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Review

The cornerstone of integrating circulating tumor DNA into cancer management

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ABSTRACT

Recent circulating tumor DNA (ctDNA) research has demonstrated its potential as a non-invasive biomarker for cancer. However, the deployment of ctDNA assays in routine clinical practice remains challenging owing to variability in analytical approaches and the assessment of clinical significance. A well-developed, analytically valid ctDNA assay is a prerequisite for integrating ctDNA into cancer management, and an appropriate analytical technology is crucial for the development of a ctDNA assay. Other determinants including pre-analytical procedures, test validation, internal quality control (IQC), and continual proficiency testing (PT) are also important for the accuracy of ctDNA assays. In the present review, we will focus on the most widely used ctDNA detection technologies and the key quality management measures used to assure the accuracy of ctDNA assays. The aim of this review is to provide useful information for technology selection during ctDNA assay development and assure a reliable test result in clinical practice.

1. Introduction

Circulating cell-free DNA (cfDNA) molecules are double-stranded nucleic acid fragments; they are released from cells and exist in bodily fluids (e.g., blood and urine) [1]. The presence of cfDNA in human blood was first discovered in 1948 [2], yet the exact mechanism of its origins, structures, and biological properties remain unclear. Recent studies have shown that cfDNA fragments exhibit a characteristic nucleosomal size profile with a predominant length of approximately 166 bp [3]. Tumor-derived circulating cell-free DNA (ctDNA) fragments are shorter than the cfDNA molecules derived from normal cells [4]. The fragmentation pattern of cfDNA is nonrandom and is related to nucleosome positioning, which makes cfDNA naturally different from naked genomic DNA [5]. These findings suggest that cfDNA molecules are likely to be generated by apoptosis-associated enzymatic digestion [5].

In the past decades, researchers have recognized the great potential of ctDNA as a non-invasive biomarker for cancer management [6,7]. Analysis of ctDNA mutational profile can provide invaluable information to assist oncologists in deciding on therapy, monitoring treatment responses, and quantifying minimal residual disease [6–8]. Recent studies have also demonstrated that ctDNA analysis can help predict response of patients receiving anticancer immunotherapy [8,9]. However, owing to the low concentration, high fragmentation rate, and high degree of admixture with normal DNA fragments, the ctDNA fraction can be rather low and highly variable among patients ($< 0.01\%$ to $> 50\%$ of the total cfDNA) [1,6,7,10]. Therefore, the main challenge facing the detection of tumor-specific (somatic) mutations in ctDNA is to achieve acceptable levels of sensitivity and specificity. Although existing ctDNA detection technologies are highly sensitive and specific

Abbreviations: ctDNA, circulating tumor DNA; cfDNA, circulating cell-free DNA; IQC, internal quality control; PT, proficiency testing; EQA, external quality assessment; EDTA, Ethylenediaminetetraacetic acid; SOP, standardized operating procedure; WT, wild-type; SNV, single nucleotide variations; indel, small insertions and deletions; PCR, polymerase chain reaction; dPCR, digital PCR; NGS, next-generation sequencing; ARMS, amplification refractory mutation system; EGFR, epidermal growth factor receptor; PNA, peptide nucleic acid; LNA, locked nucleic acid; TM, melting temperature; NaME-PrO, Nuclease-assisted Minor Allele Enrichment with Probe Overlap; DSN, double-strand nuclease; BEAMing, beads, emulsions, amplification, and magnetics; UMI, unique molecular identifier; Safe-SeqS, the Safe-Sequencing System; TAm-Seq, tagged-amplicon deep sequencing; cSMART, circulating single-molecule amplification and resequencing technology; iDES, integrated digital error suppression; PER, position-error rate; SHERLOCK, Specific High-sensitivity Enzymatic Reporter unlocking; DETECTR, DNA Endonuclease-Targeted CRISPR Trans Reporter; HPV, human papilloma virus; LDTs, laboratory-developed tests; LoD, lower limit of detection; CAP, the College of American Pathologists; NCCL, the China National Center for Clinical Laboratories; PBCs, peripheral blood cells; CH, clonal hematopoiesis

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[6,7], the performance of a particular ctDNA assay system is laboratory-dependent, and technical variations exist in all assay processes. Recent studies have indicated that there is insufficient evidence of clinical validity and utility for the majority of ctDNA assays in advanced cancer [11], and the results are striking discordance between different ctDNA assays [12]. This raises important questions about the accuracy and reproducibility of ctDNA assays. The selection of an appropriate analytical technique is crucial for ctDNA assay development. Other determinants, including pre-analytical procedures, test validation, internal quality control (IQC), and continual proficiency testing (PT) or external quality assessment scheme (EQA), are also important for maintaining the accuracy of ctDNA assays [6,7,11,13].

In the present review, we describe the determinants that affect the accuracy of ctDNA assays. We particularly focus on the most widely-used ctDNA detection technologies and key quality management measures that assure the accuracy of ctDNA assays. We propose that a detailed understanding of the technologies, and key quality management measures could provide useful information for ctDNA assay development and assure a reliable test result in clinical practice.

2. Pre-analytical considerations

Pre-analytical procedures, including blood collection, transport, processing, storage, and cfDNA extraction, are crucial for ctDNA detection (Fig. 1A) [6,11]. A comprehensive discussion of pre-analytical variables of ctDNA assays is beyond the scope of the present review. In this section, we will only focus on procedures likely to impact the performance of the analytical system. Previous publications on pre-analytical considerations can be reviewed [11,14–17]. Current evidence suggests that plasma is preferable to serum for ctDNA analysis because leukocyte lysis during serum sample preparation can release DNA from non-cancerous cells [16,18]. Ethylenediaminetetraacetic acid (EDTA) tubes are suitable for blood storage if the sample is centrifuged within 4–6 h of collection, whereas longer periods (up to 48 h to 1 week) of storage or transportation (4 °C or room temperature) warrant leukocyte stabilization tubes [17,19,20]. Centrifuging the blood twice at low and high speeds within a few hours of collection is recommended for the removal of blood cells, which may lyse and release germline DNA [19,21]. Storage of frozen plasma for longer periods before cfDNA isolation has no effect on subsequent ctDNA analysis. However, freezing unprocessed whole blood should not be done [11]. However, long-term storage and multiple freeze-thaw cycles may result in nucleic acid degradation [16,22]. Several methods are available for cfDNA extraction; affinity column-based, magnetic bead-based, and polymer-based methods vary in their ability to recover cfDNA in terms of yield, purity, and particular fragment size [6,15,23]. Several attempts have been made to reduce DNA loss and improve cfDNA yield [24,25]. Therefore, careful comparison is warranted when determining the optimal cfDNA isolation procedure. Various current efforts are directed towards standardization of the pre-analytical workflow of ctDNA analysis. These include the European FP7 consortium SPIDIA4P (Standardization of generic Pre-analytical Procedures for In vitro DIAgnostics for Personalised Medicine, http://www.spidia.eu/). SPIDIA4P focuses on the standardization and improvement of pre-analytical procedures for in-vitro diagnostics, and technical specifications are already available for extraction of cfDNA from plasma [26]. Although suggestions for sample handling are available [11,14–17], there is no consensus standardized operating procedure (SOP). Therefore, a careful evaluation of pre-analytical variables and a solid pre-analytical validation is an absolute prerequisite to ensure that only samples of optimal quality will be analyzed [27]. Examples of pre-analytical validation study have been performed to evaluate pre-analytical variables in establishing the analysis of ctDNA as a diagnostic tool [28].

3. Analytical technologies for ctDNA analysis

Current methods to distinguish ctDNA from the wild-type (WT) cfDNA originating from normal cells are mainly based on the presence of specific and/or multiple somatic mutations, including single nucleotide variations (SNVs), small insertions and deletions (indels), gene fusions, and gene amplifications. Various technologies have been developed for somatic mutation profiling, and they generally fall into one of the three categories (Fig. 1B): enrichment polymerase chain reaction (PCR)-based, digital PCR (dPCR)-based, and next-generation sequencing (NGS)-based technologies [29–32] (Table 1).

3.1. Enrichment PCR

Enrichment PCR is a strategy for enriching low-abundance mutant alleles during the amplification of mixtures containing numerous background WT DNA molecules, to facilitate subsequent mutation detection [33]. Following the enrichment PCR, existing downstream mutation detection technologies (such as the TaqMan assay, Sanger sequencing, pyrosequencing, and high-resolution melting analysis) can be used to detect specific PCR products. Multiple approaches have been developed to enrich mutation alleles, including allele-specific PCR, blocker PCR, and NaME-PrO (Nuclease-assisted Minor Allele Enrichment with Probe Overlap).

Allele-specific PCR—which is also known as the amplification refractory mutation system (ARMS)—is based on the use of sequence-specific PCR primers that only allow the amplification of target DNA (i.e., mutant or minority alleles) [33–35]. It relies on the mismatch of the primers at the 3′-end, and is designed to permit amplification only if the nucleotides at the 3′-end of the primer perfectly complement the bases of the target sequence (Fig. 2A). Many modified techniques have been developed that combine allele-specific PCR with real-time PCR; they have sensitivity rates of 0.05–0.1%, and are used to detect various epidermal growth factor receptor (EGFR) mutations in the ctDNA of cancer patients [7,36–38]. It is worth noting that several ARMS-PCR-based assays have been approved as companion diagnostic tests in the USA and Europe [39,40]. Generally, allele-specific PCR methods are relatively convenient to use and the results are accurate and reliable. However, the analytical sensitivity of these technologies is limited by inadvertent amplification of WT DNA. Therefore, they are often not able to detect extremely low-level DNA mutations in the plasma.

An alternative strategy called blocker PCR—also known as PCR clamping—is used for low-abundance mutation detection, and suppresses WT amplification using blocker oligonucleotides. In blocker PCR, nucleic acid clamps, including peptide nucleic acid (PNA) [41,42] and locked nucleic acid (LNA) probes [43,44], are used to selectively block WT DNA molecule amplification, thereby enhancing the selective amplification of target mutated alleles. LNA and PNA are synthetic nucleotide analogs that bind tightly to their complementary WT templates [45]. However, LNA/PNA-DNA duplexes are much more susceptible to destabilization by a single base mismatch than DNA-DNA duplexes. The melting temperature (Tm) may decrease by 10–18 °C when only a single base mismatch between the LNA/PNA oligonucleotide and the complementary target template is present (Fig. 2B) [46]. Combined with real-time PCR or melting curve analysis, the use of LNAs or PNAs in ctDNA analysis has enabled the identification of mutants at a frequency of 0.1% [42,47]. Although, these assays can achieve higher sensitivity, a subsequent detection procedure is often needed, which can be laborious and time-consuming. Another limitation is that only a few loci can be simultaneously interrogated using these methods.

Another ingenious method to the enrichment of mutation alleles is nuclease-assisted minor allele enrichment with probe overlap (NaME-PrO) [48]. NaME-PrO is based on the selective digestion of WT alleles using a double-strand nuclease (DSN) and mutation-overlapping oligonucleotide probes. The DSN preferentially cleaves perfectly matched

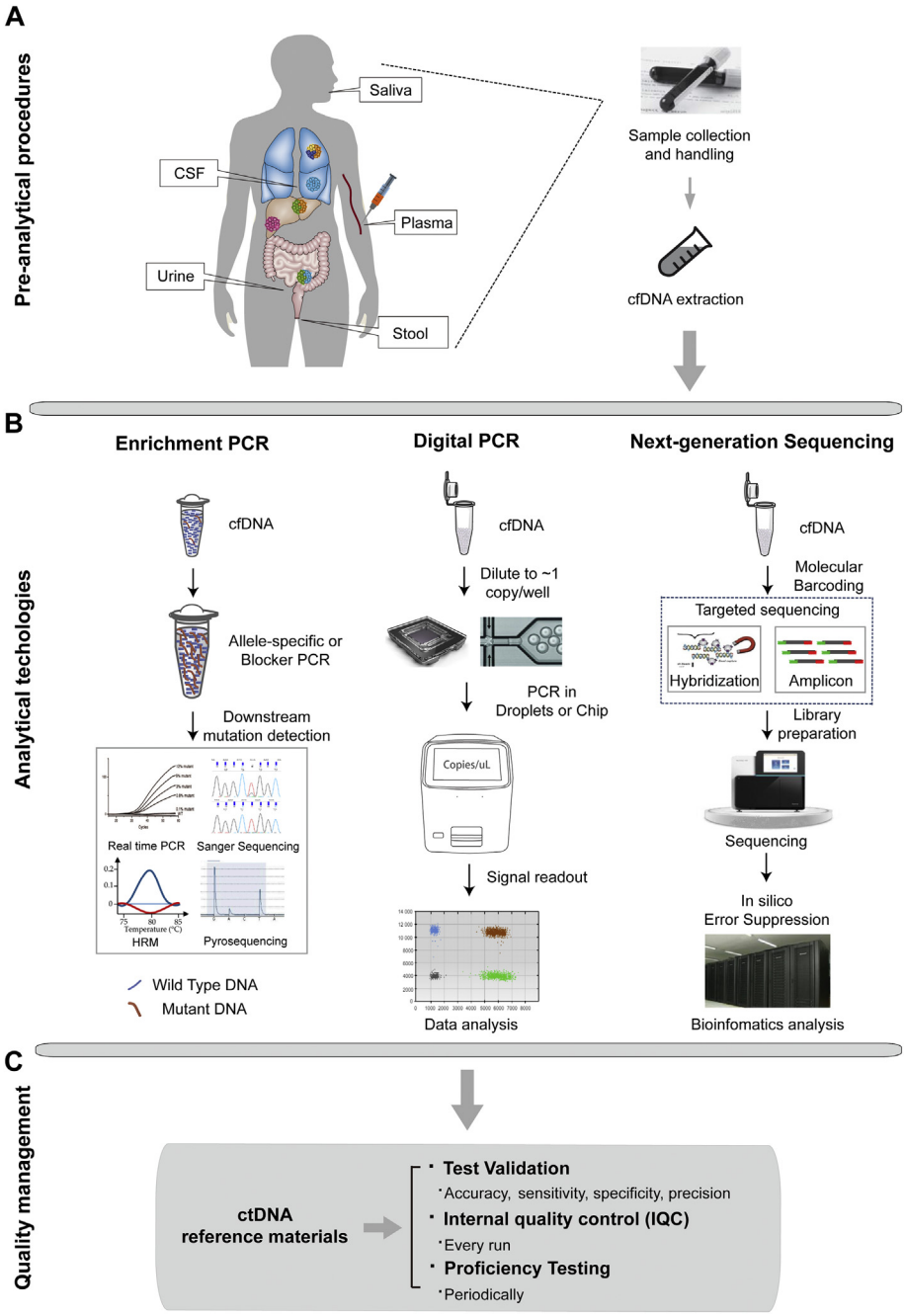

Fig. 1. Schematic overview of ctDNA analysis workflow in clinical laboratories. (A) The pre-analytical procedures of ctDNA assays. (B) The analytical technologies widely used for ctDNA detection, including enrichment PCR, digital PCR and next-generation sequencing. (C) Quality management measures that assure the accuracy of ctDNA assay results.

oligonucleotide-template DNA duplexes, while duplexes containing mutations in the template are relatively insensitive to this cleavage. Subsequent PCR amplification magnifies the target mutant DNA (Fig. 2C). This method is highly multiplexable and can improve detection limits for any downstream read-out technologies, which enables routine mutation detection at 0.01% abundance [48]. Recently, the same group presented a modified procedure of NaME-PrO which was called no denaturation NaME-PrO (ND-NaME-PrO) [49]. ND-NaME-PrO allows mutation enrichment to be performed in a closed-tube without the need for DNA denaturation, which effectively avoids the risk of cross-contamination during manual tube opening and addition of reagents in the pre-PCR setting. Although the ND-NaME-PrO methodology is conceptually attractive, more work is required to optimize this novel approach before use in clinical practice [50].

3.2. Digital PCR

dPCR is based on the strategy of separately amplifying DNA at the single-molecule level, and the resultant PCR products are therefore homogeneous (completely mutant or completely WT) [51,52]. Therefore, by a combination of limiting dilution, end-point PCR, and Poisson statistics, dPCR can be used to accurately detect rare mutations by directly counting the number of target DNA molecules present in a sample (Fig. 2D). At first, the limited number of compartments and the large reaction volume restricted its application in clinics. However, recent advances in nanofabrication and microfluidics have enabled the

Table 1
Comparison of circulating tumor DNA detection technologies.

Technology	Methods	Loci interrogated	LoD	Variant type	Advantages	Limitations
PCR-based						
Enrichment PCR	Allele-specific or ARMS PCR [33–38]	Single locus	0.05–0.1%	SNVs, indels	• Approved companion diagnostics kits • Rapid turnaround-time • Cost-effective • Simple workflows	• Prior knowledge of mutations • Limited sensitivity • Limited loci
	LNA/PNA blocker PCR [41–47]	Single locus	0.01–0.1%		• High sensitivity	• Prior knowledge of mutations • Need to optimize • Limited loci
	NaME-PRO [48,49]	Multiple loci	0.01%		• High sensitivity • High multiplexable	• Prior knowledge of mutations • Need to optimize
Digital PCR	BEAMing [29,54,56,57]	Single locus	0.01%	SNVs, indels	• High sensitivity • Quantification • Rapid turnaround-time • Simple workflows	• Prior knowledge of mutations • Limited loci
	Droplet- or Chip-based dPCR [55,58–65]		0.001%	SNVs, indels, CNVs		
NGS-based						
Molecule barcoding	Safe-sequencing [32,72]	Custom panels with large number of target loci	0.1%	SNV, indels, CNVs, gene fusions	• Does not require any prior knowledge of mutations • High sensitivity • Detect thousands of loci simultaneously	• Longer turnaround time • Need Bioinformatics analysis • High cost
	Duplex Sequencing [68]		0.001%			
	cSMART [75]		0.01%			
	digital sequencing [76]		0.1%			
	TAm-Seq. [74]		2%			
	Bias-Corrected Targeted NGS [77]		0.1%			
	Circle sequencing [73]		NA			
In silico error suppression	iDES-enhanced CAPP-Seq [31,67]		0.004%			
	BPER [69]		0.1–0.3%			
New technologies						
CRISPR-Cas13a/C2c2	SHERLOCK [78,79]	1–10 loci	NA	SNVs	• Rapid and ease of operation • Cost-effective • High sensitivity • Without the need for special equipment	• Prior knowledge of mutations • Need to optimize • Limited loci
CRISPR-Cas12a/Cpf1	DETECTR [81]	1–10 loci	NA			

creation of thousands of nanoliter-sized partitions with reduced reaction volumes, resulting in high sensitivity and precision [53–55].

BEAMing (beads, emulsions, amplification, and magnetics) is the first high-throughput dPCR system that allows accurate ctDNA analysis in plasma [32,54]. It combines emulsion PCR with magnetic beads and flow cytometry, in which templates are clonally amplified in the presence of beads [54,56,57]. BEAMing has allowed the detection of mutant and WT sequences in cancer patients, even when those sequences were present at ratios of < 0.01% [29,57]. Currently, BEAMing is commercially available for ctDNA detection. However, the high risk of contamination due to the extreme high sensitivity and complex workflow, and high cost limits its adoption in routine clinical settings [55]. Droplet-based dPCR uses a water–oil emulsion instead of magnetic beads. This system takes advantage of microfluidic circuits and surfactant chemistry to form water–oil partitions that separate the template DNA molecules, and partition target and background DNA into thousands of nanoliter-sized droplets [55,58]. Following PCR amplification, the droplets are transferred to an automated droplet reading machine, which acts like a flow cytometer and analyzes each droplet to determine whether a reaction has occurred. As with chip-based dPCR methods, DNA molecules are partitioned into thousands of independent small-volume, solid reaction partitions on a high-density nanofluidic chip [59]. After mixing, the sample is loaded onto the chip and partitioned into multiple reaction wells. Once the PCR amplification is complete, the chip is read on an instrument to determine whether amplification has occurred in each partition based on the detection of fluorescence.

Nowadays, several dPCR systems are commercially available, and involve either chips or water-in-oil emulsions [55,59,60]. Following sample mixing, these commercialized instruments automate the dPCR workflow of droplet generation or chip loading, thermal cycling, reading, and data analysis, making this technology accessible in clinical settings [61]. dPCR has been widely used for ctDNA detection and quantification, with reports of detection sensitivity below 0.001% [62–65]. Although dPCR technology has been used extensively in ctDNA detection, and has a high level of analytical sensitivity and specificity, it requires primers or probes that target specific known mutations. Therefore, dPCR methods are generally limited to the investigation of a single known hot-spot mutation (or the simultaneous investigation of a small number of such mutations).

3.3. Next-generation sequencing

PCR-based methods that target specific known hotspot mutations, which are known as “actionable” alterations, deliver satisfactory analytical sensitivity. However, NGS technology is now greatly preferred over PCR for investigations of large numbers of loci and other types of somatic alterations, such as gene amplifications and gene fusions. Currently, target panel sequencing in which genomic regions are selectively captured from a sample before sequencing is widely used in ctDNA detection [6,7,31]. Owing to errors introduced during PCR amplification or base misincorporation, which are required for sample library preparation and sequencing (for example, the Illumina sequencing platform consistently displays a random error rate of 0.1–0.5%), somatic mutations with a minor allele frequency < 0.5% cannot be reliably distinguished from artifacts by traditional NGS methods [66]. To correct these errors and enable rare mutations to be detected accurately by NGS, a variety of strategies, including molecular barcoding [32,67,68] and in silico error suppression [67,69–71], have been developed to tackle artifacts and permit ultrasensitive ctDNA assessment.

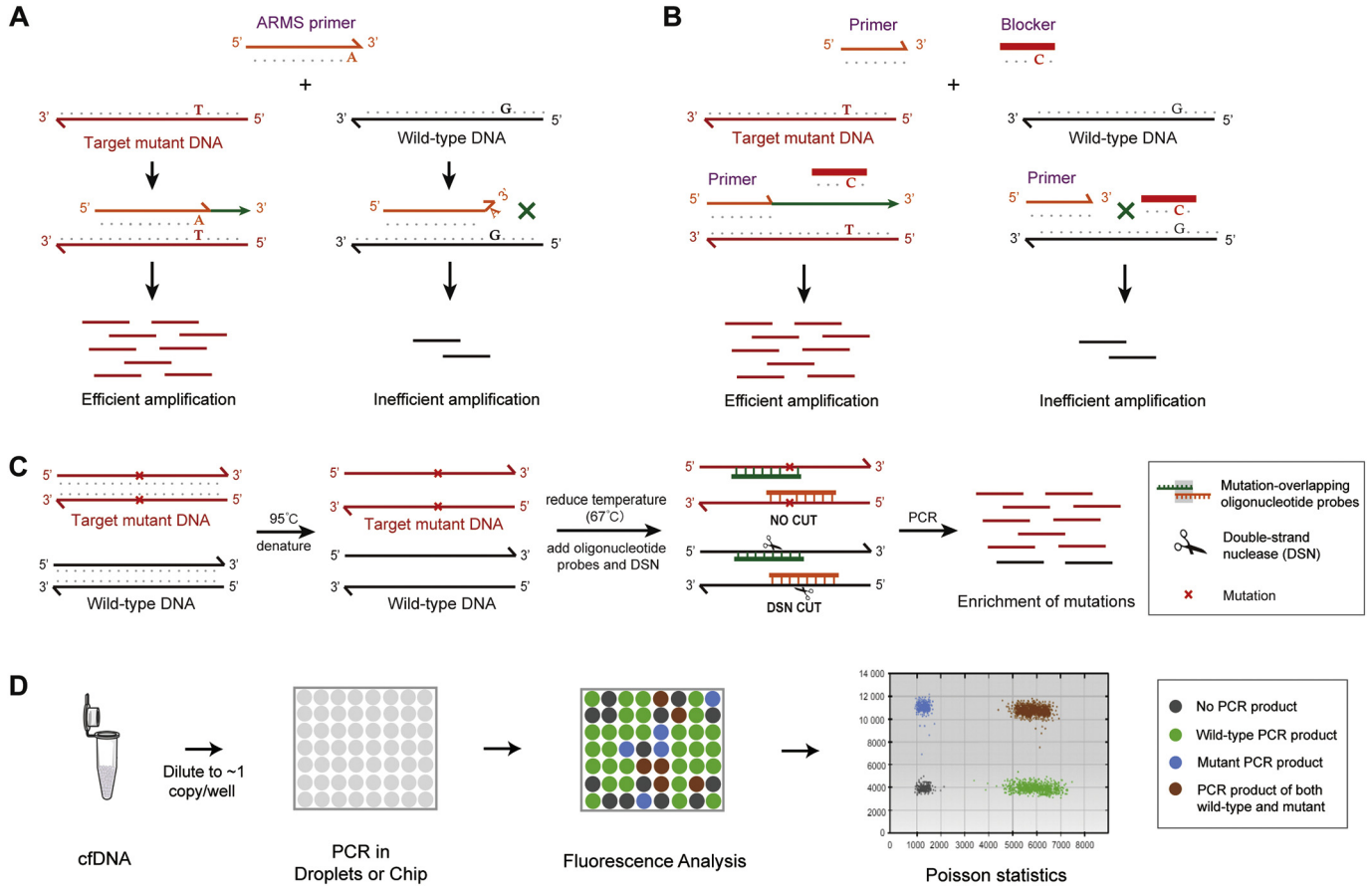

Fig. 2. Schematic diagram of Allele-specific PCR, blocker PCR and digital PCR. (A) The 3′ nucleotide of ARMS primers is designed to hybridize perfectly to the target variant template, but is mismatched to the wild-type (WT) template. The amplification proceeds with full efficiency, when the primer is fully matched. In contrast, only low-level background amplification occurs when the 3′ base is mismatched. (B) In blocker PCR, the primer is designed to binds upstream of the variant locus. The blocker binds tightly to the WT template with high affinity and specificity, but more destabilized by a single base mismatch. Polymerase extension of the primer halts when it reaches the blocker on the WT template. In addition, the blocker is not recognized by the polymerase and cannot serve as a primer for polymerization. Thus, blocker PCR can discriminate match and mismatch target sequence with as little as one base pair difference. (C) The mutation-overlapping oligonucleotide probes are designed to bind the positive and negative target DNA strands of the WT sequence. The overlap region of the probes defines the DNA region targeted for mutation enrichment. Following fragmented DNA denaturation, the overlapping probes bind to complementary target DNA strands. Double-strand nuclease (DSN) then digests the fully matched templates, but not when there are mutation-caused mismatches, thereby enriching the mutated DNA target upon subsequent PCR amplification. (D) In digital PCR, the DNA is diluted into hundreds or even millions of separate reaction plates to distribute either one or zero DNA molecule per PCR reaction. Then every individual well can be analyzed to identify the presence of mutant (blue dots) and WT (green dots) sequence by using fluorescent probes or other standard techniques, as the PCR products are homogeneous in sequence. In reality, some compartments will receive both WT and mutated fragments (brown dots) or no DNA template (grey dots) during the partitioning stage. Thus, a Poisson-based statistics model is used to correct for this effect on the number of positive and negative wells that are identified. This correction enables all target DNA molecules in the sample could be accurate quantified. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3.1. *Molecular barcoding*

Because errors in PCR amplification and base misincorporations are randomly distributed along the reads, strategies that create a consensus sequence for each DNA template can correct base errors at each position. The most widely used approach is the molecular barcoding strategy that utilizes a consensus analysis of independently sequenced fragments by incorporating with exogenous unique molecular barcodes (also called unique molecular identifiers, UMIs) before the PCR amplification. UMIs are short molecular tags (consisting of 6–14 random nucleotide sequences) linked to universal sequence adaptors, which can be introduced to targeted templates by ligation or PCR. In 2011, Kinde et al. first described the Safe-Sequencing System (Safe-SeqS) [32] using single-strand UMIs (Fig. 3A). The Safe-SeqS method has been used for ctDNA detection in various kinds of cancers, and has a detection limit of 0.1% [32,72]. Theoretically, the Safe-SeqS approach eliminates PCR errors and substantially improves the accuracy of NGS. However, DNA damage artifacts or errors introduced at the initial round of PCR amplification are indistinguishable from true mutations if the base changes are propagated to all subsequent copies. To overcome the inherent limitations of Safe-SeqS, Schmitt et al. [68] developed the Duplex Sequencing approach by identifying and analyzing both strands of the DNA template based on double-strand molecular barcoding (Fig. 3B). With highly redundant sequencing, this strategy results in high detection sensitivity down to one mutant molecule per 10,000 WT molecules [68]. Although Safe-SeqS and particularly Duplex Sequencing are robust with regard to error suppression, they both suffer from several technical drawbacks. First, they both rely on highly redundant sequencing, which is expensive and inefficient. Second, errors may be introduced into the molecular barcode sequences during PCR amplification and sequencing, which presents a challenge for consensus analysis in UMI families. Another challenge is the UMI incorporation efficiency of library construction protocol need to improve. The conversion efficiency is currently between 30% and 50% [67,68]. Low conversion efficiency will result in limited sensitivity in certain amounts of cfDNA input. In addition to UMIs, an ingenious strategy called circle sequencing can be applied to cfDNA to overcome these technical shortcomings

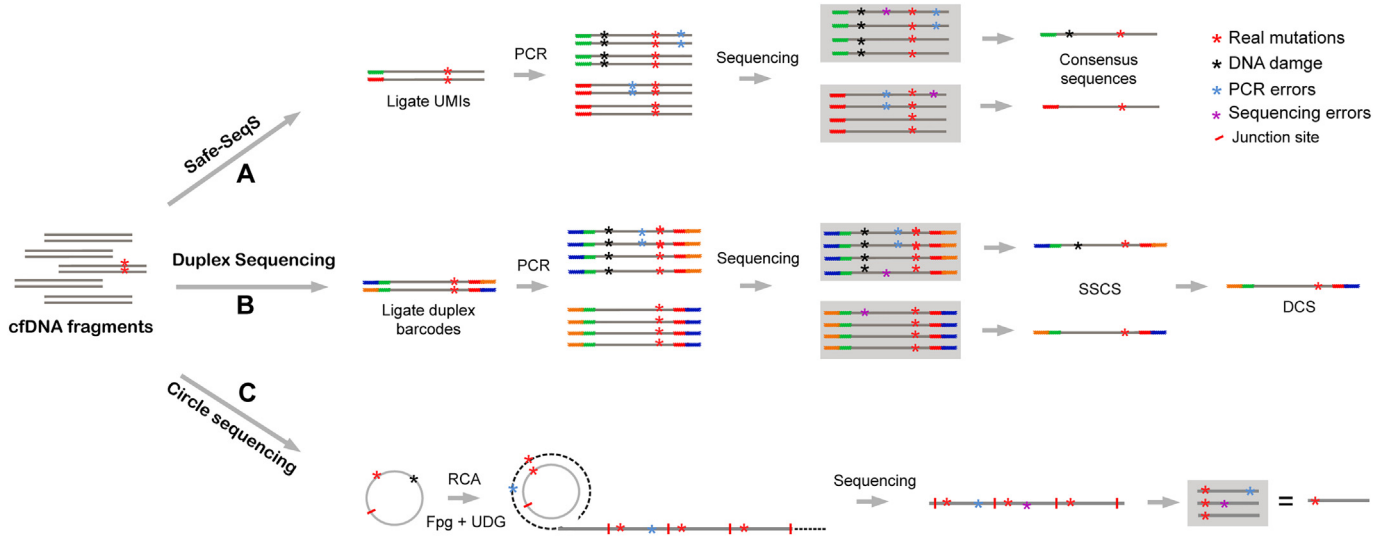

Fig. 3. Schematic diagram of molecular barcoding strategies for error suppression. (A) **Safe -SeqS.** At the beginning of library preparation, UMIs (different color) are assigned to each DNA template before PCR amplification. Each uniquely barcoded DNA template is then amplified to produce "read families" that each member has the identical UMI. If a mutation exists in the origin DNA template, the mutation should also be present in each member of the "read family" which has the identical UMI. On the contrary, errors occurring during library preparation and sequencing process should only present in several read family members. After sequencing of the amplification products, "read families" are collapsed into consensus sequences according to UMIs to identify the true rare mutations. (B) **Duplex sequencing.** Each of the two strands is tagged by duplex UMIs independently in the initial step, and then followed by amplification, and sequencing. Similar to the Safe-SeqS, members of each "read family" are identified according to the duplex barcodes to create a single-strand consensus sequence (SSCS). Next, SSCSs representing two complementary strands are identified based on the complementary molecular tags among SSCS reads and used to form the so-called duplex consensus sequence(DCS), where mutations are accepted only if they present at the same position on both DNA strands. (C) **Circle sequencing.** In brief, DNA molecular is size-selected to approximately 1/3 of the anticipated read length. Then double-stranded DNA fragments are denatured, circularized, and isothermally amplified as rolling circle amplification (RCA) using the Phi29 polymerase. The amplified DNA products each containing ~3 copies of initial template and sequenced on Illumina MiSeq platform. Following the data analysis, the repeating copies are collapsed into a consensus sequence. Thus, sequencing and amplification errors are eliminated.

[73]. Circle sequencing takes advantage of rolling circle amplification to ensure redundant sequencing of individual DNA fragments (Fig. 3C), and has an approximately 100-fold lower sequencing error rate with lower cost than molecular barcoding strategies [73].

As the need for accurate ctDNA detection has increased, other similar strategies, such as tagged-amplicon deep sequencing (TAm-Seq) [74], circulating single-molecule amplification and resequencing technology (cSMART) [75], digital sequencing [76], and Bias-Corrected Targeted NGS [77] have also been developed.

3.3.2. *In silico error suppression*

Although the molecular barcoding strategies mentioned above can efficiently remove background noise, their use in clinical settings is limited because they are expensive and are not equally effective for all nucleotide substitutions encountered in ctDNA [67]. To overcome these limitations, several in silico error suppression approaches based on bioinformatics algorithms have been developed to further improve assay sensitivity [67,69].

According to the sequencing artifact patterns in healthy adult cfDNA, certain sets of sequencing errors are much more likely to occur at specific places in DNA molecules, and the majority of background errors are due to G > T transversions, which are much more prevalent than C > A changes [67]. These errors reflect oxidative damage arising during the hybrid capture step. Newman et al. [67] developed a computational approach termed "integrated digital error suppression" (iDES) by modeling position-specific errors in a training cohort of control samples. When iDES is applied to cfDNA samples from healthy subjects, the global error rates are similar to those obtained using a molecular barcoding strategy alone [67]. Notably, iDES-enhanced CAPP-Seq developed from the researchers' previously published CAPP-seq [31], which combines iDES with the molecular barcoding strategy, achieved a ctDNA detection limit of 0.004% [67]. Moreover, iDES-enhanced CAPP-Seq only requires a median cfDNA input of 32 ng, which is an advantage bearing in mind the tiny amounts of cfDNA present in plasma. It is important to note that the sensitivity of 0.004% of iDES-enhanced CAPP-Seq is achieved by integrating multiple classes of somatic alterations. This sensitivity cannot be achieved when targeting a single mutation with limited cfDNA input (e.g., 32 ng).

Other researchers have developed a statistical method based on the quantification of the error rate of each base position [69]. By quantifying the position-error rate (PER) at each base position independently in a set of negative controls, the minor-allele frequency in each plasma sample was compared with the measured PER at each base position using a binomial test to determine whether this mutation was true. The base-PER (BPER) method detected mutations with an allele frequency as low as 0.3% for SNVs and 0.1% for indels (> 2 bp) without molecular barcoding. This method has been used to detect ctDNA in patients with pancreatic cancer and non-small cell lung cancer [69].

3.4. *New technologies for ctDNA analysis*

Recently, researchers have created a new Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-based tool called SHERLOCK (Specific High-sensitivity Enzymatic Reporter unLOCKing) for DNA detection [78,79]. In SHERLOCK, the RNA-targeting CRISPR enzyme, which is now called Cas13a, is harnessed as a sensitive detector to detect the presence of a single molecule of target RNA or DNA [78,80]. Researchers have demonstrated the method's versatility in a range of applications, including identifying cancerous mutations in simulated cfDNA fragments. This tool offers the sensitivity to detect an extremely small amount of ctDNA in a patient's blood. Moreover, SHERLOCK has been developed as a miniature paper test without the necessity of complicated upstream experimental work or the need for specialized equipment [79]. Another CRISPR-based method known as DNA Endonuclease-Targeted CRISPR Trans Reporter (DETECTR) has also been reported [81]. Instead of Cas13a, DETECTR relies on Cas12a—an RNA-guided, DNA-targeting enzyme—as a sensitive detector [82]. By combining Cas12a, the guide RNA, a fluorescent reporter

molecule, and recombinase polymerase amplification, DETECTR achieves attomolar sensitivity for DNA detection. DETECTR has been demonstrated to successfully distinguish between human papilloma virus (HPV) types 16 and 18. Together, both these two methods provide a new generation of diagnostic tools for ctDNA detection that may be more rapid, robust and sensitive than current technologies.

3.5. Analytical technology selection

Current technologies for ctDNA detection differ significantly in sensitivity, specificity, and throughput; each has its own merits and limitations. For a given ctDNA assay to be used in clinical settings, several considerations typically determine the choice of technology, including intended clinical use, desired turnaround time, costs, and available expertise [83]. Cross-platform comparison studies have been conducted on leading technology platforms [84–87], and emerging studies suggest that dPCR- and NGS-based methods are the most promising technologies for the detection of rare mutations in ctDNA [6,7,84–87]. Despite several advantageous features (including high sensitivity, simple workflow, and low cost), dPCR-based methods are limited to interrogating individual mutations, and require prior knowledge of the mutations likely to be present in the primary tumor. In contrast, NGS-based methods are capable of interrogating more targets, ranging from individual exons to the entire exome; they are inexpensive and enable the detection of SNVs, indels, gene fusions, and gene amplifications. The cfDNA amounts are usually extremely low. Thus, the amount of input DNA (the number of genome equivalents entering the assay) is another limiting factor for assay sensitivity. For instance, the typical yield of cfDNA from 10 mL of blood in a normal human is about 30 ng (approximately 10,000 genome equivalents), which means that approximately 10,000 unique molecules are expected at any given genomic site. This conversion is based on the assumptions of uniform genome representation in a sample and a human haploid genome weight of 3.3 pg [88]. If all 10,000 molecules were assayed, the analytical sensitivity could theoretically be achieved as low as 0.01%. However, in reality no assay will recover all input molecules. Therefore, the effective cfDNA input sets the absolute detection limit of a particular ctDNA assay [89].

4. Quality management considerations

Standardization and validation of integrated analytical workflows is important for the implementation of ctDNA analysis in daily clinical practice. Public-private consortiums have been launched with a focus on standardizing the methods and technologies for ctDNA analysis. The CANCER-ID (www.cancer-id.eu) consortium is supported by the European Innovative Medicines Initiative. It aims to establish standard protocols and clinical validation of blood-based biomarkers. Key contributions of the consortium include best practice recommendations for pre-analytical sample processing and the evaluation of different analysis technologies [26]. The US based Blood Profiling Atlas in Cancer (BloodPAC) (https://www.bloodpac.org/) is another public-private partnership launched with the aim of collecting and harmonizing data to standardize protocols for sample processing and analysis of current liquid biopsy tests. BloodPAC has released an initial public accessible dataset to accelerate the development of blood-based assays [90]. However, there are no integrated, internationally recognized workflows available covering the requirements for the clinical setting [11,26]. Each clinical laboratory providing molecular pathology services should establish quality management measures to assure the analytical validity of assays that conform to the international clinical laboratory accreditation standards (e.g., ISO15189 [91]) and local regulations (e.g., the US Food and Drug Administration) [83,92]. In this section, four main components of quality management, including test validation, IQC, PT, and ctDNA reference materials, will be discussed (Fig. 1C).

4.1. Test validation

Currently, nearly all ctDNA assays developed in-house are considered laboratory-developed tests (LDTs). ISO15189 requires proof-of-concept validation of LDTs with established performance metrics before introducing into the clinical laboratory [83]. Analytical validation represents establishing the performance metrics of the assay based on its intended use to demonstrate the fitness of an assay [93]. The analytical validity of an assay result is usually assessed through performance metrics including accuracy, precision, analytical sensitivity, analytical specificity, lower limit of detection (LoD), and other relevant metrics.

Although guidance and standardized frameworks on the validation of molecular genetic tests are currently available [93–96], there is currently no clear guidance on how to validate a ctDNA assay. Depending on the intended clinical use and the analytical technology deployed, ctDNA assays may be subject to different levels of validation [9]. For example, owing to the complexity of NGS technology, the performance metrics defined in the available standards do not apply to NGS-based ctDNA testing systems, and both wet laboratory and bioinformatics analysis pipeline should be included [13,97]. Thus, guidance on the validation of NGS-based assays [13,96,97] should be reviewed when validating a NGS-based ctDNA assay. Since an NGS-based ctDNA assay typically targets multiple genes with various variant types at different genomic regions, the performance will likely vary by mutation type and sequence contexts (e.g., low complexity, GC-rich, and homopolymeric sequences). Therefore, the performance metrics should be determined for each class of genomic alterations (e.g., SNVs, indels, gene fusions, and gene amplifications) and different genomic regions [96]. In addition, validation of ctDNA assays should include testing a variety of samples containing known variants at specified variant allele fractions, and statistical metrics can be determined when analyzing the validation data (Fig. 4). In general, both well-characterized clinical samples or ctDNA reference materials can be used to assess the

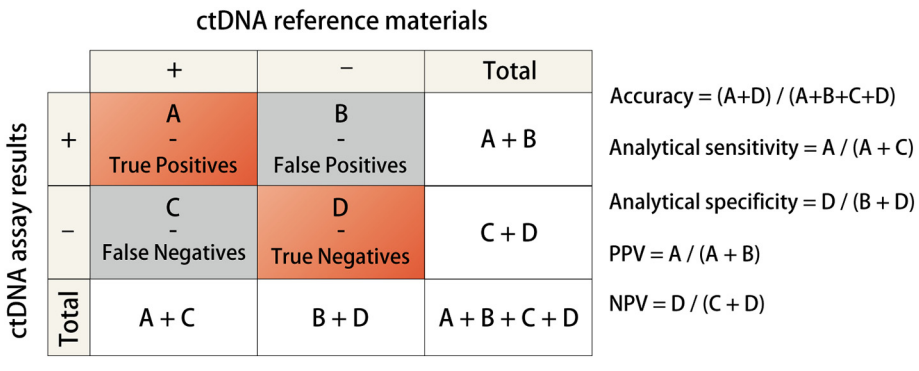

Fig. 4. Statistical metrics in analytical validation of ctDNA assays.
To illustrate these metrics, consider the use of ctDNA reference materials serve as the ground truth to evaluate the performance to identify single nucleotide variations (SNVs) of a ctDNA assay. The number of true-positive(A), false-positive(B), false-negative (C) and true-negative(D) predictions for a ctDNA assay can be calculated by comparison to ctDNA reference materials. These data are then used to calculate a range of statistical metrics that represent different aspects of ctDNA assay performance, including accuracy, analytical sensitivity, analytical specificity, positive predictive value(PPV), and negative predictive value (NPV). Equations for calculating each metric are encoded graphically in terms of the quantities in the confusion matrix.

analytical performance of the assay. Several studies have used tumor tissues as a gold standard for the validation of ctDNA assays by comparing concordance between variants detected in tumors and plasma [98,99]. Such comparisons may not be valid because many biological factors affect concordance (e.g., tumor type, stage, and temporal heterogeneity) [6,7]. To overcome this issue, several analytical validation studies have been performed using ctDNA reference materials with known variants at specified variant allele fractions [100,101]. Such reference materials also allow documentation of the lower limit of detection for various types of mutations. These analytical validation studies could provide laboratories with alternative approaches for improvement of their validation of ctDNA assays.

4.2. Internal quality control and proficiency testing

To ensure accuracy, all ongoing ctDNA assays should be monitored with IQC and PT programs [11]. IQC procedures must be implemented to monitor the performance of every run of the total testing processes, including pre-analytical, analytical, and post-analytical phases of testing [11,83,102]. Each component of the testing process should have established QC materials and metrics during the validation procedure. The QC materials and metrics depend on the chosen technology and instrument. For example, specimen quality, cfDNA quality, depth of coverage, uniformity of coverage, GC bias, strand bias, and base quality scores are QC metrics that should be evaluated for NGS-based ctDNA assays [13,96]. In the meanwhile, control samples, including positive and negative ctDNA reference materials, and no template controls (blank), should be incorporated in every run to detect sources of error and exclude false-negative results. PT is a process for assessing laboratory performance by which blinded samples are sent to participating laboratories for analysis, and then results are reported for performance evaluation [9]. Participation in PT programs permits laboratories to assess their ability to detect variants of interest, and provides an independent comparisons of inter-laboratory performance [13]. Currently, PT programs for ctDNA assays are offered by the College of American Pathologists (CAP) and the China National Center for Clinical Laboratories (NCCL) [103]. In addition, Haselmann et al. also launched the first EQA scheme for both isolation and analysis of ctDNA in Europe [104].

4.3. ctDNA reference materials

Well-characterized ctDNA reference materials are central to demonstrating the validity of an assay, and ensuring that the assay is well developed, validated, and monitored [11,105]. However, the production of reference materials that are capable of authenticating ctDNA has been a challenge owing to a number of factors including the fact that ctDNA is not fragmented randomly [5,106]. Several commercial cfDNA reference materials produced by mechanical shearing methods are available, but these synthetic methods perform comparatively poorly [106]. Recently, synthetic cfDNA quality control materials that are better at emulating biological reality than currently available products have been developed [103,106]. These synthetic cfDNA quality control materials are suitable for various ctDNA testing methods—including ARMS-PCR, dPCR, and NGS—and are appropriate for method comparison, test validation, IQC, and PT. In 2016, the NCCL first launched a pilot PT program for somatic mutation detection in ctDNA using these synthetic cfDNA QC materials [103].

5. Future directions and conclusions

ctDNA have great potential for the management of cancer and are currently being incorporated into preclinical and clinical studies [107,108]. Although promising, the implementation of ctDNA assays will only occur in practice after their clinical utility has been demonstrated [11]. For a ctDNA assay to be truly clinically applicable, the first step is to ensure that patients receive a timely and accurate results on which their treatment will be based. Pre-analytical considerations are important, and the standardization of sample handling processes is necessary. A solid pre-analytical validation allows laboratories to define the quality criterion for sample acceptance. In this review, we have described a range of technologies that have been widely used clinically for ctDNA detection. Each detection technology has its own relative merits and limitations, a detailed understanding of the ctDNA detection technologies is a prerequisite to make the appropriate choice of optimum analytical method which can achieve desired sensitivity to meet the intended clinical use. Although several commercial kits are approved based on safety and effectiveness criteria, the limited sensitivity and specificity of these kits may render them not suit for early stage patients or patients with low tumor burden, since the mutant alleles could be extremely low. In contrast, dPCR- and NGS-based methods have the ability to detect extremely low-level mutations in plasma. The dPCR enjoy several advantageous features including high sensitivity, simple workflow, and low cost, but can only interrogate individual mutations and require prior knowledge of the mutations. NGS has the ability to detect multiple mutations simultaneously and enable the discovery of novel mutated variants, but with higher costs. Thus, in the future, NGS and dPCR could both be used complementarily for ctDNA detection. In addition, we have also described the main quality management considerations, including test validation, IQC, and PT, that can assure the quality of a reliable test result of ctDNA assays. Another critical issue to be aware of is that not all somatic mutations identified in cfDNA originate from the cancer. Somatic mutations have been detected in normal tissues and peripheral blood cells (PBCs) [109,110]. Several studies have demonstrated that somatic mutations can be identified in cfDNA from healthy individuals due to the complex origins of plasma cfDNA including tumor and normal tissues, PBCs, and others, albeit at lower frequency [111,112]. The increased analytical sensitivity of ctDNA assays (e.g., BEAMing, dPCR, NGS) will increase the chance of background somatic mutations identification, which adds an additional layer of complexity of ctDNA analysis in clinical practice [113]. Since the majority of cfDNA is derived from PBCs, mutations derived from non-malignant hematopoietic cells, known as clonal hematopoiesis (CH) [114], could be the main source of background somatic mutations in cancer patients and healthy individuals. Therefore, several studies recommend that concurrent sequencing of paired PBCs will help to identify CH-derived somatic mutations [112,113]. Other researchers believe that baseline spectrum of background somatic mutations in healthy individuals may also be important for accurate ctDNA detection in cancer patients [112,115]. However, these background somatic mutations still make the interpretation of ctDNA analysis results more complex. Thus, caution is needed when interpreting ctDNA analysis results, and further work is needed to determine how to interpret and report ctDNA variants when background somatic mutations are identified [116].

Once analytical validity has been demonstrated, well-designed prospective clinical trials or equivalence studies are needed to demonstrate the clinical utility of the assay according to its intended use. With the advent of ultrasensitive and highly specific technologies and increasing standardization, ctDNA applications are moving into the clinic. We anticipate that robust prospective clinical trials with analytically valid ctDNA assays will demonstrate the clinical utility of more ctDNA assays.

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Disclosure

The authors have declared no conflicts of interest.

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