

Current and Emerging Applications of Droplet Digital PCR in Oncology

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Published online: 5 May 2017
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Abstract The clinical management of cancer has evolved in recent years towards more personalized strategies that require a comprehensive knowledge of the complex molecular features involved in tumor growth and evolution, and the development of drug resistance mechanisms leading to disease progression. Droplet digital PCR (ddPCR) has become one of the most accurate and reliable tools for the examination of genetic alterations in a wide variety of cancers because of its high sensitivity and specificity. ddPCR is currently being applied for absolute allele quantification, rare mutation detection, analysis of copy number variations, DNA methylation, and gene rearrangements in different kinds of clinical samples. This methodology has proven useful for the evaluation of archival tumor tissues, where poor DNA quality and limited sample availability are major obstacles for standard methods, providing less subjective and more automated quantitative results. However, most applications of ddPCR in cancer are focused on liquid biopsies (including cell-free DNA as well as circulating tumor cells) because these represent non-invasive alternatives to tissue biopsies that can more accurately reflect intratumoral heterogeneity and track the dynamic changes in tumor burden that occur in response to treatment at different times during follow-up. A

broad spectrum of molecular markers have been interrogated in blood using ddPCR for diagnostic, predictive, and monitoring purposes in various malignancies. Emerging alternative approaches using other body fluids such as cerebrospinal fluid and urine are also currently being developed. This article aims to give a complete overview of ddPCR applications for molecular screening in oncology.

Key Points

Droplet digital PCR (ddPCR) is a sensitive, specific, and accurate method for the molecular characterization of a wide variety of clinical samples in many human malignancies.

Molecular biomarkers to monitor treatment response, associated with disease progression and survival, are detectable and quantifiable at both the DNA and RNA level by ddPCR methodologies.

Larger prospective studies and validations are required, and issues related to experimental artifacts need to be addressed prior to ddPCR implementation into daily clinical practice.

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1 Introduction

Tumor genotyping has become crucial for the clinical management of cancer. There are many types of human malignancies, and their origin and evolution are related to a

broad spectrum of genetic alterations [1]. On the one hand, these molecular features play a significant role in tumor responses to treatment and their ability to acquire resistance to targeted therapies. Mutations that are initially present in minute amounts or arise secondarily in response to therapy, as a mechanism of drug resistance, are particularly challenging for standard analytical methods [2]. On the other hand, many difficult-to-detect genetic biomarkers have also been proposed for disease monitoring, as a tool for surveillance and for early detection of tumor progression. In all these clinical scenarios, extremely sensitive and specific techniques for detection are needed [3]. Droplet digital PCR (ddPCR) has become one of the most important methodologies applied in cancer research for clinical purposes because of its superior sensitivity and accuracy in comparison to conventional standard methods [4–8].

As Morley reviewed in 2014 [9], the first attempts of approaches similar to the currently known digital PCR technology were published in the early nineties, and were referred to as “single molecule PCR” [10, 11] or “limiting dilution PCR” [12, 13]. At the time these methodologies were described, they were hindered by technical and economic limitations that were later circumvented by the development of improved nanofluidic devices and emulsion-based formulations for sub-partitioning [14].

Although most studies reporting digital PCR have been published in the last 5 years, this term was actually coined by Vogelstein and colleagues in 1999 [15]. These authors developed one of the pioneering digital PCR platforms applied in oncology and, particularly, in colorectal cancer (CRC): BEAMing (beads, emulsions, amplifications and magnetics) [16]. Since it was described in 2003, this methodology has been used for molecular screening in a wide variety of cancers [17], including breast cancer [18], gastrointestinal stromal tumors [19], lung cancer [20–23] and CRC [24–28]. However, alternative digital PCR methodologies have been developed, such as the microfluidic chamber-based BioMark™ Digital PCR from Fluidigm [29], the chip-based Quantstudio 12k/3D digital PCR System from Thermo Fisher Scientific [30], the droplet-based QX-100/QX-200 Droplet Digital PCR Systems from Bio-Rad Laboratories [31], and RainDrop® Digital PCR from RainDance® Technologies [32]. In this regard, Bio-Rad announced the acquisition of its droplet-based competitor RainDance® in January 2017. These systems have proven useful for molecular analysis in different kinds of cancer, showing similar results in terms of effectiveness for DNA copy number quantification when correction for partition volume is performed [33]. BEAMing and ddPCR also provide a comparable sensitivity and specificity [23, 112], but ddPCR is simpler as it has instrumentation and protocols that are easily transferable to, and adoptable by, any lab [34] and is less time consuming and labor

intensive; these factors may have facilitated more widespread experimentation using this technology.

Of note, among all the currently commercially available digital PCR platforms, ddPCR has gained increasing interest, particularly for cancer applications. More precisely, the Bio-Rad ddPCR platform was the most widely used when we searched the literature of this field in PubMed. For these reasons, ddPCR will be the focus of this review.

Consisting of a massive sub-partitioning of the PCR mixture into thousands of nanoliter droplets through a water-in-oil emulsion, ddPCR represents an enrichment strategy that allows for the detection of low-level mutations by amplification of single DNA molecules [4]. DNA samples (together with ddPCR Mastermix, primer, and probes in a final volume of 20 μ L) and droplet generator oil are loaded into the wells of the droplet generator cartridge. A vacuum ensures that both samples and oil pass through the microfluidic circuits to form a dispersion of 1-nL droplets. In each well, the 20- μ L mixture of sample and reagents is divided into 20,000 highly uniform droplets or partitions. These droplets are stabilized by the use of surfactant chemistry. Once transferred to a 96-well PCR plate, amplification is carried out in a conventional thermal cycler [4]. Since the sample is compartmentalized into single droplets, it is considered that an independent PCR reaction takes place in each droplet [35]. Subsequently, the plate is introduced into a droplet reader, where droplets are aspirated from the wells and pass through a two-color detector. Depending on their fluorescence amplitude, droplets are classified as positive or negative using a binary threshold (one positive, zero negative) that gives ddPCR its name of “digital” [4] (Fig. 1). The known droplet volume and the proportion of positive droplets relative to the total number of droplets are used to estimate the target concentration applying a Poisson correction. This is considered a form of “absolute quantification” that makes calibration curves unnecessary for ddPCR [4, 7, 36]. The limit of blank (LOB) and the limit of detection (LOD) for each ddPCR assay have to be determined [37]. LOB is defined as “the frequency of positive droplets measured in normal control DNA samples without mutant DNA present” [37], whereas the LOD is defined as “the true mutant concentration within a sample that will be detected within stated α (false positive) and β (false negative) error limits” [38]. A detailed method for the determination of LOD in ddPCR assays has already been described [38]. In 2013, an article providing some basic guidelines for reporting the results of quantitative ddPCR experiments was published [36]. Among these detailed recommendations, which might be of help to standardize experimental protocols, the necessity of including appropriate controls is highlighted. Thus, negative controls are mandatory for monitoring false-positive

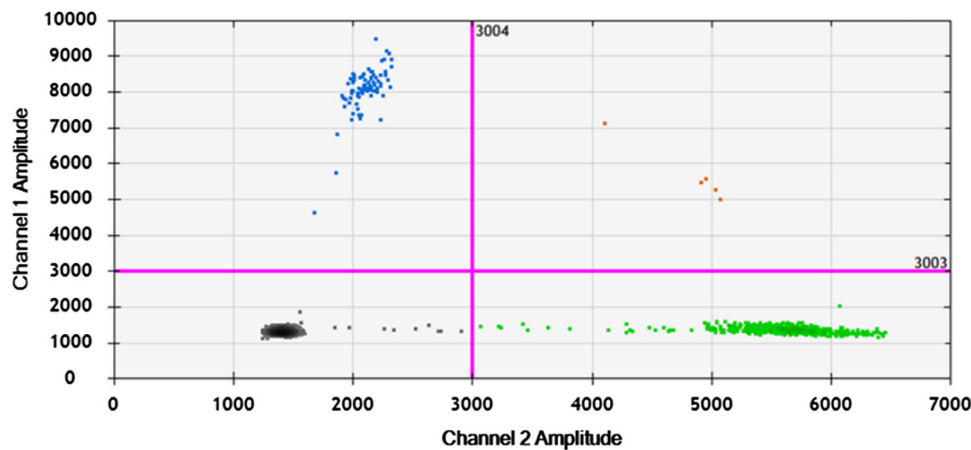


Fig. 1 Representative 2D scatter plot of a ddPCR result, corresponding to *KRAS* G13D-mutated plasma from a CRC patient analyzed using the QX200™ Droplet Digital™ PCR System (Bio-Rad). The y-axis shows the fluorescence amplitude of the FAM probe, designed to hybridize only to the mutant allele (blue). The HEX probe, which

hybridizes only to the wild-type reference allele (green), is plotted on the x-axis. Double-positive droplets carrying both types of molecules are represented in orange, while double-negative droplets (no amplification) are shown in grey. CRC colorectal cancer, ddPCR droplet digital PCR

reactions, which could be derived from non-specific binding of probes, primer dimer formation, or cross-contamination between samples. A threshold must be set in order to distinguish positive from negative partitions and determine false-positive and false-negative rates. A wild-type (WT) control containing exclusively the wild-type sequence should also be included. In the case of rare event detection assays, the use of controls with different known proportions of wild-type and mutant sequences is also recommended [36].

2 Potential Benefits of ddPCR in the Analysis of Formalin-Fixed Paraffin-Embedded Tissues

ddPCR poses as an attractive alternative option to the established standard methods currently used in clinical routine for the measurement of molecular markers in formalin-fixed paraffin-embedded (FFPE) tissues. Firstly, DNA from FFPE archival tumors is often degraded, making poor sample quality a major obstacle for downstream molecular assays [39]. Also, the amount of available sample is sometimes limited in the clinical setting, and the presence of normal tissue mixed in with the tumor tissue in the original sample represents a potential source of “contamination” for mutant DNA detection [5]. ddPCR, based on the analysis of short DNA fragments, provides high sensitivity and specificity for detection and quantification even in degraded samples with a high wild-type background [39]. Additionally, this technology allows the examination of a minute amount of DNA extracted from clinical samples, as was demonstrated in a study where *FGFR2* amplifications were determined in FFPE gastrointestinal adenocarcinomas, with a superior sensitivity to

standard real-time PCR [5]. Another example of the ddPCR application in archival tumor tissues is loss of heterozygosity (LOH) assessment of the *BRCA2* gene, a relevant genetic feature in breast cancer, whose determination seems to be severely affected by the above mentioned shortcomings of conventional methods [39]. Requiring low amounts of DNA input, using very short amplicon sizes (<100 bp)—suitable for the fragmented FFPE-derived DNA—and interrogating individual DNA molecules, ddPCR has unique features that make it superior to normal PCR methods; it achieves a precise measurement of copy number differences below twofold, which is actually the lowest LOD of conventional real-time quantitative PCR (qPCR). In a similar way, conventional PCR followed by Sanger sequencing has been proven inadequate for determining allelic ratios and LOH using FFPE-derived DNA [39].

A ddPCR workflow for copy number variation (CNV) analysis and other ddPCR applications has been thoroughly described by Mazaika and Homsy [40]. In this compilation of detailed protocols, a comparison to standard real-time PCR methods can be found. These authors state that systematic errors are usually introduced when standard real-time PCR is used to measure CNVs, for example, during normalization of standard sample DNA concentrations or at cycle threshold measurements. In ddPCR, quantification is performed without using standard samples, based on the number of positive droplets rather than cycle thresholds, which involves a lower probability of error [40]. ddPCR is faster and shows less technical variability between experiments, providing a better throughput, since more accurate, reproducible and less ambiguous results are obtained in comparison to standard qPCR [6, 40].

In various studies, *HER2* status has been examined by ddPCR in FFPE tissues from breast and gastric cancers,

obtaining concordant results with fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC), which are commonly used reference techniques for these purposes [41–46]. In all cases, ddPCR showed good performance for precise measurement of *HER2* copy number at both DNA and RNA levels [41, 47]. The advantages offered by ddPCR over FISH and IHC include a more automated procedure with a comparable sensitivity and specificity, that is conducted in a more quantitative and less subjective manner, which is readily usable for a large number of clinical samples allowing the evaluation of *HER2*, a relevant biomarker for targeted therapy with monoclonal antibodies in these cancer types [42, 44]. Similarly, ddPCR has also allowed measurement of certain CNVs of *FGFR1* in dysembryoplastic neuroepithelial tumors that could not be detected by comparative genomic hybridization (CGH) array and FISH because these techniques lacked the required sensitivity [48].

Of note, in all these studies, the analysis of copy number alterations (CNAs) in FFPE samples was restricted to the quantification of amplifications in a single target. However, it would be of great interest if a panel of biomarkers could be analyzed as a multiplex assay in order to obtain maximal information from the limited amount of starting material available in clinical samples, without increasing costs. Efforts are currently being made to design multiplexed ddPCR strategies in which experimental artifacts could be minimized. Recently, a novel method has been proposed that includes primer and probe design optimization strategies, DNA fragmentation correction, selection of panels of CNA-neutral reference loci for normalization as well as improved analytic algorithms in order to reduce bias and allow for an accurate quantification of CNAs [49]. Using this method, 15 biomarkers have been analyzed for CNAs in paired samples of frozen and FFPE tissues from squamous cell carcinoma, with a good correlation, despite the low quality and quantity of DNA obtained from FFPE samples [49]. Other examples of the multiplex ddPCR show the detection of *KRAS* mutations using a small amount of input DNA extracted from a FFPE sample of a non-small cell lung cancer (NSCLC) patient [50] and the simultaneous detection and quantification of *ALK*, *ROS1* and *RET* fusions in lung cancer [51]. Other studies on multiplexed detection of molecular markers have been conducted for liquid biopsies and will be discussed in the next section.

Although the vast majority of publications retrieved when a search was performed using the terms “droplet digital pcr” and “formalin-fixed paraffin-embedded” were focused on the analysis of CNAs (probably because this is the application where ddPCR offers major advantages for FFPE tissues in comparison to standard methods), some studies reporting the detection of single nucleotide variants

and gene rearrangements were also found. Some examples are the detection of somatic activating mutations in *GNAQ* and *GNAI1* [52] or *MAP3K3* [53], associated with congenital hemangiomas, the quantification of recurrent somatic mutations in FFPE samples from diffuse large B-cell and follicular lymphoma [54], *BRAF* V600 somatic point mutations in FFPE tissues from CRC patients [55] or *EGFR* T790M mutation [56] and *EML4-ALK* rearrangements in NSCLC [57, 58]. Beaver et al. [59] reported that a point mutation (*PIK3CA* E545K), detected at a fractional abundance of 28.9% in the primary tumor tissue by ddPCR, could not be identified by Sanger sequencing. These authors suggest that, when FFPE DNA is used for PCR and Sanger sequencing, a phenomenon of “allelic drop out” can occur, probably due to sample degradation. ddPCR is less prone to this phenomenon because of the lower amounts of input DNA and smaller amplicon sizes used in this technique [59].

However, the poor integrity of DNA in FFPE samples, which is usually highly fragmented, and the presence of inhibitors, sometimes give rise to the appearance of the so-called “rain,” a factor that can affect quantification in ddPCR assays. This term refers to the presence of subpartitions that have higher fluorescence intensity than the negative droplets but lower signal than the positive droplets [60]. Thresholds can be either automatically set by the ddPCR software or manually set by the user. In the latter case, known negative controls could be the best reference to define rain partitions that should be excluded from the analysis. However, to standardize the analysis procedure and limit the subjectivity of manually established thresholds, several methods have been proposed [61–63]. One of these strategies, for example, is the “definetherain” software, an algorithm based on *k*-nearest neighbor clustering of a positive control sample to set the cut-offs that define positive and negative droplets, removing rain droplets, decreasing the LOD, and reducing the number of false positive droplets at low target inputs [61].

3 Applications of ddPCR in Liquid Biopsy

Intratumoral heterogeneity, i.e., the existence of tumor regions carrying different genetic profiles, is a major hurdle for effective tumor genotyping, since there is increasing evidence that single biopsies are not representative of the whole tumor [64]. Taking serial biopsies would be mandatory for the proper management of cancer, but it involves additional risks for the patient and could be unfeasible in certain clinical situations [3, 65]. Consequently, in the last few years, there has been a tendency towards the development of non-invasive strategies for the analysis of many cancer-related genetic features. The use

of tumor DNA fragments present in blood as an alternative source for tumor genotyping has given rise to the approach known as “liquid biopsy” [66], comprising the analysis of cell-free DNA (cfDNA)—and more precisely, circulating tumor DNA (ctDNA)—as well as circulating tumor cells (CTCs) [3]. cfDNA and CTCs could be used as complementary approaches, although it has been suggested that cfDNA serial samples could provide more clinically useful information than CTC-based monitoring [67]. In fact, a recent study has compared both sources for tumor genotyping, and ctDNA proved much more sensitive than CTCs for the detection of *KRAS* mutations in lung adenocarcinomas (78 vs. 34%, respectively) [68].

On the other hand, molecular screening in many hematologic disorders has been performed using mononuclear cells from peripheral blood and bone marrow aspirates, which could be considered, to some extent, liquid biopsies. In all these kinds of clinical samples, molecular markers have been examined at both the DNA and RNA level. This wide range of liquid biopsy approaches will be described in detail below.

3.1 Cell-Free DNA

It is worth mentioning that the vast majority of publications found in a PubMed search using the terms “droplet digital pcr” and “cell-free dna” and “cancer” correspond to studies using plasma as a source of cfDNA. However, there are also some studies where cfDNA has been extracted from serum. ctDNA in the serum of patients with gynecologic cancers has been tested by ddPCR for disease surveillance. Interestingly, patients with undetectable levels of ctDNA in the serum after treatment initiation showed a better clinical outcome and survival, and ctDNA elevation anticipated recurrence months earlier than computed tomography (CT) scanning [69]. Other studies reporting the detection of *PIK3CA* mutations in metastatic biliary cancers [70] or *HER2* amplifications in gastric cancer [43] also exemplify that serum could be a suitable source of cfDNA.

In a study by Combaret et al. [71], either plasma or serum were indistinctly utilized to detect hotspot mutations in the *ALK* gene, showing that analyzable ctDNA could be obtained from both fluids. Future studies should be conducted to further elucidate whether the differential amounts of cfDNA obtained from plasma and serum could significantly affect the results of ddPCR assays.

Regarding the analysis of molecular markers in cfDNA from plasma, lung cancer is one of the most prevalent malignancies where ddPCR has a major application. Detection of *KRAS* mutations in NSCLC patients by ddPCR is of great interest as a non-invasive method for surveillance, since the mutational load dynamics are

closely associated with changes in tumor burden monitored by CT during treatment [68, 72]. The clinical outcome of targeted therapies with *EGFR* tyrosine kinase inhibitors (TKIs), a major therapeutic strategy against this type of cancer, is highly dependent on the presence of activating mutations and resistance mutations, especially *EGFR* T790M [73–75]. A high number of studies reporting the detection of *EGFR* mutations in the plasma of lung cancer patients have been published in the last few years [31, 67, 73–83]. In these studies, different analytical sensitivities have been reported; 0.005–0.01% (equivalent to 5–50 mutant copies in a background of 10,000 wild-type copies) was obtained by Oxnard et al. [31], depending on the mutation assayed, which is in the same range as the 50 mutant to 50,000 wild-type copies of Wei et al. [78] for T790M, and the 0.003 and 0.005% mutant allele frequencies found by Lee et al. [84] for L858R and ex19del, respectively. In some cases, sensitivities of 0.032% [82] or 0.04% have been reported [75, 76, 85]. Apart from analytical sensitivity, many studies report their results in terms of clinical sensitivity, defined by Lee et al. [84] as “the number of samples positive in both tissue and plasma out of the positive tissue samples,” whereas “specificity was calculated from the number of plasma- and tissue-negative samples out of the total negative tissue samples.” In the abovementioned studies, clinical sensitivities ranged from 61 to 82% and specificities from 63 to 100% [75, 77, 81–84, 86].

In a study by Watanabe et al. [56], the T790M mutation was detected by ddPCR in FFPE tumors from 373 NSCLC patients who participated in the Japan Molecular Epidemiology for Lung Cancer (JME) study. The authors reported an analytical sensitivity of 0.001%, which allowed the detection of the T790M mutation in one patient (0.3%) at an extremely low frequency (0.009%), but its clinical significance remains unknown. The clinical relevance of harboring the T790M mutation before treatment in patients with early-stage NSCLC had not been previously analyzed in large scale studies using a methodology as sensitive as ddPCR. The 10-year follow-up results of this study will shed light on the meaningfulness of these low-abundance mutations in the clinical setting.

Thus, the use of ddPCR to detect *EGFR*-activating mutations and T790M mutant alleles in a quantitative manner has been considered particularly relevant for clinical management and disease monitoring in NSCLC, since mutated copy levels increase with tumor stage and correlate with worse progression and survival [74], whereas undetectable levels of *EGFR* mutations after treatment with TKIs correlate with longer progression-free and overall survival [84].

This approach has also proven useful in reflecting tumor heterogeneity, a frequent event in NSCLC tumors, since

double *EGFR* mutations have been detected in patients whose tumors only presented a single mutation [74]. Similarly, ddPCR has also unveiled intratumoral heterogeneity in ctDNA in other cancers, such as hepatocellular carcinoma [87] and neuroblastoma [71].

In a similar manner to *EGFR*, which plays a major role in the development of resistance mechanisms against monoclonal antibodies in lung cancer, other hotspot mutations detectable in plasma by ddPCR have been related to drug resistance, associating changes in mutation detection and allele frequency with response to treatment. This is the case with *ESR1* mutations in estrogen receptor-positive metastatic breast cancer, which lead to endocrine therapy resistance [88–91]. Increased levels of *ESR1* mutations have been correlated to a shorter effectiveness of therapy and survival [90, 92, 93]. *PIK3CA* mutations determined by ddPCR, on the other hand, are good prognostic factors in triple-negative breast cancer patients [94]. *PIK3CA* mutations have been detected in pre-surgery blood samples from early-stage breast cancer patients, with 93.3% sensitivity and 100% specificity. Some of these patients had T1-T1b tumors of less than 1–2 cm in size, pointing to the possibility of using liquid biopsy for mutation detection in early-stage patients, without the need for a tissue biopsy [59]. Mutation tracking in serial samples from early breast cancer patients has also proven useful for the identification of patients at high risk of recurrence, anticipating relapse 7.9 months before being clinically detectable [95]. Another example of a relevant biomarker detectable by ddPCR with predictive and prognostic value is the *BRAF* V600E mutation in cutaneous melanoma, the detection of which is a determinant for the selection of patients for therapy with *BRAF* inhibitors [96]. In this cancer type, circulating levels of *BRAF* and *NRAS* mutations change during disease evolution, depending on response to treatment, and are associated with clinical outcome [34, 96–98]. In light of all these examples, the potential use of ctDNA analysis for companion diagnostics for directing targeted therapy seems increasingly feasible in the near future.

Most publications regarding ddPCR for the analysis of cfDNA are focused on rare variant detection, such as the above mentioned *EGFR* T790M single nucleotide polymorphism. However, this methodology also brings the possibility of examining other genetic alterations, including genomic rearrangements. *HER2* amplifications [99] and copy numbers of tumor-specific rearrangements [100] have been detected by ddPCR in the plasma of metastatic breast cancer patients. Thus, the quantification of fragments of tumor-specific chromosomal rearrangements in cfDNA in plasma by ddPCR is able to anticipate relapse and progression months earlier than traditional imaging techniques in breast cancer [100].

Novel ddPCR techniques for hotspot mutation screening have recently been developed. Bidshahri et al. have described a ddPCR-based WT-negative screening strategy using model-designed probes [55]. This method consists of a two-well assay. Well 1 contains a HEX-labeled locked nucleic acid (LNA)-substituted probe designed to selectively hybridize to *BRAF*^{V600E1} that does not cross-react to other clinically relevant *BRAF* alleles. The second well contains a FAM-labeled probe that hybridizes only to WT *BRAF*, allowing investigators to distinguish WT V600 from all *BRAF* V600 MT alleles. Through the detection of *BRAF*^{V600E1} in CRC tumors, this methodology identifies those metastatic patients who are not candidates for therapy with anti-epidermal growth factor receptor (anti-EGFR) antibodies. Additionally, this strategy also gives valuable information to clinicians, unveiling less common, rare V600 variants that might require further analysis of CRC biomarkers, more personalized or aggressive treatment, and closer patient monitoring [55].

“Inverted” ddPCR assays also deserve to be mentioned as newly developed ddPCR approaches [54]. In these assays, a combination of wild-type probes labeled with different fluorophores targeting the wild-type alleles of two genes of interest is used to detect any single nucleotide variant affecting the two hotspots, thus creating an inverted assay. This methodology has allowed the detection of variants in fresh tumors and FFPE DNA samples from non-Hodgkin lymphoma patients with known mutations, providing an accurate assessment of which samples carried either an *EZH2* or *STAT6* mutation (or both) and which samples lacked mutations at either hotspot [54].

A recent study has proposed an alternative application of ddPCR in cancer: quantification of methylation markers, an approach called droplet digital methylation-specific PCR (ddMSP) [101]. In this work, breast cancer epigenetic markers were first selected from genome-wide screening by methylation array analysis of FFPE tumors, cell lines and blood samples from healthy volunteers. The methylation array identified 12 epigenetic markers, which were used to build a ddMSP panel of 16 markers, including four additional internal control markers needed to evaluate the concentration of cfDNA in plasma samples. ddPCR was employed for absolute quantification of the amount of cfDNA and methylated DNA fragments. A detection model was then developed by support vector machine using this panel in a training dataset including cfDNA from healthy volunteers and breast cancer patients, which was subsequently validated in an independent set of samples. This ddMSP-based model has shown potential for early detection of breast cancer, with a similar effectiveness to mammography [101]. Another recent example of this ddPCR approach is the use of a previously known panel of differentially methylated genes quantified by ddMSP in

pancreatic cyst fluid to predict the grade of dysplasia [102]. Changes in methylated ctDNA have also been proposed as a biomarker to monitor tumor evolution of CRC patients at different stages [103].

As mentioned above, attempts to develop improved multiplex ddPCR screening assays for the analysis of cfDNA are currently being undertaken. The fundamentals of ddPCR multiplexing have been recently reviewed by Whale et al. [60]. Thus, *KRAS* mutations have already been screened in cfDNA from the plasma of advanced cancer patients, including CRC, by ddPCR assays in a multiplex format [104, 105]. ddPCR has been also applied to detect *KRAS* mutations with a multiplex assay in cfDNA and exosome-derived DNA from pancreatic ductal adenocarcinoma patients. This study highlights the potential of exosomes as an alternative source of tumor DNA that may provide complementary information to cfDNA. These authors found a higher prevalence of mutated *KRAS* in exosome-derived DNA of early stage pancreatic cancer than previously reported for cfDNA, suggesting that it may be helpful for early detection of this extremely aggressive type of cancer [106].

Multiplex assays for *ESR1* mutations in breast cancer patients have also been developed [89, 92]. The screening of mutation hotspots with assays lacking wild-type probes in non-Hodgkin lymphoma [54] is an additional example of multiplex ddPCR.

A recent study has reported how different amounts of DNA input can influence the sensitivity of detection of *EGFR* mutations by ddPCR in cfDNA from the plasma of NSCLC patients: 82.6% when cfDNA input is ≥ 5 ng per reaction, 60% with 2–5 ng and 46.7% for cases with < 2 ng per reaction [86]. These authors suggest that higher plasma volumes should be used for cfDNA extraction (4 mL instead of 2 mL, which is the most common volume described in the literature) and that the cfDNA concentration in plasma must be carefully verified prior to ddPCR testing, in the same way as tumor tissue is subjected to pathologic evaluation before molecular analysis.

Importantly, increasing evidence suggests that ctDNA-based monitoring could have a limited clinical utility in the early stages, of, for example NSCLC [74]. It must be noted that, although ctDNA detection reflects the dynamic changes in the circulating tumor burden throughout the disease course in the majority of advanced cases, there are also patients in whom mutated fragments cannot be detected in plasma, probably because their tumors do not shed DNA continuously into the circulation, so these mutations could be undetectable regardless of the method's sensitivity [67, 76]. The risk of false-negative results when ctDNA is analyzed in plasma samples by ddPCR still represents a problem that needs to be addressed. These shortcomings point to the need for a combination of

detection strategies including imaging tests, tissue biopsies, and liquid biopsies, whenever possible, to have a complete overview of the complex landscape of tumor molecular characteristics and their relationship with disease course. In spite of their extremely high sensitivity and specificity, ddPCR methodologies are still under development, and their use should not completely replace other complementary approaches as yet.

The most relevant studies reporting molecular screening of biomarkers by ddPCR in cfDNA from plasma and serum of cancer patients are summarized in Table 1.

3.2 Circulating RNA Biomarkers

MicroRNAs (miRNAs) have been proposed as relevant cancer biomarkers [107]. Quantification of miRNA biomarkers in the serum of prostate and lung cancer patients has been performed by ddPCR, obtaining comparable sensitivity, similar or superior precision, and reproducibility with respect to standard real-time PCR [7, 108]. One of the main advantages of ddPCR over qPCR is that no reference or standard calibration for quantification are required [108]. As an example of the clinical utility of ddPCR-based circulating RNA biomarkers determination, dysregulation of miRNAs has been reported in breast cancer patients versus controls, associating higher levels of serum miR-10b-5p with a poor prognosis [109].

Absolute quantification of cell-free miRNAs by means of ddPCR in several types of malignancies, including melanoma and lung, colorectal, and breast cancers, has revealed that different concentrations of these biomarkers are present in plasma and serum [110]. As mentioned above, this finding raises concerns about how different sources of genetic material for molecular screening could affect the results of ddPCR assays.

Plasma-derived exosomes have also been used as a source of RNA for the absolute quantification of AR-V7, an androgen receptor splice variant, which represents an important biomarker in castration-resistant prostate cancer (CRPC), associating AR-V7 positivity with a shorter progression-free and overall survival [111]. Similarly, messenger RNA (mRNA) has been isolated from serum extracellular vesicles of glioma and glioblastoma patients to detect and quantify *IDH1* transcripts, yielding higher copy numbers in tumor-bearing patients [112].

3.3 Circulating Tumor Cells

CTCs have been proposed as an alternative and/or complementary source of genetic material to cfDNA in liquid biopsies for tumor genotyping. Thus, CTCs have been used to detect *KRAS* mutations in CRC patients before surgery, predicting those mutations that were later found in the

Table 1 ddPCR analysis of cancer molecular markers in cfDNA from plasma and serum

Molecular marker	Tumor type	cfDNA source	ddPCR platform	References
<i>PIK3CA</i> mutations	Breast cancer	Plasma	Bio-Rad	Beaver et al. [59]
<i>ESR1</i> , <i>PIK3CA</i> , <i>TP53</i> mutations	Breast cancer	Plasma	Bio-Rad	Guttery et al. [88]
<i>ESR1</i> mutations	Breast cancer	Plasma	Bio-Rad	Fribbens et al. [89], Takeshita et al. [90], Wang et al. [91], Schiavon et al. [92], Clatot et al. [93]
<i>PIK3CA</i> mutations	Breast cancer	Plasma	Bio-Rad	Takeshita et al. [94]
<i>PIK3CA</i> , <i>TP53</i> , <i>ANK3</i> , <i>APOBEC1</i> , <i>CDH1</i> , <i>ERBB2</i> , <i>ESR1</i> , <i>FGFR1</i> , <i>GATA3</i> , <i>KMT2C (MLL3)</i> , <i>RBI1</i> , <i>SYNE-1</i> , <i>TBX3</i> , <i>XIRP2</i> mutations	Breast cancer	Plasma	Bio-Rad	García-Murillas et al. [95]
<i>HER2</i> amplifications	Breast cancer	Plasma	Bio-Rad	Gevensleben et al. [99]
Chromosomal rearrangements	Breast cancer	Plasma	Bio-Rad	Olsson et al. [100]
Methylation markers	Breast cancer	Plasma	Bio-Rad	Uehiro et al. [101]
Methylation markers	Colorectal cancer	Plasma	RainDance	Garrigou et al. [103]
<i>KRAS</i> mutations	Colorectal cancer	Plasma	RainDance	Taly et al. [104]
<i>KRAS</i> G12V	Colorectal cancer	Plasma	Bio-Rad	Olmedillas López et al. [151]
<i>ATM</i> , <i>ATRX</i> , <i>BRAF</i> , <i>ERBB2</i> , <i>IKZF1</i> , <i>NRAS</i> , <i>IGF1R</i> , <i>MED12</i> , <i>TP53</i> , <i>CCND1</i>	Early lung neoplasms (atypical adenomatous hyperplasia and minimally invasive adenocarcinoma)	Plasma	Bio-Rad	Izumchenko et al. [140]
<i>HER2</i> amplification	Gastric cancer	Serum	Bio-Rad	Kinugasa et al. [43]
ctDNA levels	Gynecologic cancers	Serum	RainDance	Pereira et al. [69]
<i>TP53</i> , <i>TERT</i> , <i>CTNNB1</i>	Hepatocellular carcinoma	Plasma	Bio-Rad	Huang et al. [87]
<i>BRAF</i> mutation	Leptomeningeal disease from metastatic melanoma	Plasma	Bio-Rad	Li et al. [126], Momtaz et al. [128]
<i>EGFR</i> , <i>KRAS</i> , <i>TP53</i>	Lung cancer	Plasma	RainDance	Zonta et al. [80]
<i>BRAF</i> V600E	Melanoma	Plasma	Bio-Rad	Sanmamed et al. [96]
<i>BRAF</i> and <i>NRAS</i>	Melanoma	Plasma	Bio-Rad	Chang et al. [97], Gray et al. [98], Tsao et al. [34]
<i>PIK3CA</i> mutations	Metastatic biliary cancers	Serum	Bio-Rad	Kim et al. [70]
<i>ALK</i> mutations	Neuroblastoma	Serum and plasma	Bio-Rad	Combaret et al. [71]
DEL, INS, INV, ITX, CTX	NMIBC	Plasma	Bio-Rad	Birkenkamp-Demtröder et al. [130]
<i>EGFR</i> , <i>KRAS</i> , <i>BRAF</i> mutations	NSCLC and melanoma	Plasma	Bio-Rad	Oxnard et al. [31]
<i>KRAS</i> mutations	NSCLC	Plasma	Bio-Rad	Guibert et al. [68]
<i>EGFR</i> mutations	NSCLC	Plasma	Bio-Rad	Yanagita et al. [67], Chia et al. [73], Alegre et al. [74], Zheng et al. [75], Zhu et al. [76], Takahama et al. [77], Wei et al. [78], Zhao et al. [79], Ishii et al. [82], Lee et al. [84], Yang et al. [85], Zhang et al. [86]
<i>EGFR</i> mutations	NSCLC	Plasma	RainDance	Seki et al. [81]
<i>EGFR</i> and <i>KRAS</i> mutations	NSCLC	Plasma	Bio-Rad	Sacher et al. [83]
<i>KRAS</i> mutations	Pancreatic cancer	Plasma	RainDance	Takai et al. [150]
<i>KRAS</i> mutations	Pancreatic cancer	Plasma	Bio-Rad	Allenson et al. [106]

cfDNA cell-free DNA, ctDNA circulating tumor DNA, CTX interchromosomal translocations, ddPCR droplet digital PCR, DEL deletions, INS insertions, INV inversions, ITX intrachromosomal translocations, NMIBC non-muscle invasive bladder cancer, NSCLC non-small cell lung cancer

FFPE tumors [113]. In that study, whole genome amplification of DNA from CTCs was performed prior to the determination of *KRAS* status using a multiplex ddPCR screening assay for the seven most frequent *KRAS* mutations.

ddPCR has proven useful as a reference technique to validate mutations found in CTCs of lung cancer patients previously uncovered by targeted deep sequencing [114]. Similarly, *BRAF* mutations have also been detected, in combination with whole-genome amplification, in CTCs from patients with metastatic melanoma [115].

Using ddPCR, a small number of CTCs (up to five cells) is sufficient to detect AR-V7 in advanced CRPC patients [116]. In this recent study, considerable intratumoral heterogeneity was identified; one patient with a high CTC recovery showed in turn a very low AR-V7 expression, whereas another patient with as few as ten CTCs yielded a high number of AR-V7 copies, suggesting that not all tumor cells contribute to AR-V7 expression in CRPC.

3.4 Peripheral Blood Mononuclear Cells and Bone Marrow Aspirates

Mononuclear cells obtained from peripheral blood and bone marrow aspirates could also be, in some way, considered liquid biopsies that have been interrogated by ddPCR. Thus, acquired mutations within the *JAK2* [8] and *CALR* [117] genes detectable by ddPCR in patients with myeloproliferative neoplasms have been proposed as molecular markers of minimal residual disease (MRD) [118]. ddPCR has been compared to qPCR for monitoring MRD using patient-specific *IGH* rearrangements and the *BCL2/IGH MBR* translocation in patients with multiple myeloma, mantle cell lymphoma, and follicular lymphoma, showing similar sensitivity, reproducibility, and accuracy [35]. Immunoglobulin and T-cell receptor gene rearrangements have also been quantified by ddPCR for MRD monitoring in acute lymphoblastic leukemia (ALL) [119]. In these malignancies, ddPCR offers several advantages over the standard qPCR-based methods, mainly that ddPCR allows an absolute quantification of target sequences without the need for standard reference curves, in a more applicable, less labor-intensive, and more cost-effective manner [35]. Other examples of molecular biomarkers tested by ddPCR in hematologic disorders are the *BRAF* V600E mutation in bone marrow from patients with hairy cell leukemia (HCL) [120], *NOTCH1* mutations in chronic lymphocytic leukemia (CLL) [121], and the quantification of *BCR-ABL1* and *PML-RARA* fusion transcripts in chronic myeloid leukemia (CML) [122] and acute promyelocytic leukemia (APL) [123], respectively. Interestingly, the amount of *PML-RARA* transcript before treatment has been identified as a prognostic factor for APL relapse [123].

One further example of ddPCR application in hematologic disorders is the examination of point mutations in *DNMT3A* and *IDH1/2* genes, which have been investigated in bone marrow samples from patients with acute myeloid leukemia (AML) in an attempt to improve early detection of relapse after allogeneic hematopoietic stem cell transplantation [124].

4 Clinical Utility of Other Body Fluids Examined by ddPCR

Most applications of ddPCR in oncology and, particularly, in liquid biopsies for different kinds of cancers, employ plasma or serum as the main sources of nucleic acids for molecular characterization and tumor genotyping. However, there are other body fluids that provide sufficient amounts of DNA and RNA to be analyzed.

4.1 Cerebrospinal Fluid

ddPCR has been applied to the detection of hotspot mutations in CSF of patients with different types of solid brain tumors (primary tumors as well as brain metastases from melanoma and lung and colon adenocarcinoma) [125]. In a similar manner, *IDH1*-mutated transcript was previously detected and quantified by two different digital PCR methods (BEAMing and ddPCR) in the extracellular vesicles present in CSF from patients bearing gliomas and glioblastomas, without the need for tumor biopsies [112]. Quantitative analysis of the *BRAF* V600E mutation fraction in cfDNA from CSF allowed treatment response monitoring in metastatic melanoma leptomeningeal disease, as this fraction fluctuates in parallel with clinical symptoms [126]. Along this line, various studies have reported that changing levels of tumor-derived cfDNA in CSF of patients with central nervous system (CNS) involvement could act as an indicator of tumor burden and treatment response (or unresponsiveness to therapy) [127, 128].

These publications suggest that mutations originating from brain tumors are more readily detectable in CSF than in plasma, and their concentrations in plasma could be dependent on metastatic burden and the level of systemic involvement of disease [125]. The difference between CSF and plasma in terms of ctDNA detection in CNS metastases could also be a consequence of the limited drug penetrability across the blood–brain barrier [79]. Thus, the analysis of ctDNA in CSF by ddPCR could be a useful biomarker to monitor treatment response and disease progression. It represents an alternative and/or complementary assay to tissue biopsy and cytology, being less invasive than the former and more sensitive than the latter

[125, 128]. In fact, this approach has been shown to complement cytopathologic assessment for the diagnosis of leptomeningeal carcinomatosis [127].

CNS tumors are continuously evolving and experience genomic alterations in response to treatment, so there is a need to track their dynamic molecular features. This information could help physicians in selecting the best therapeutic options, and patients could benefit from more personalized therapies and achieve improved clinical outcomes.

4.2 Urine

TERT and *FGFR3* mutations have recently been tested in a prospective blinded study by ddPCR in urinary DNA from patients with gross hematuria, in order to detect urothelial bladder carcinoma [129]. These DNA-based biomarkers could represent an alternative or a complementary analysis to cystoscopy for the diagnosis and surveillance of patients at high risk of developing bladder tumors. ddPCR has also been applied in combination with next-generation sequencing (NGS) to detect patient-specific variants in non-muscle invasive bladder cancer (NMIBC) [130]. Of note, in that study, elevation of tumor DNA levels in urine was an early event in disease progression, even in patients without detectable tumor DNA in plasma. Furthermore, these levels were also able to distinguish patients with metastasis from those with superficial bladder tumors, showing a close relationship with tumor invasiveness [130].

In another recent study, absolute quantification of miRNA biomarkers in urine-derived exosomes from bladder cancer patients was validated by ddPCR [131]. These authors report that miRNA shedding from bladder tumor cells into different body fluids from the same patient is variable, since they found some upregulated miRNAs in the tumor tissue that were also detectable in urine exosomes, but not in plasma. miRNA analysis of matched tumor and urine exosome samples showed a good correlation in six miRNAs and one transfer RNA fragment (tRF), two of which have not previously been reported in bladder cancer. The potential application of a newly developed panel of biomarkers including the identified miRNAs and tRF for clinical management of NMIBC patients should be further evaluated [131].

Surprisingly, the analysis of cfDNA in urine is not only applicable for tumors of the genitourinary tract, but also for systemic malignancies such as Langerhans cell histiocytosis (LCH) and Erdheim-Chester disease (ECD) [132]. In these histiocytic disorders, *BRAF* V600E mutation interrogation in DNA from urine gives a chance to non-invasively determine tumor genotype with more feasibility than bone biopsies, which, for example, are the most common

available source. Besides, tracking quantitative dynamic changes in allele burden could be a complement to radiographic follow-up for serial monitoring of response to therapy [132].

Another non-genitourinary-related malignancy where urine analysis has proven a potential clinical application is NSCLC. *EGFR* mutation status has recently been determined in urinary cfDNA from NSCLC patients by ddPCR with a comparable sensitivity and specificity to ctDNA in plasma [133]. In that study, urinary cfDNA concentration fluctuated at different time points during the disease course and accurately tracked the dynamic changes that occurred in response to treatment with *EGFR* TKIs. It also allowed investigators to actively uncover the emergence of a secondary mutation that confers drug resistance, *EGFR* T790M. These changes were closely related to disease progression and survival [133]. Similar results have also been observed with *KRAS* mutations in NSCLC patients [134]. Taken together, these studies support the usefulness of urinary cfDNA analysis by ddPCR for non-invasive monitoring of response to therapy, with a potential predictive and prognostic value.

Previous attempts to use the analysis of urinary cfDNA through real-time PCR-based methods were made for detection of bladder cancer. In most cases, differences in cfDNA quantity and integrity between healthy individuals and bladder cancer patients were pursued. For example, in 2007, Chang et al. [135] proposed measuring urinary cfDNA concentration (adjusted for urine creatinine) as a non-invasive screening tool for bladder cancer. Their real-time PCR-based assay detected the amplification of 400-bp beta-actin (named 400-bp ucf-DNA/UCr) with a sensitivity and specificity of 86.1 and 72.0%, respectively. Zancan et al. [136], in 2009, compared several methods for urinary cfDNA quantification including real-time PCR and found that this strategy was not able to accurately distinguish bladder cancer patients from healthy individuals.

In another study following this line, sequences longer than 250 bp of three genes known to be frequently amplified in bladder cancer (*c-Myc*, *BCAS1*, and *HER2*) were quantified by real-time PCR to verify urinary cfDNA integrity, showing a sensitivity of 79% and a specificity of 84% [137]. However, subsequent studies revealed that urinary cfDNA integrity is not a good biomarker for prostate cancer, suggesting that more specific alterations, such as mutations and epigenetic markers may be more informative for early diagnosis of this disease [138].

The introduction of ddPCR technology has considerably improved the sensitivity and specificity of urinary cfDNA assays. The newly developed ddPCR-based assays are designed to detect specific mutations, and their concordance rates with the mutational status of the matched tumor tissues range from 88 to 100% [132–134]. Dahmcke et al.

reported a sensitivity of 97.0% and a specificity of 76.9% [129]. In the study by Hyman et al., urinary *BRAF* V600E cfDNA showed a sensitivity of detection of 92.9% and a specificity of 100% [132]. These results support the utility of urinary cfDNA analysis by ddPCR with diagnostic and monitoring purposes in different types of cancer, although it requires further clinical validation in future studies.

4.3 Sputum

Sputum could be also used as a non-invasive source of nucleic acids for molecular analysis, since it contains exfoliated cells from the airway epithelium. A study by Li and colleagues in 2014 demonstrated that miRNAs could be efficiently and reproducibly quantified by ddPCR using RNA purified from the cell pellets of sputum [139]. Thus, they found that miR-31 and miR-210 copy numbers were significantly elevated in lung cancer patients in comparison to cancer-free controls. These results suggest a potential application of absolute quantification of miRNA copy number in sputum by ddPCR as a clinical biomarker for lung cancer. Another approach that has already been explored is the analysis of point mutations; in a study aimed at identifying predictive markers of malignant transformation, mutations affecting seven different genes were detected in sputum from patients with early lung neoplasms [140].

4.4 Pleural Effusions and Ascites Fluid

Pleural effusions and ascites fluid have been successfully analyzed by ddPCR in a recent clinical trial with NSCLC patients where these malignant fluids were used as an alternative source of DNA for *EGFR* T790M mutation testing [77]. Interestingly, the frequency of mutation detection in these fluids was high, suggesting that they could be better candidates, when available, for molecular marker screening than plasma (where some cases of false negative results were found) in this type of cancer.

4.5 Stool

Stool has been proposed as an alternative source of DNA for molecular screening in CRC due to the exfoliation of tumor cells to the bowel lumen. *KRAS* mutations have been detected in stool DNA from CRC patients using BEAMing [28], but, to our knowledge, ddPCR has not been applied to stool DNA mutation analysis to date. Of note, our group has recently detected the *KRAS* G12D mutation in stool DNA from CRC patients, using a commercially available ddPCR platform (manuscript in preparation).

Cancer molecular biomarkers analyzed by ddPCR in different body fluids are listed in Table 2.

5 Other Applications of ddPCR Related to Cancer Research

ddPCR is considered an established and reliable method to accurately determine DNA and RNA quantities at several steps throughout current next-generation sequencing workflows as well as for the final library quantification [141]. Of note, ddPCR offers a much faster and cost-efficient platform with superior sensitivity to next-generation sequencing for application in a wide variety of cancer studies. Advantages of ddPCR over next-generation sequencing also include less probability of errors, the necessity of a lower DNA input, and, furthermore, analytic bioinformatics pipelines are not required [39]. In fact, in a recent study, ddPCR was selected rather than massive parallel sequencing for detecting rare alleles as molecular markers in pre- and post-operative plasma samples of CRC patients because of its remarkably superior sensitivity [142]. It has been recognized that NGS-based strategies applied to plasma samples for mutation detection face serious problems derived from polymerase error rates [95]. This enzyme introduces mistakes during amplification, generating point mutations. An estimated error rate of 1% gives rise to hundreds of millions of sequencing mistakes in a single experiment [143]. In a recent publication, 0.08% was the lowest allelic fraction detectable for *KRAS* mutations in cfDNA from pancreatic cancer patients using an NGS pipeline, suggesting that some true mutations might be overlooked because their allelic fraction would be indistinguishable from the sequencing background [144]. In another publication, the performance of NGS and ddPCR was compared for quantification of *EGFR* mutant alleles in ctDNA from NSCLC patients, showing LODs of 5 and 0.04%, respectively [85].

However, some advanced NGS methods have been developed in the last few years that are expected to challenge ddPCR in terms of sensitivity and specificity, such as error suppression methods and duplex sequencing (DS). Integrated digital error suppression (iDES) relies on a computational approach for elimination of background artifacts and a molecular barcoding system to improve the recovery of cfDNA molecules. iDES has already been applied to the analysis of *EGFR* mutations in NSCLC patients, with 92% sensitivity and 96% specificity, which allowed the detection of four in 10^5 cfDNA molecules [145]. DS is another approach aimed at reducing sequencing errors by tagging the two complementary strands of the DNA fragments [143, 146]. After sequencing, true mutations will be located at the same point in both strands, whereas mutations present only in one strand will be identified as sequencing errors and easily discarded. Thus, the background error rate of DS has been established

Table 2 Body fluids used as alternative sources for ddPCR analysis of cancer biomarkers

Molecular marker	Tumor type	Body fluid	ddPCR platform	References				
<i>IDH1</i> transcript	Glioma, glioblastoma	CSF	RainDance	Chen et al. [112]				
<i>TP53</i> R114C	Glioblastoma, medulloblastoma, brain metastases from lung and breast cancer, leptomeningeal carcinomatosis	CSF	Bio-Rad	De Mattos-Arruda et al. [127]				
<i>IDH1</i> R132H								
<i>AHRR</i> G353C								
<i>ADAMTS12</i> T982K								
<i>EGFR</i> C620S, R108K, L858R								
<i>PTEN</i> D162V								
<i>ANK2</i> K2337X								
<i>FTH1</i> I146T								
<i>OR51D1</i> R135C								
<i>POLE</i> E318K								
<i>PCDH1</i> S190C								
<i>CD9</i> W22L								
<i>ARID5B</i> E572K								
<i>NF2</i> R57					Vestibular schwannoma, atypical meningioma, brain metastases from melanoma, colon and lung adenocarcinoma	CSF	Bio-Rad	Pan et al. [125]
<i>AKT1</i> E17K								
<i>BRAF</i> V600E								
<i>NRAS</i> Q61L								
<i>KRAS</i> G12D								
<i>EGFR</i> L585R								
<i>EGFR</i> E746_A750del								
<i>BRAF</i> V600E	Leptomeningeal disease from metastatic melanoma	CSF	Bio-Rad	Li et al. [126]				
<i>BRAF</i> V600E, V600K								
<i>EGFR</i> E19-Del, L858R, T790M	Leptomeningeal disease from metastatic NSCLC	CSF	Bio-Rad	Zhao et al. [79]				
miRNAs absolute quantification	Bladder cancer	Urine	Bio-Rad	Armstrong et al. [131]				
<i>BRAF</i> V600E	Langerhans cell histiocytosis, ECD	Urine	RainDance	Hyman et al. [132]				
DEL, INS, INV, ITX, CTX	NIMBC	Urine	Bio-Rad	Birkenkamp-Demtröder et al. [130]				
<i>EGFR</i> L858R, L861Q, T790M	NSCLC	Urine	Bio-Rad	Chen et al. [133]				
<i>KRAS</i> mutations	NSCLC	Urine	Bio-Rad	Wang et al. [134]				
<i>TERT</i> C228T, C250T	Urothelial bladder carcinoma	Urine	Bio-Rad	Dahmcke et al. [129]				
<i>FGFR3</i> R248C, S249C, G370C, Y373C	NSCLC	Sputum	Bio-Rad	Li et al. [139]				
miRNAs absolute quantification								
miR-31, miR-210								

Table 2 continued

Molecular marker	Tumor type	Body fluid	ddPCR platform	References
<i>ATM</i> p.2455L>M <i>ATRX</i> p.929Q>E <i>BRAF</i> p.466G>V, p.469G>A <i>ERBB2</i> p.243G>S <i>NRAS</i> p.13G>R <i>IGF1R</i> p.983A>T <i>CCND1</i> p.293V>M <i>EGFR</i> T790M	Early lung neoplasms (atypical adenomatous hyperplasia and minimally invasive adenocarcinoma)	Sputum	Bio-Rad	Izumchenko et al. [140]
<i>KRAS</i> G12D	Colorectal cancer	Pleural effusions and ascites fluid Stool	Bio-Rad	Takahama et al. [77] Olmedillas-López et al. (manuscript in preparation)

CSF cerebrospinal fluid, *CTX* interchromosomal translocations, *ddPCR* droplet digital PCR, *DEL* deletions, *ECD* Erdheim-Chester disease, *INS* insertions, *INV* inversions, *ITX* intrachromosomal translocations, *miRNA* microRNA, *NMIBC* non-muscle invasive bladder cancer, *NSCLC* non-small cell lung cancer

as less than one mutation per billion nucleotides sequenced. This methodology has been applied to the analysis of mutational patterns in human mitochondrial DNA [143].

The combination of NGS and ddPCR could provide complementary information. As we have already mentioned, in some recent publications ddPCR has been used as a reference technique to validate the results and verify the presence of mutations detected by other methodologies, including NGS strategies [88, 114, 147–149]. It has been suggested that combined approaches using both ddPCR and targeted deep sequencing could be feasible for clinical translation, for example, in pancreatic [150] and breast cancer [88, 100].

6 Concluding Remarks

The number of publications reporting ddPCR applications in oncology is rapidly increasing, and this field has experienced a huge growth, especially in the last year. Superior sensitivity and specificity compared to other techniques, as well as demonstrated accuracy and reproducibility in a broad range of scenarios, have made ddPCR a powerful tool for cancer research. Many studies have proven its ability to evaluate a wide variety of genetic alterations that range from the detection of rare mutations to precise quantification at both DNA and RNA levels, using minute amounts of starting material obtained from different clinical samples, including plasma, CSF, and urine. Molecular markers determined by ddPCR provide information of clinical utility for monitoring response to treatment, or unveiling the complex mechanisms

implicated in the development of drug resistance in several human malignancies. In some cases, these biomarkers correlate with progression and survival, showing a predictive and prognostic value, but additional studies in larger cohorts of patients are needed for further validation. Furthermore, ddPCR is also able to detect MRD, allowing an early detection of disease progression, much earlier than standard imaging methods. This could impact clinical decision making, shortening the time needed by physicians to switch to an alternative therapeutic option. For all these reasons, ddPCR has enormous possibilities with regard to its translation into daily clinical practice in the near future.

However, this methodology still has some limitations that need to be addressed. For example, detection of molecular markers in circulating cfDNA at early cancer stages or in patients with low DNA-shedding tumors is still challenging, so the use of other complementary systems for patient follow-up and surveillance should not be fully replaced by ddPCR yet. False-negative as well as false-positive results, although relatively infrequent, have not been fully eliminated from ddPCR assays, raising concerns about the risks of making clinical decisions exclusively based on this methodology. Thus, there are still shortcomings related to experimental artifacts that need to be addressed. Full automation and multiplexing are major current and future challenges of ddPCR with regard to achieving maximal implementation into the clinical routine. Finally, the combination of ddPCR with other next-generation methodologies could help in gaining further insight into the complex landscape of cancer genetic features.

Acknowledgements The authors gratefully acknowledge the editors and reviewers for their valuable comments and suggestions, which contributed to improve the quality of this article.

Author Contributions SO-L wrote the manuscript. MG-A and DG-O reviewed the manuscript. All authors agreed on the final version.

Compliance with Ethical Standards

Funding Susana Olmedillas-López is a post-doctoral researcher at the Foundation Health Research Institute-Fundación Jiménez Díaz University Hospital (FIIS-FJD), Madrid, from the RETIC Program of ISCIII-FEDER (RD12/0019/0035). This work is part of a grant from “Fondo de Investigaciones Sanitarias-FEDER”, Ministry of Health, Spain (FIS; PI13/01924).

Disclosure of potential conflicts of interest Susana Olmedillas-López, Mariano García-Arranz, and Damián García-Olmo declare that they have no conflicts of interest.

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