patients from healthy individuals and might even be applied as generic tumor markers (79).

#### REFERENCE MATERIAL

Parallel testing of patient-matched reference samples is essential to eliminate calling of germline and CHIP-related variants, at least for tissue-agnostic approaches. However, to prevent false-positive and false-negative calling, it is of utmost importance to include reference samples that are both positive and negative for the analyzed variants to assess the assay performance, particularly during validation and for external quality assessment (EQA) programs (80). Well-characterized commercially available reference materials containing (spiked-in) mutations seem the most appropriate samples to serve as references (26, 29, 81). However, development of suitable reference materials covering unique variants with low variant allele frequency (VAF) (<0.01%) required for certain applications (e.g., screening, MRD) is challenging. Currently, quality requirements and (international) guidelines regarding reference samples for the clinical approaches of liquid biopsy testing are lacking.

#### ANALYTICAL OUTCOME

In current diagnostics, the measure of analytical outcome is becoming increasingly important as accumulating studies show the relation between ctDNA levels and tumor load (8, 16, 82). PCR-based assays and targeted panels are generally semiquantitative and therefore suitable for the identification of mutations related to the primary tumor or acquired resistance (30). Methodologies with strong quantitative potential (e.g., digital PCR- and NGS-based assays) are applicable for monitoring purposes as well (73). The results of quantitative assays are frequently presented as the number of mutant molecules per milliliter of plasma or the VAF. The VAF is the ratio between the mutant and wild-type copies at a certain genomic region and thereby represents the tumor fraction in the plasma while taking the total ccfDNA input into account. However, the wild-type ccfDNA concentration is highly variable, related to physiological conditions (see section Blood collection procedure) and might negatively affect accurate quantification of ctDNA dynamics. Previous studies have shown that monitoring of mutant molecules is superior to VAF in treatment response prognosis and prediction of progressive disease (76, 83).

## Postanalytical Contemplations

In the postanalytical phase, there are several factors that affect the sensitivity and specificity of tumor-derived variant calling. Although the analysis software of many tests has incorporated algorithms for variant calling, the raw data collected potentially contains additional valuable information. Fragmentomic and other bioinformatic pipelines can significantly improve the accuracy of ctDNA

detection (15). Additionally, combining biomarkers assists in identifying cancer patients and treatment response predictions (1). Postanalytical analyses have an effect on how detected variants are interpreted and ultimately adopted in molecular diagnostic reports.

#### VARIANT INTERPRETATION

Besides proper sample handling and application of appropriate analytical algorithms, postanalytical evaluation of the detected variants is required for accurate interpretation. A major complication in variant calling is due to artifacts that accrue during sample preparation and NGS (84). Specifically, low ctDNA abundance associated with non-metastatic and early-stage lesions may be indistinguishable from sequencing errors. Therefore, previous studies filtered variants with few unique reads or at low VAFs (76, 85). Bioinformatic curation enables in silico size-selection to enrich for ctDNA and improve the detection of tumor-derived mutations (84). After removal of sequencing artifacts, germline, and CHIP-related variants (see section Tumor-agnostic analysis), each remaining mutation should be critically assessed for clinical and biological relevance. Variant interpretation protocols have already been established for tissue-based routine diagnostics (86). It is recommended to apply similar strategies in tumor-agnostic primary molecular profiling. Commercial decision support tools for variant interpretation of plasma-derived tumor-specific variants are in development. However, a recent assessment of the concordance of tier classification approaches between 3 platforms [Roche NAVIFY Mutation Profiler; QIAGEN Clinical Insight (QCI) Interpret, and CureMatch Bionov] using the same NGS data from cell-free DNA of patients with metastatic breast, colorectal, and NSCLC, revealed unfavorable deviations in treatment recommendations (87). For the selection of the most appropriate and reliable decision support approach for ctDNA results, additional development and validation of platforms is required. During longitudinal monitoring, however, the biological relevance of a variant is less important as long as it is assured that the variant is tumor-derived, although a driver mutation is preferred.

#### REPORTING

Diagnostic molecular reports should encompass all relevant information to enable accurate clinical characterization and treatment decision-making, including the detected variants with clinical significance and VAF, methodological and metrical data, limit of detection, analytical sensitivity, clinical specificity, coverage, and reference range (11, 27, 30, 88). Accurate and harmonized reporting of variants is necessary to facilitate correct interpretation using the appropriate nomenclature according to the Human Genome Variation Society (HGVS) guidelines. In current tumor tissue-based diagnostics, many laboratories solely report variants in genes with therapeutic relevance. For liquid biopsy approaches, which types of variants should be reported remains to be determined. Of note, since the ctDNA abundance is uncertain, negative results should be interpreted with caution as plasma tumor fraction could be below the analytical sensitivity of the assay. Therefore, such findings should be regarded as not detectable instead of ctDNA negative. As described earlier (see chapter ccfDNA qualification), the assessment of the plasma tumor fraction using sWGS might assist to correctly interpret true-positive and -negative results. Distinguishing known predictive mutations detected at low VAF (<0.05%) from sequencing artifacts or false-positive calls in patients with a high plasma tumor load is challenging. An international guideline to accurately interpret and report ctDNA results is urgently needed. Molecular tumor boards (MTBs) can assist with appropriate interpretation of questionable results (89).

## Standardization

Accumulating research has shown the clinical validity of many liquid biopsy approaches. However, implementation in clinical practice has not been achieved so far due to the divergent use of protocols and the lack of evidence regarding clinical utility. For clinical implementation, standardized protocols and work flows have to be developed and validated in interlaboratory studies in attempts to establish consensus guidelines. Interlaboratory comparative studies are required to assess the effect of discrepancies in work flows on ctDNA detection. Previous studies have shown that divergent preanalytical conditions affect the total ccfDNA yield and the recovery of ctDNA molecules (26, 53, 90). EQAs analyzing several aspects of (pre) analytical procedures are required to develop criteria that assays have to meet to be implemented in a clinical work flow and ultimately lead to the development of universally applicable guidelines and SOPs. Collaboration in (inter)national consortia and societies [e.g., CANCER-ID, BLOODPAC, ISLB, ELBS (91) and ctDNA on the way to implementation in the Netherlands (COIN) (53)] should pursue a leading role in EQAs and have ambition to harmonize (pre)analytical work flows for clinical applications. In addition, to ensure equal access for all patients to high-quality ctDNA testing in clinical practice following most recent developments and guidelines, implementation profits from close collaboration within regional networks involving multiple peripheral hospitals around a centralized expert laboratory.

**Table 1. Summary of the most critical factors and recommendations in the preanalytical, analytical, and postanalytical phase of ctDNA analysis.[a]**

| Chapter | Critical factor | Recommendation | Evidence-based relevance[b] | Supportive literature |
|---|---|---|---|---|
| ***Preanalytical considerations*** | | | | |
| *Blood collection* | | | | |
| Blood collection procedure | Hemolysis during phlebotomy | Use of butterfly needles | *** | Ungerer et al. (28), Chiu et al. (35) |
| | Biological features hampering ccfDNA integrity | Minimize physical divergences prior to phlebotomy | * | Ungerer et al. (28) |
| Sample quantity | Plasma availability for ccfDNA extraction | 2 × 10 mL BCTs per blood collection | ** | Heitzer et al. (30) |
| Blood collection tubes | ccfDNA stability after blood collection | Proper handling of BCTs following protocol | *** | Rolfo et al. (27), Ungerer et al (28), Heitzer et al. (30) |
| Sample transport and handling | Cellular degeneration due to improper handling | Minimize mechanical stress and samples agitation | * | Ungerer et al. (28) |
| Storage of blood specimens | Hemolysis and evaporation during BCT storage | Use preservative BCTs when storage >4 h is required | *** | Rolfo et al. (27), Heitzer et al. (30) |
| *Cell-free plasma processing* | | | | |
| Centrifugation | Remnant cells in plasma | Apply double centrifugation | *** | Ungerer et al. (28) |
| Centrifuge braking | Cell resuspension during braking of centrifuge | Avoid heavy braking | — | — |
| Storage of cell-free plasma | ccfDNA stability during storage | Prolonged storage of cell-free plasma at −80 °C | *** | El Messaoudi et al. (51), Bronkhorst et al. (52) |
| | Efficient ccfDNA recovery after plasma storage | Use low-bind virgin polypropylene tubes | ** | Bronkhorst et al. (52) |
| *ccfDNA extraction* | | | | |
| Thawing conditions | Reactivation of nucleotide-degrading enzymes | Thaw stored cell-free plasma on ice | ** | Bronkhorst et al. (52) |
| Plasma and elution volumes | Concentration of the ccfDNA sample | Highly concentrated eluates are preferred | * | Ungerer et al. (28) |
| | Inter-run variation during ctDNA monitoring | Consistent use of plasma and eluate volumes | ** | van der Leest et al. (53), Weber et al. (76) |

*Continued*

**Table 1. (continued)**

| Chapter | Critical factor | Recommendation | Evidence-based relevance[b] | Supportive literature |
|---|---|---|---|---|
| ccfDNA extraction chemistry | Efficient ccfDNA extraction | Apply silica membrane-based ccfDNA extraction methods | ** | Ungerer et al. (28). van der Leest et al. (53) |
| ccfDNA extraction work flow | Well-organized ccfDNA extraction work flows | Optimize (semi) automatic methodologies | * | Lampignano et al. (26), van der Leest et al. (53) |
| *Patient-matched reference samples* | Exclude germline and CHIP-related variants | Use patient-matched PBMCs during ctDNA analysis | *** | Moser et al. (14), Chiu et al. (35) |
| ***Critical factors in the analytical work flow*** | | | | |
| *ccfDNA quantification and analytical input* | Accuracy of ctDNA analysis | Accurately quantify the ccfDNA prior to execution | *** | Lampignano et al. (26) |
| | Analytical sensitivity of the performed assay | Only analyze samples with sufficient concentrations | ** | van der Leest et al. (53) |
| *ccfDNA qualification* | gDNA contamination of the ccfDNA sample | Review fragment size distribution of ccfDNA | | |
| | Interpretation of negative results | Estimate plasma tumor fraction using sWGS | ** | Moser et al. (14) |
| *ctDNA analysis method* | | | | |
| Tumor-informed testing | Cost-efficient monitoring of molecular response | Limited target monitoring of tumor-derived mutations | ** | Chan et al. (16), Larribère et al. (72), Duffy et al. (73) |
| Tumor-agnostic analysis | Removal of uninformative variants | Use patient-matched PBMCs during ctDNA analysis | *** | Moser et al. (14), Chiu et al. (35) |
| *Reference samples* | Assess ctDNA assay performance | Include positive and negative reference samples | ** | Ungerer et al. (26), Weber et al. (29), Geeurickx et al. (80), Fairley et al. (81) |
| *Analytical outcome* | Fluctuations in ccfDNA concentrations | Report results as mutant copies per mL of plasma | ** | Ungerer et al. (28), Weber et al. (76), van der Leest et al. (83) |
| ***Postanalytical contemplations*** | | | | |
| *Variant interpretation* | Consider ctDNA-derived variants accurately | Critically assess biological relevance | * | Li et al. (86) |

***Continued***

**Table 1. (continued)**

| Chapter | Critical factor | Recommendation | Evidence-based relevance[b] | Supportive literature |
|---|---|---|---|---|
| *Reporting* | Discrepancies in variant reporting | Harmonized reporting according to HGVS guidelines | *** | Pasqual et al. (11), Rolfo et al. (27), Heitzer et al. (30), Dufraing et al. (88) |
| | Reports encompassing all relevant information | Define required elements to report in guidelines | ** | Pasqual et al. (11), Dufraing et al. (88), Van Casteren et al. (90) |
| | Interpretation of negative results | Report as not detectable instead of ctDNA negative | ** | Dufraing et al. (88), Van Casteren et al. (90) |
| ***Standardization*** | Harmonization of (pre) analytical work flows | Establish standardized protocols and consensus guidelines | *** | Pasqual et al. (11), Rolfo et al. (27), Ungerer et al. (28), Heitzer et al. (30) |

[a]Abbreviations: BCT, blood collection tube; ccfDNA, circulating cell-free DNA; CHIP, clonal hematopoiesis of indeterminate potential; ctDNA, circulating tumor DNA; gDNA, genomic DNA; HGVS, Human Genome Variation Society; PBMCs, peripheral blood mononuclear cells; sWGS, shallow whole genome sequencing.
[b]The available evidence-based relevance of the provided recommendations are rated as: ***strong support in current literature; **moderate support in current literature; *little support in current literature; and —no specific support in current literature.

## Conclusion

Although blood-based ctDNA analysis has shown great promise in molecular diagnostics for various cancer types, the clinical implementation of such assays has been delayed. The most prominent reasons for this are the significant divergences in preanalytical work flows and missing standardization thereof for analytical applications. Due to improper handling during blood collection and processing, ccfDNA can be contaminated with gDNA, thereby diluting the ctDNA and inducing uninformative variants that complicate molecular interpretation. Quantitative and qualitative assessment of the ccfDNA and the use of patient-matched reference samples are required to determine suitability for molecular analysis. The broad variety of commercially available products and lack of universally applied reference standards intrinsically induces deviations in the analytical procedures of different laboratories in the absence of cooperation, which has detrimental effects on interlaboratory comparability and quality assurance. In addition, these deviations hamper the provision of evidence on clinical utility of liquid biopsy testing. Consensus on bioinformatical pipelines and data analysis to distinguish true mutations from sequencing artifacts is key for appropriate variant interpretation. The current evidence-based recommendations on how to address these issues are summarized in Table 1. Initial collaborative efforts in (inter)national consortia and societies have led to guidelines that pave the way towards harmonization; however, they lack stringent quality measures and assessment protocols. Criteria that define what quality standards the critical factors in preanalytical (e.g., choice of BCTs, plasma processing, and ccfDNA extraction method), analytical (e.g., choice of assay, use of [patient-matched] reference samples, and analytical outcome), and postanalytical (e.g., variant interpretation and reporting) work flows must meet has to be pursued. Interinstitutional cooperation through EQAs can assist in the development of standardized guidelines and SOPs, required for the clinical implementation and reimbursement of ctDNA-based liquid biopsy testing.

**Nonstandard Abbreviations:** ctDNA, circulating tumor DNA; BCT, blood collection tube; ccfDNA, circulating cell-free DNA; MRD, minimal residual disease; SOP, standard operating procedure; CTC, circulating tumor cell; HMW, high molecular weight; gDNA, genomic DNA; CHIP, clonal hematopoiesis of indeterminate potential; PBMC, peripheral blood mononuclear cell; NGS, next-generation sequencing; EQA, external quality assessment; VAF, variant allele frequency.

**Human Genes:** *KRAS*, KRAS proto-oncogene, GTPase; *TP53*, Tumor Protein P53.

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