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Review

Application of Digital PCR in Detecting Human Diseases Associated Gene Mutation

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Key Words
Digital PCR • Gene mutation • Cancer • Infectious disease • Genetic disease • Mitochondrial DNA • Genome editing

Abstract
Gene mutation has been considered a research hotspot, and the rapid development of biomedicine has enabled significant advances in the evaluation of gene mutations. The advent of digital polymerase chain reaction (dPCR) elevates the detection of gene mutations to unprecedented levels of precision, especially in cancer-associated genes. dPCR has been utilized in the detection of tumor markers in cell-free DNA (cfDNA) samples from patients with different types of cancer in samples such as plasma, cerebrospinal fluid, urine and sputum, which confers significant value for dPCR in both clinical applications and basic research. Moreover, dPCR is extensively used in detecting pathogen mutations related to typical features of infectious diseases (e.g., drug resistance) and mutation status of heteroplasmic mitochondrial DNA, which determines the manifestation and progression of mtDNA-related diseases, as well as allows for the prenatal diagnosis of monogenic diseases and the assessment of the genome editing effects. Compared with real-time PCR (qPCR) and sequencing, the higher sensitivity and accuracy of dPCR indicates a great advantage in the detection of rare mutation. As a new technique, dPCR has some limitations, such as the necessity of highly allele-specific probes and a large sample volume. In this review, we summarize the application of dPCR in the detection of human disease-associated gene mutations.

Introduction

Gene mutation refers to any permanent alteration of the nucleotide sequences in the genome. To date, it has been confirmed to be closely related to the occurrence and progression of many disorders, including genetic diseases, cancer and infectious diseases. Additionally, mutations affect therapeutic options and prognosis of these diseases. Currently, identification of mutations is considered to be a promising direction in the screening

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of disorders. Several conventional methods have been developed to detect mutations, including sequencing, real-time quantitative PCR (qPCR) and their derivative methods such as single molecular real-time sequencing, amplification refractory mutation system-based PCR (ARMS-qPCR) and nested-qPCR. These methods contribute to the identification of gene mutations. However, there are still significant limitations of these methods. In recent years, digital PCR (dPCR) has emerged as the third generation of PCR, which has been developed to meet the demands of absolute quantification. In this review, we summarize the biomedical applications of digital PCR in detecting gene mutations in cancer, genetic diseases, infectious diseases, mitochondrial diseases and genome editing with a comparative analysis of its advantages and technical limitations. Our review provides reliable guidance for the further use of digital PCR.

Digital PCR

Before amplification, the template is diluted to a certain concentration and dispersed to a number of micro-reaction units, which results in zero or one target DNA sequence(s) in each unit. After amplification, the units containing copies of target DNA sequences show positive signals (defined as "1"), whereas only background fluorescence (defined as "0") is observed in the units with no target sequence (Fig. 1). A typical result is presented in Fig. 2. Poisson distribution is then applied to quantify the mean number and fraction of positive units to reduce the errors generated by the presentation of more than one copy of target sequence in some units [1]. On this basis, the initial copy number and concentration of target DNA can be obtained.

As the essential part of dPCR, the distribution of the template is mainly based on microwell chip, water-in-oil droplet and microfluidic techniques [2]. Among these methods, the water-in-oil droplet technique is the most frequently used, and the related droplet digital PCR (ddPCR) has been commercialized. This method distributes template into 20, 000 droplets before amplification, presenting high sensitivity to distinguish mutations in DNA with a detection limitation of 0.001% [3].

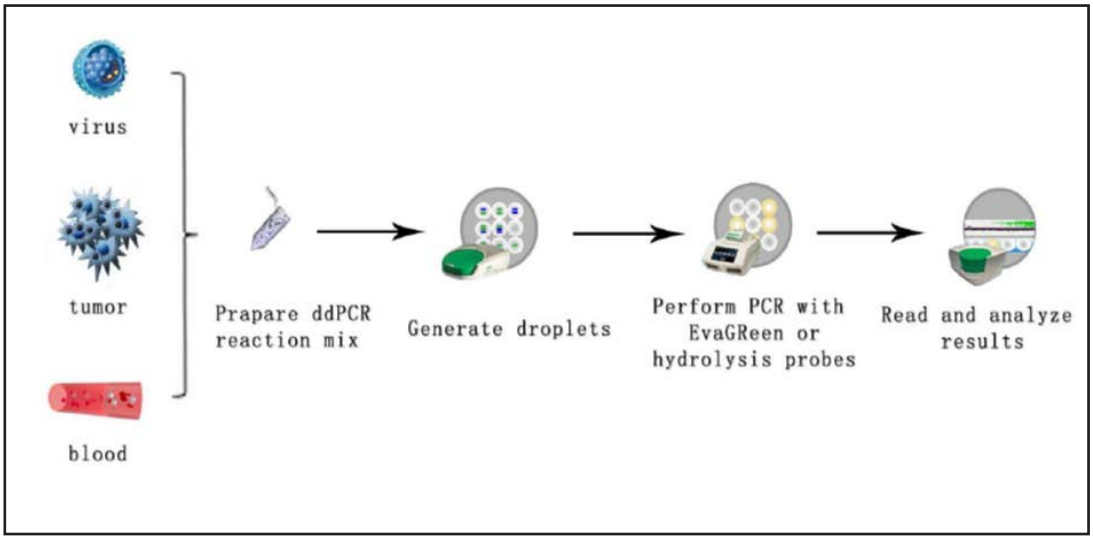

Fig. 1. Technical route of ddPCR. Template extracted from pathogens, tumors or plasma is distributed into 20,000 droplets by the water-in-oil droplet technique. After amplification, signals are detected by a photomultiplier, followed by data analysis.

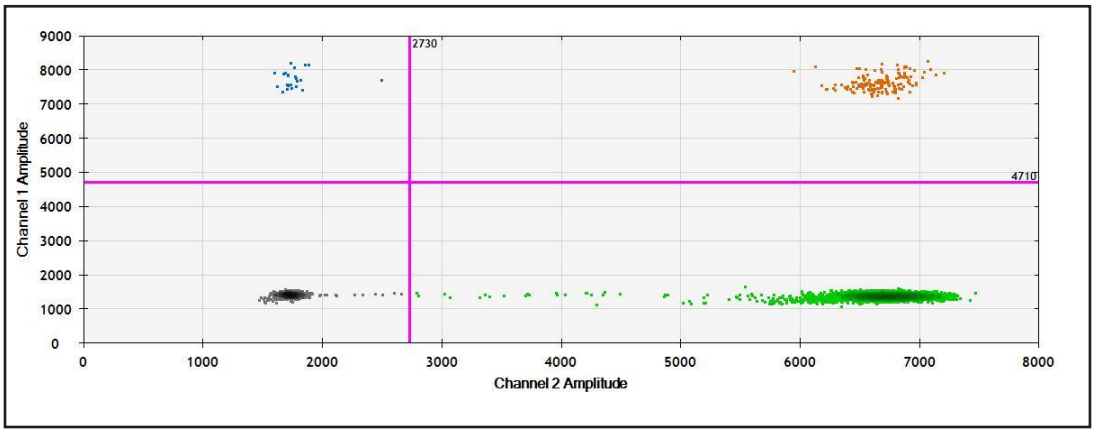

Fig. 2. Representative 2D scatter plot of ddPCR results of a standard sample using the QX200 Droplet Digital PCR System (Bio-Rad) The y-axis shows the fluorescence amplitude of the FAM probe, which is designed to hybridize only to the mutant allele (blue). The VIC probe hybridizes only to the wild-type reference allele (green) and is plotted on the x-axis. Double-positive droplets carrying both types of molecules are shown in orange, while double-negative droplets (no amplification) are shown in gray.

Medical application of dPCR in detecting gene mutation

Recently, dPCR has been extensively utilized for DNA detection in several fields, such as cancer biomarker screening, pathogen detection, gene expression analysis and environmental and food monitoring. Importantly, gene mutation analysis is a crucial aspect for all of these fields.

Cancer-associated gene mutation

Compared with qPCR, dPCR is more effective in detecting rare mutations in which a variant single-nucleotide polymorphism is present among the predominantly wild-type sequences [4]. For example, dPCR has been commonly used for the detection of epidermal growth factor receptor (*EGFR*) mutation in non-small-cell lung cancer (NSCLC) [5], *KRAS* mutation in colorectal cancer [6] and *ESR1* mutation in breast cancer [7]. It has been shown to be superior and is highly relied upon for its capability to remarkably enhance the limit of detection in cfDNA samples [8], such as blood, sputum [9], stool [10] and cerebrospinal fluid [11] containing low-level cancer cells, as well as in formalin-fixed paraffin-embedded (FFPE) tumor samples in which DNA may be partially degraded [12]. Therefore, dPCR is considered to be a crucial tool in cancer studies, diagnosis, personalized treatment and patient monitoring and follow-up management.

Diagnosis and basic research

Genetic markers have been shown to reflect the pathogenesis, progression and prognosis of cancer. Indeed, some markers are specifically expressed in certain cancer types. These markers are considered promising candidates in the diagnosis and basic research of cancer.

A recent study shows that ddPCR is a robust approach to distinguish *IDH1* mutation from a very large background of wild-type sequences in cerebrospinal fluid [11]. Thus, it could be an effective method for the diagnosis of brain gliomas, as biopsy is very difficult to perform. In 2017, Farkkila et al. [13] detected a *FOXL2 402C > G* (C134W) mutation in the circulating tumor DNA (ctDNA) in patients with adult granulosa cell tumor (AGCT) using a specific ddPCR assay with a low sensitivity threshold of 0.05%. Given that this mutation is specific to AGCTs, ddPCR can be beneficial for both diagnosis and relapse monitoring of AGCTs.

In FFPE brain tumors, ddPCR has been shown to be superior for detecting *FGFR1-ITD* mutations compared with whole-genome sequencing. This is a valuable tool for basic

research on the genetic characteristics of dysembryoplastic neuroepithelial tumors (DNT) and low grade neuroepithelial tumors (LGNTs) [14].

Targeted therapy and its monitoring

Targeted therapy acts on specific molecular targets associated with cancer. Mutation of certain genes is one of the most important causes of drug resistance.

EGFR over-expression has often been detected in NSCLC [15], and *EGFR* tyrosine kinases inhibitors (TKIs) are an *EGFR*-targeted cancer therapeutic for NSCLC. However, the *EGFR T790M* mutation may induce drug resistance [16]. In a previous study, Zhang et al. compared the efficiency of ddPCR and ARMS-qPCR in detecting *EGFR* mutations and found that ddPCR was able to detect plasmid samples with lower mutation rates than ARMS-qPCR, specifically citing the advantages of low DNA concentration requirement and the independence of C_t values [17]. This promises the ability for early diagnosis of acquired resistance to TKIs. In addition, the approval has been granted for investigating the clinical utility of ddPCR to detect *EGFR* mutations in plasma to evaluate the treatment response and predict drug resistance in advanced cases [18]. For comparison of sensitivity, ddPCR can detect *EGFR* mutations at a level of ~0.001% [3], which is far more sensitive than the threshold of direct sequencing (20%) [19], NGS (5%) [20], ARMS-qPCR (1%) [21] and scorpion ARMS (0.1%) [3]. Moreover, Chen et al. [5] quantified *EGFR* mutations in urine, blood and matched primary cancer samples with ddPCR before and after TKIs in 150 patients with an activating *EGRF* mutation. The data indicated that urinary ctDNA yielded a close agreement of 88% on detection of the *EGRF* mutation compared with the primary tissue at baseline. Additionally, analysis of urinary ctDNA showed a strong correlation between *EGFR* mutation and treatment efficacy at several post-treatment time points. On this basis, it is reasonable to conclude that ddPCR can be an alternative method for the non-invasive monitoring of TKI therapy through detecting *EGFR* mutation in urinary samples.

Cancer progression and treatment response monitoring

Cancer-associated gene mutations change in a dynamic manner with the progression of the disease progression and/or treatment. Therefore, we speculate that detection of gene mutations can be used to monitor the metastasis and/or relapse of cancer.

To assess the sensitivity and reproducibility of ddPCR in detecting plasma *KRAS G12V* mutation in colorectal cancer patients, Olmedillas et al. [6] showed that the copy numbers of wild-type *KRAS* and *KRAS G12V* mutation were remarkably elevated in plasma from colorectal cancer patients compared with healthy controls. Further, the mutant copy numbers were even higher in cases of metastasis. After taking into consideration the correlation between *KRAS G12V* mutation and poor prognosis, such as worse progression, high rate of post-operative complications and short survival time, it is desirable to detect *KRAS G12V* mutation in the plasma of colorectal patients by ddPCR. Thereafter, the mutation could be used as a non-invasive biomarker for disease progression monitoring in colorectal cancer. In the future, ddPCR can be developed in this field when considering the superior detection threshold of ddPCR for *KRAS* mutations in colorectal cancer (0.025%) [6] compared with Sanger sequencing (10–20%) [22], targeted NGS (1%) [23] and TagMeltPCR and High-resolution melt (HRM) (0.5%) [24].

In 2016, Takeshita et al. [7] demonstrated that the ratio of cfDNA *ESR1* mutations in estrogen receptor-positive breast cancer patients changed during treatment and that an increase in the frequency of cfDNA *ESR1* mutations was associated with a poor treatment outcome. Accordingly, ddPCR monitoring of recurrent *ESR1* mutations in cfDNA is a practical method to predict estrogen therapy response. Its higher sensitivity has been confirmed to have a detection limit of 0.05% [25] compared with 1% in multiplex allele-specific and real-time PCR [26] and 3.1% in NGS [27].

To date, ddPCR has been widely applied in the field of hematological malignancies. In 2016, Minervini et al. [28] revealed a higher incidence of *NOTCH1* mutations using ddPCR in chronic lymphocytic leukemia (CLL) rather than ARMS-qPCR as well as a lower detec-

Table. 1. Applications of dPCR in detecting cancer related gene mutations. Abbreviations: NSCLC: Non-small-cell lung cancer, FFEP: Formalin-fixed paraffin-embedded, se: sensitivity, sp: specificity, con: concordance rate, lim: limitation, ct DNA: circulating tumor DNA, CTCs: circulating tumor cells, LoD: limit of detection, sb: sensibility

Mutations	Diseases	Samples	Platforms	Relevant data	References
EGFR	NSCLC	Plasma	Bio-Rad QX100	Selective sensitivity: 0.04%, Se: 46-82%; sp: 90-99% vs tissue Con: 94% with tissue)	[20] [60]
EGFR T790M	NSCLC	FFEP	RainDance Technologies, Lexington, MA	Lim: 0.001%	[3]
EGFR	NSCLC	Plasmid and FFEP	Bio-Rad QX200	0.1-5% vs 1-5% (ARMS)	[17]
EGFR	NSCLC	Urine	Bio-Rad QX200	Con: 88% with tissue 98% with plasma ctDNA	[5]
EGFR	NSCLC	Pleural effusions and ascites fluid	Bio-Rad QX100	Con: higher than plasma (vs tissue)	[61]
EGFR	Leptomeningeal disease from metastatic NSCLC	CSF	Bio-Rad		[62]
KRAS G12V	Colorectal cancer	Plasma	Bio-Rad QX200	Lim: 0.025%	[6]
KRAS G12D		Stool	Bio-Rad		[63]
KRAS	NSCLC	Blood	Bio-Rad QX200	ctDNA (se: 78% sp: 100%) vs tissue CTCs (se: 34.3%; sp: 100%) vs tissue Total (se: 81%; sp:100%) vs tissue	[64]
KRAS	NSCLC	Urine	Bio-Rad	Con: 95% with tissue	[65]
KRAS	Pancreatic cancer	plasma	Bio-Rad	Lim: 0.01%	[66]
ESR1	Breast cancer	plasma	Bio-Rad QX200		[7]
ESR1	Breast cancer	Frozen tumor tissue(primary and metastases), Blood	Bio-Rad QX100	Lim: 0.05-0.16% (depend on mutation types)	[25]
PIK3CA	Breast cancer	Plasma	RainDance	Se: 93.3% Sp: 100% Accuracy: 96.7% vs FFPE	[67]
PIK3CA	Metastatic biliary cancers	Serum	Bio-Rad QX100	LoD: 0.1% Sb: at least 0.1%	[68]
TP53	Breast cancer	Plasma	Bio-Rad QX100	Sp: 100%	[69]
TP53 R114C	Glioblastoma, Medulloblastoma, brain metastases, leptomeningeal	CSF	Bio-Rad QX200		[70]
BRAF	Leptomeningeal disease from metastatic melanoma	Plasma	Bio-Rad		[71]
BRAF	Melanoma(V600E)	Plasma	Bio-Rad QX100	Sb: 0.005% Con: 84.3% with tissue	[72]
BRAF	HCL(V600E)	Bone marrow	Bio-Rad QX100	Sb: 0.005%	[30]
BRAF	Langerhans cell histiocytosis	Urine	RainDance	Threshold: 0.094% Se: 92.9%, Sp: 100% vs tissue	[73]
NARS	Early lung lung adenocarcinoma	Sputum	Bio-Rad	Lim 0.1%	[74]
NOTCH1	CLL	Blood	Bio-RadQX200	LoD: 0.03%	[28]
CALR (type1 and 2)	Myeloproliferative neoplasms	Blood	Bio-RadQX200	Sb: 0.01%	[32]
CALR type1 and 2	Myelofibrosis (CALR2+)		Duplex-dPCR assay (CALR type2-specific, new)	Sb: 0.02%	[34]
FGFR1	Brain tumor	FFEP	Bio-RadQX100		[14]
IDH1 A395	Glioma	Serum CSF (RNA)	Raindrop(ddPCR)		[11]
FOXL2 402C > G (C134W)	Adult granulosa cell tumor (AGCT)			Lim: 0.05%	[13]
ALK	Neuroblastoma	Serum and plasma	Bio-Rad QX100	F1174L(3520TC, 3522CA), R1275Q (mutations): Se: 100%, 85.7%, 92.3% Sp: 100%, 92.4%, 97.9% vs tissue	[75]

tion threshold with ddPCR (0.03%) than ARMS-qPCR (0.1%) and Sanger sequencing (10–20%). Using ddPCR, the *NOTCH1*^{mut} allelic burden in CLL has been shown to be reduced after treatment. However, *NOTCH1*^{mut} allelic burden was elevated in CLL relapses compared with cases with complete or partial remission (CR/PR) [28]. Thus, the clinical follow-up and disease monitoring in the "watch and wait" interval after chemotherapy in CLL could be accomplished more easily and accurately.

Minimal residual disease (MRD) refers to a remaining small number of cancer cells in patients during and/or after treatment, which is a commonly used term in hematological malignancies. MRD is the major cause of relapse in cancers [29]. Recently, ddPCR has been shown to have promise in MRD monitoring where assays with high sensitivity are

Table. 2. Applications of digital PCR in detecting human diseased associated gene mutation. Abbreviations: se: sensitivity, sp: specificity, con: lim: limitation, LoD: limit of detection, LoQ: limit of quantification. CV: Coefficient of Variance, NIPD: Non-invasive prenatal diagnosis

Fields	Mutations	Disease	Sample	Platform	Relevant data	Reference
Pathogens mutations	Mutations in Core a.a.70	HCV	Serum	Bio-RadQX100	LoD: 0.005% Sp: 100% Repeatability (CV): 0.04-1.76% for mutant (log copies/mL)	[36]
	Oseltamivir drug resistance mutation (SNP. p.H275Y; C-to-T transition)	influenza virus model of resistance to oseltamivir; clinical samples	An influenza virus	Bio-RadQX100/QX200	Lim: 0.1% SNP abundance	[37]
NIPD	Hemophilia mutations	hemophilia	Maternal plasma	BioMark System (Fluidigm)		[43]
	Common and rare deletions	Alpha thalassemia	DNA samples	Bio-RadQX100	Se: 1 ng	[45]
Mutations in mtDNA	Age-related mtDNA Mutations		Human brain tissue		Lim: 10^{-7} per genome (frequency)	[49]
	m.11778G > A	Leber's disease	Plasmid samples			[48]
Mutations in genome editing	Mediated gene disruption	HIV provirus	MegaTAL-treated cells containing plasmid-derived HIV	Bio-RadQX100	Threshold: 0.02% LoD: 1.06% LoQ: 2.19%, CV: 20%	[54]

urgently needed. For instance, Guerrini et al. [30] proposed that ddPCR would be applicable in the monitoring of MRD and follow-up of patients with Hairy cell leukemia, as it showed higher sensitivity in detecting *B-RAF* mutation compared with qPCR (0.005% vs. 0.025%). Moreover, the detection limit in *B-RAF* mutations was 0.0005% when ddPCR was combined with whole genome amplification [31]. Furthermore, Luisa et al. [32] developed a ddPCR assay for the absolute quantification of calreticulin (*CALR*) gene mutations ($CALR^{mut}$). This method provided a new protocol for the MRD monitoring of myeloproliferative neoplasms, with a sensitivity of 0.01% [33] and confirmed its utility in monitoring the $CALR^{mut}$ load of essential thrombocythemia (ET) and primary myelofibrosis (PMF). Badbaran et al. [34] developed a duplex-dPCR assay detecting the *CALR* type-2 mutation with a sensitivity of 0.02% and demonstrated its ability in the monitoring of deep molecular remission and MRD analysis in *CALR*2+ myelofibrosis patients after allogeneic stem cell transplantation. In Table 1, we summarize detailed data in the applications of dPCR for cancer-related gene mutations.

Pathogen mutations

Initially, dPCR was first used for quantification of pathogens such as HBV [35] in infectious disease. In recent years, an increasing number of studies have been carried out to focus on its application in detecting pathogenic mutations.

In 2014, Mukaide et al. [36] designed a ddPCR-based assay to detect HCV *core a.a.70* mutations in plasma samples from HCV-1b infected patients. The results showed that ddPCR was effective for quantifying mutations in polymorphic viral genomes (Table 2). Moreover, ddPCR was able to identify the mutations around the target points.

To assess the efficiency of dPCR for SNP detection, Whale et al. [37] compared dPCR with qPCR in detecting clinically relevant SNPs in an influenza virus model of resistance to Oseltamivir. The data showed that dPCR could identify samples with extremely low mutation concentrations (0.1%), which was superior to the threshold of qPCR (5%). This indicated that dPCR is useful for detecting rare drug-resistant sequence variants, which plays a vital role in guiding clinical research and patient management. Additionally, this approach could be used in drug resistance monitoring of numerous infectious diseases such as HIV and viral hepatitis and bacterial infectious diseases including tuberculosis and gonorrhea.

Prenatal diagnosis of genetic disease

Prenatal diagnosis is a subfield of clinical genetics and gynecology exemplifying the integration of theoretical and clinical medicine. Invasive prenatal diagnosis, such as amniocentesis and chorionic villus sampling, involves certain risks that inevitably cause psychological stress in parents. Currently, non-invasive prenatal tests (NIPTs), based on identification of cell-free fetal DNA (cffDNA) in maternal circulation, are commonly used in clinical practice [38, 39].

dPCR contributes to the prenatal diagnosis of monogenic disease [40]. When the mother carries mutations, qualitative analysis of the concerned mutations is not sufficient to determine the mutational status of the fetus, as it is difficult to distinguish fetal alleles from maternal DNA background in the presence of maternal and fetal DNA in the maternal plasma [39]. However, after pregnancy, the cffDNA is released into the maternal plasma DNA pool, causing a slight elevation of the ratio of mutant or wild-type DNA [41]. Compared with the traditional methods, ddPCR could more precisely detect the subtle difference of mutant DNA in maternal plasma before and after pregnancy. Based on the principle of dPCR, the digital relative mutation dosage (RMD) approach has been developed to discern the balance between the mutant and wild-type causative genes [42]. Subsequently, the RMD approach was utilized in the NIPT of β thalassemia [42] and hemophilia [43].

In 2015, Debrand et al. [44] used ddPCR to detect paternal *CFTR* mutations in cffDNA from the plasma of pregnant mothers from families known to carry various mutant *CFTR* alleles. The data showed that ddPCR could precisely recognize the *ΔF508-MUT CFTR* allele in cffDNA of all proband fetuses and exclude unaffected control fetuses with high sensitivity and cost-effectiveness. In 2016, Lee et al. detected both common and rare deletions in α thalassemia using ddPCR, which showed a detection limit of ~1 ng and a rapid detection of α thalassemia variants in a Malaysian population [45].

Mitochondrial DNA (mtDNA) mutations

mtDNA mutations are associated with many pathological processes. Clinical manifestation and disease progression may present upon the mutant mtDNA reaching a certain threshold [46]. However, detection of mutant mtDNA is still a challenge owing to heteroplasmy, which is defined as coexistence of mutant and wild-type mtDNA. Additionally, the diagnosis of mitochondrial disease remains difficult, as the symptoms are typically non-specific [47]. As the clinical manifestations and severity of mitochondrial-associated diseases are strongly determined by the level of mutant mtDNA, it is a fundamental requirement to develop methods for detecting mutations in heteroplasmic mtDNA with outstanding sensitivity and accuracy, which may contribute to the early diagnosis and dynamic monitoring of mutant mtDNA during disease progression [48].

In 2014, Taylor et al. [49] developed a new method known as Digital Deletion Detection (3D) based on ddPCR, which has the ability to precisely quantify deletions in mtDNA. The method was used to analyze the dynamic change of age-related mtDNA mutations in human brain. In the same year, Rebolledo-Jaramillo et al. [50] used ddPCR to validate heteroplasmy and confirmed mutations in mtDNA with reliable outcomes. In 2016, Sofronova et al. [48] combined ddPCR with allele-specific probes and specially designed primers to detect Leber's disease-related *m.11778G > A* mutation and optimized the process with lowered temperatures during the annealing step.

According to these data, ddPCR meets the requirements of data reproducibility and high sensitivity in detecting heteroplasmy. In addition, it contributes to the simultaneous detection and quantitative analysis of mtDNA mutations [48].

Mutations in genome editing

Genome editing, represented by technical systems such as TALEN and CRISPR, can precisely modify the genome in a targeted fashion. It has been rapidly adopted in research and treatment of several diseases including genetic diseases [51] and viral diseases [52]. These genome editing technologies heavily rely on the delivery of sequence-specific designer nucleases, which induce deletion, insertion or SNP mutations in target sequences. Surveyor or T7 endonuclease I mismatch cleavage assay (T7 MCA) are commonly used to quantify the mutation rate of samples processed by designer nucleases in order to assess the effects of genome editing [53]. However, these methods fail to meet the sensitivity requirements of quantitative screening. As an alternative method, next generation sequencing (NGS) is frequently used in the screening assay and currently serves as the gold standard in the field. However, this method is time-consuming and technically demanding [54].

ddPCR has been employed to detect endonuclease-mediated gene disruption in the HIV provirus, with higher accuracy (Table 2) than T7 MCA. In addition, it generated highly concordant results with clonal amplicon sequencing and NGS [54]. Moreover, ddPCR is relatively easy to perform and requires less time and equipment. It is hopeful that the application of ddPCR as an assessment method can contribute to optimize and accelerate the advancement of genome editing technology.

Advantages

The sequencing technique is regarded as the gold standard for the detection of mutations. However, its extensive application is limited because of its high cost and relatively low sensitivity, especially for the identification of rare mutations. Compared with the sequencing, dPCR is superior in sensitivity and feasibility. As the most common mutation-detecting technique, qPCR satisfies the requirements in most cases. However, its accuracy is not high owing to its semi-quantitative characteristics. In contrast, dPCR can accurately detect the copy number of target DNA independent of C_t values [17] and exhibits excellent sensitivity [8] and accuracy [54]. Additionally, dPCR reduces background fluorescence [55], which makes it less susceptible to inhibitors [56]. These characteristics suggest the promise of the application of dPCR in detecting rare mutations with precise quantification.

Technical limitations

Indeed, there are still some technical limitations that have to be overcome to finalize the application of dPCR in clinical practice. dPCR requires highly allele-specific probes to reduce cross-reactivity and false positives [57]. Studies with a large sample size are also required to cover the targeted mutations. These factors are significant in rare mutation detection. Moreover, some sources of bias and variance, such as DNA denaturation during partitioning, will result in separation of single strands to two different units, which consequently causes overestimation. Conversely, underestimation may be caused by the presence of factors such as "molecular dropout," template linkage and sample inhomogeneity, as well as partition volume variance.

Perspectives

Although dPCR is only used to detect mutations in known sequences, researchers have equipped it with co-amplification at lower denaturation temperature PCR (COLD-PCR),

another highly sensitive PCR method. Researchers have validated COLD-ddPCR in detecting multiple mutations in *TP53* and *EGFR* with limits as low as 0.2% [58]. Thus, COLD-ddPCR has been demonstrated to easily and rapidly detect multiple mutations and identify unknown variants in the target sequences. In 2016, Arbeithuber et al. [59] demonstrated that DNA repair enzymes could reduce the artificial mutations caused by long heat incubation and the subsequent error in ultrasensitive technologies such as ddPCR. This finding provides direction in optimizing dPCR performance.

Altogether, dPCR has been applied in many fields with high sensitivity and accuracy, which contributes to the detection of rare mutations under complex backgrounds. This will help to promote the development of personalized cancer treatment, drug resistance research, NIPD, mitochondrial disease management and gene-modifying techniques. We are confident that dPCR systems have promising prospects, as they will hopefully lead the frontier of gene research and can be adopted as routine clinical assays in the near future.

Abbreviations

dPCR (Digital polymerase chain reaction); qPCR (Real-time quantitative PCR qPCR); ddPCR (Droplet digital PCR); EGFR (Epidermal growth factor receptor); NSCLC (Non-small-cell lung cancer); cfDNA (Cell-free DNA); ctDNA (Circulating tumor DNA); AGCT (Adult granulosa cell tumor); FFEP (Formalin-fixed paraffin-embedded); DNT (Dysembryoplastic neuroepithelial tumors); LGNTs (Low grade neuroepithelial tumors); AMARS-qPCR (Amplification refractory mutation system-based PCR); CLL (Lymphocytic leukemia); MRD (Minimal residual disease); HCL (Hairy cell leukemia); SNP (Single nucleotide polymorphism); NIPT (Noninvasive prenatal tests); cffDNA (Cell-free fetal DNA); RMD (Relative mutation dosage); mtDNA (Mitochondrial DNA); T7 (MCA, T7 endonuclease I mismatch cleavage assay); NGS (Next generation sequencing); COLD-PCR (Co-amplification at lower denaturation temperature PCR).

Disclosure Statement

The authors confirm that there are no conflicts of interest.

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