Review Article
Principles of digital PCR and its applications in current obstetrical and gynecological diseases

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Abstract: Digital PCR (dPCR) is a revolutionary technique to precisely quantify nucleic acids. For its high sensitivity and specificity, this technique has been widely replicated worldwide. To verify its applicability, we reviewed all the related articles in PubMed database published before May 10, 2019. Original articles and reviews on the topics were selected. Entered key words included “digital PCR/dPCR”, “advantage”, “combined use”, “microfluidic chip”, “gynecological cancer/tumor”. We found that dPCR has shown great potential in clinical operations, like tumor liquid biopsy, non-invasive prenatal diagnosis, microorganism detection, and next-generation sequencing library quality-control.

Keywords: dPCR, advantages, combined use, microfluidic chip, clinical use, gynecological tumors

Introduction

Polymerase chain reaction (PCR) has been modified by three generations. The first generation of PCR relies on gel electrophoresis to analyze PCR products, but always challenged by low detection limit, laborious operation, and single application (qualitative). The second generation of PCR, also called real-time quantitative PCR (RT-qPCR), can quantify the products with standard curves, but also show low tolerance to interfering substances [1]. Digital PCR (dPCR) is the third generation of PCR that enables absolute quantification through partitioning the reaction. Highly sensitive and accurate in molecular detection, this technology has demonstrated applications like trace DNA detection, rare mutation detection and copy number variation [2].

History and advantages

History

In 1992, Sykes et al. detected the genes with mutated heavy chains through limiting dilution and end-point signals quantification, a method that lays out today’s dPCR [3]. In 1999, to measure the K-RAS mutation, Kenneth KZ and Bert VS carried out limiting dilution in increased reaction chambers; in this way, the error from body cells can be eliminated and trace K-RAS mutation detected. Therefore, dPCR, as a new technique, was first brought out. However, it still needs laborious manual dilution of DNA samples in which bias may arise. In 2003, Liu et al. reformed the technique by adding microfluidic elements. Micropumps and microvalves were introduced to partition liquids from DNA templates, thus increasing the accuracy of dilution and reducing the operation-derived error; but the technique was still restricted by expensive chips that can only be specifically manufactured [5]. In 2011, droplet digital PCR (ddPCR) was invented based on water-oil emulsion droplet technology in Quantalife Co, LTD. featuring high-throughput, automaticity, and cheapness. Since then, dPCR was ushered into clinical work.

Fundamental concept

In dPCR, the PCR system of DNA or RNA is separated into numerous small volume compart-
ments (nanometer precision), in which molecules are randomly distributed. Each compartment has zero, one or many molecules. After amplification, the absorbance in each compartment is counted [6]. A reaction with no target molecule is counted as a 0, and a reaction that has one target molecule is counted as a 1. The initial copy number and density of target DNA are calculated with Poisson statistics and the number of PCR-positive reactions. Theoretically, when the assay mixture has a low density of target DNA and the microreactions are large enough, only 0 or 1 should be counted. In this condition, the number of compartments showing bright fluorescence equals that of target DNA molecules. However, one compartment often contains more than one target molecule, the count of which should be corrected with Poisson statistics (Figure 1).

Advantages

Small volume of sample

dPCR still achieves high accuracy even though only a small volume of sample is available, especially in detecting hard-to-be-taken samples or the samples showing degraded nucleic acid. To provide a volume large enough, the clinical samples should be amplified when to carry out other genomic analyses, like comparative genomic hybridization (CGH) chip, next-generation sequencing (NGS), but dPCR can be performed based on a minimum of samples, reaping reliable results by eliminating the error generating from preamplification [8].

High sensitivity and absolute quantification

Compared to Real time fluorogenic quantitative PCR, dPCR is more sensitive and clinically applicable. In dPCR, the standard PCR system is accomplished by numerous microunits, which largely increases the tolerance of PCR system to inhibitors. The sensitivity of traditional PCR can reach 1%, but dPCR can achieve a sensitivity up to 0.1%, sometimes 0.001%, making it an ideal tool for trace DNA detection, rare mutation detection, especially circulating tumor DNA detection. Moreover, dPCR has been technologically modified into a tool more operable and compatible, and its two-dimensional data is highly readable [9] (Table 1).

Independence on amplification efficiency and high repeatability of results

dPCR can realize absolute quantification. Amplification refractory mutation system (ARMS) only provides qualitative results. The absolute or relative quantification of traditional PCR analyses is highly dependent on standard curves or reference genes. Therefore a 100% absolute quantification cannot be achieved. dPCR defines the amplification results with “1” and “0” and quantifies the results with counts (sometimes corrected with Poisson statistics), which makes absolute quantification possible [10] (Table 1).
### Table 1. Comparison of digital PCR and real-time fluorescence quantitative PCR

<table>
<thead>
<tr>
<th>Methods</th>
<th>Principle</th>
<th>Application</th>
<th>Advantages/disadvantages</th>
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<tbody>
<tr>
<td>Digital PCR</td>
<td>The PCR reaction system is divided into small volume compartments. After amplification, and the numbers of positive and negative reactions are counted to obtain the copy number of samples directly.</td>
<td>Copy number variation analysis; Absolute quantification of viral load; Detection of rare mutations; Quantification of gene expression; Second generation sequencing library analysis</td>
<td>Advantages: absolute quantification, no standard curve required, high sensitivity, better resistance to PCR inhibitors Disadvantages: narrow dynamic range and high cost</td>
</tr>
<tr>
<td>Real-time fluorescent quantitative PCR</td>
<td>The amount of amplified product is proportional to the fluorescence signal intensity, and the sample is quantified through using the reaction cycle threshold and standard curve.</td>
<td>Detection and quantification of pathogens; Relative detection of gene expression; Single nucleotide polymorphism analysis; Detection of tumor markers, etc</td>
<td>Advantages: wide dynamic range, wide application range, low cost Disadvantages: amplification efficiency is susceptible to PCR inhibitors</td>
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</table>
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*dPCR can retest the results of NGS*

Given that amplification efficiency is decided by various factors (like enzymes, primers and inhibitors), cycle threshold (Ct), a value essential for quantitative analysis, is unstable in traditional PCR; as a consequence, the assays of the same design may show poor repeatability, or even contradictory results. Unlike fluorogenic quantitative PCR, dPCR calculates the expression of nucleic acids with end-point signals, rather than Ct values; therefore, its results rule out the interference of unstable amplification and become highly repeatable with an error rate below 5% (*Table 1*). In dPCR, primers and probes are reduced, which lowers the mispairing of target sequences. dPCR can be combined with NGS to quantify and interpret the data. On the one side, dPCR can retest the results of NGS. On the other side, the quality of sequencing data can be guaranteed, including adaptors, Joint dimer, error link fragment, long fusion junctions. Together, dPCR suppresses former techniques in all aspects [11].

**Devices for dPCR**

According to the methods of separating reaction mixture, dPCR can be classified into chip dPCR (cdPCR) and droplet dPCR (ddPCR).

**cdPCR**

Depending on microfluidic technology, cdPCR involves the partitioning of reaction into nanoliter reaction chambers. After cycles of reaction, fluorescence is detected with an imaging system and an inverted endoscopy. Then the copy number of target sequence is calculated with imaging software. cDPCR may be based on integrated fluidic circuits, arrayed lipid bilayer chamber system (ALBiC) and nanoliter self-priming compartmentalization chip.

Integrated fluidic circuit is made from polydimethylsiloxane (PDMS) with multiplayer soft lithography (MSL). Fluid lines, valves and reaction chambers are integrated into a single chip. Monitoring valves, the reaction can be partitioned into numerous separate microunits [12]. In ALBiC, the reaction microchambers are arrayed on the chip that allows the reaction to directly flow into through an orifice [13].

The self-priming compartmentalization chip contains two glass coverslips. Sliping these overslips can partition the reaction into microunits [14]. The droplets separated by cdPCR are even in volume, free of solvent evaporation, and stable. BioMark and QuantStudio3D are two merchandized cdPCR systems. BioMark uses integrated fluidic circuits harboring 10000 to 40000 microchambers. QuantStudio3D uses integrated chip containing 20000 microchambers [15]. The workflow of cdPCR is shown in *Figure 2*.

**Droplets of ddPCR**

ddPCR creates microreaction units with water-in-oil and microfluidic technology. The nucleic acids are randomly subdivided into water-in-oil droplets that undergo PCR separately. Then a two-color optical detection system is used to read the signals in each droplet. The chips have shapes of “+”, “T” and “Y” [16-18]. The workflow of ddPCR is shown in *Figure 3*.
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The droplets of ddPCR are created by a layer (micrometer precision) which has a cost lower than cdPCR. However the droplets of ddPCR are not even in volume. Therefore, commercialized ddPCR introduces standardized procedures. The droplets with similar volumes are selected for fluorescence detection. The results are then adjusted with Poisson statistics. QX100/200 and Rain Dro are two ddPCR vendors. The former can divide each reaction mixture into 20000 droplets and the later into 1000000 to 10000000 droplets [19].

Clinical use

Besides its advantages over other techniques, dPCR is highly efficient for the detection of low-abundance nucleic acids in 1) non-invasive prenatal testing, 2) tumor diagnosis, 3) pathogen-containing samples (like HIV detection) [20, 21].

Non-invasive prenatal testing

Prenatal testing plays a key part in the secondary prevention of birth defects. Traditional prenatal diagnosis is performed through amniocentesis and chorionic villus sampling (CVS), both being invasive and occasionally abortion-causative. In 1997, cell-free fetal DNA (cffDNA) was discovered in maternal plasma, heralding a new era of non-invasive prenatal testing (NIPT) [22]. However, the level of cffDNA in maternal plasma is extremely low, only making up 10-20% of all plasma proteins [23]. Therefore, only highly sensitive molecular diagnostic tool can trap the cffDNA sparsely suspended in maternal plasma. dPCR makes it possible. With dPCR, cffDNA can be precisely quantified, through which genetic diseases can be prenatally predicted at the genetic level. Prenatal testing usually covers chromosome aneuploids, single gene inheritance disease, and autosomal recessive disorder.

El Khattabi et al. combined dPCR and hydrolysis probes to quantify plasma circular DNA in 213 pregnant women, concluding that dPCR can diagnose trisomy 21 syndrome non-invasively [24]. Barrett et al. evaluated the possibility of dPCR to detect sickle cell disease (SCD), finding that 82% of male fetuses and 75%

Figure 3. Overview of droplet digital PCR workflow. HU SH, et al. The Principle of Digital PCR and Its Applications in Current Molecular Diagnosis [8] (A) ddPCR reactions are prepared in a tube. (B) The ddPCR reactions are partitioned into individual droplet by droplets generator. (C) The ddPCR reactions in droplets are transferred to a 96-well plate for PCR amplification. (D) Droplets with green or blue color were assigned as positive reaction. Droplets in gray color are assigned as negative. (E) Fluorescence is measured for each droplet by two channels. (F) An example of ddPCR resultes in two fluorescent probes.
female fetuses were diagnosed with SCD by dPCR using DYS14, the specific marker of Y chromosome marker [25]. SMN1 and SMN2 at 5q13 are pathogenic factors for spinal muscular atrophy (SMA). To be specific, the copy number of SMN2 can be used as a diagnostic indicator. Stabley et al. found that dPCR could detect 0-3 copies of SMN1 and 0-5 copies of SMN2, with a CV of 1.7-3.7% and 2.1-2.7%, respectively. Besides, the lower limit and variation degree were much lower than those of RT-PCR [26]. In 2018, Pei WX et al. detected the paternal pathogenic gene loci in circulating fetal nucleic acid and amniotic fluid DNA using ddPCR. Meanwhile, they confirmed the paternal pathogenic gene loci in amniotic fluid DNA using Sanger sequencing, which demonstrated the same results as in ddPCR detection. This evidenced that ddPCR could qualitatively analyze small samples and provide effective, quick and safe prenatal diagnosis of single gene inheritance disease by testing the circulating fetal nucleic acid [27]. dPCR shows a higher accuracy than the routinely used prenatal screening techniques, therefore, it has great diagnostic potentials in NIPT (Table 2).

**Chromosomal aneuploidy detection**

When using NIPT to detect aneuploidy disorders, its efficiency is restricted by the low level of cffDNA and confounded by irrelevant maternal DNA. Comparatively, dPCR shows a stable and high efficiency since it can directly count target molecules. Fan et al. found that the sensitivity of cdPCR was much higher than that of RT-PCR and fluorogenic quantitative PCR in detecting the fetuses with trisomy 21 syndrome. Through comparing the amyloid protein sequences on chromosome 21 and the copy numbers of GAPDH on chromosome 12, cdPCR accurately screened out the fetus with trisomy 21 syndrome, even when the cffDNA contributed 10% of the total cell-free DNA in plasma [28]. Tong et al. combined dPCR with comparative analysis of maternal and fetal methylation; using quantified expression of ZFY gene on chromosome Y as internal reference, their epigenetic-genetic chromosome-dosage approach successfully screened out five plasma samples with trisomy 21 syndrome [29].

Using chromosome 1 as reference and relative quantification as a method, Lo et al. detected the aneuploidies, but their experiment showed efficiency only when cffDNA made up 20% of total cell-free DNA in plasma [30]. However, given the fact that cffDNA only contributes to 6-10% of the total and no method is available to enrich cffDNA, their method cannot be introduced into the clinical practice [31]. Using dPCR, TSUI et al. quantified the expression of single nucleotide polymorphisms (SNPs) and PLAC4 in cffDNA, finding out four cases of trisomy 21 syndrome, with a sensitivity of 100% and specificity of 89% [32] (Table 2). Recently, Tan et al. developed a simple but effective NIPT method to detect chromosome aneuploidy based on the high sensitivity of dPCR in single molecule detection and its power in absolute quantification. Using this new method, the maternal peripheral blood sample was tested and the results were obtained within 4.5 h. Then using the results of 60 clinical samples, the accuracy and clinical

### Table 2. dPCR for prenatal diagnosis diseases

<table>
<thead>
<tr>
<th>Diseases</th>
<th>Target genes</th>
<th>Roles</th>
<th>Reference</th>
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<td>Sickle cell disease</td>
<td>Diagnose trisomy 21 syndrome non-invasively</td>
<td>Finding that 82% of male fetuses and 75% female fetuses were diagnosed with SCD by dPCR using DYS14, the specific marker of Y chromosome marker</td>
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</tr>
<tr>
<td>Spinal muscular atrophy</td>
<td>SMN1 and SMN2</td>
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SCD, sickle cell disease; SMA, spinal muscular atrophy; DPCR, digital PCR; DYS14, a specific marker for Y chromosome; cffDNA, cell-free fetal DNA; SNPs, single nucleotide polymorphisms.
applicability of this new NIPT method was evaluated, which demonstrated a 100% concordance rate as compared to NGS [33].

**Autosomal recessive disorder**

In 2008, it was reported that based on relative mutation dosage and digital size selection, dPCR could detect β-thalassemia; in the case of paternal and maternal mutation, quantifying maternal mutated DNA and fetal mutated DNA can help diagnose the fetus inheriting pathogenic genes [34, 35]. In 2012, based on paternal haplotypes and reformed relative mutation dosage, Lam et al. designed the probes for globins and their external SNP to pinpoint the site of interest; besides, based on SNPs divided into α and β groups, dPCR was performed to identify the genotype of the fetus; consequently, two families of β-thalassemia were screened out [36]. Also in 2012, using DYS14 (a specific marker for Y chromosome), dPCR detected 82% of male fetuses and 75% of female fetuses with SCD, indicating its precision high enough to overcome the challenge of low cffDNA and high-level maternal DNA [37]. Pornprasert et al. designed two pairs of primers to amplify wild-type α-globin and SEA genes, their ddPCR identified 15 carriers of SEA-deletion thalassemia and 8 cases of severe α-thalassemia, which verified the diagnostic efficiency of ddPCR in thalassemia [38].

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**Gynecological tumor**

ddPCR, as a high-throughput and low-cost absolute quantification technique, can be used to screen amplified HER2 gene. It has shown a high concordance with the traditional techniques. ddPCR can detect the absolute copy number of HER2 in invasive breast cancer, far outdoing semiquantitative PCR, second-generation sequencing, and hybrid chip technologies. The concordance reaches 93.9% (31/33) in the positive results and 100% (77/77) in the negative results of ddPCR and IHC+FISH test. HER2 copy number is associated with negative ER/PR, over expressed Ki-67 and high-grade histology. Therefore, a higher ddPCR value can indicate a higher malignancy and a poorer prognosis. When the copy number of each cell exceeds 3.2, the patient should be recommended to take HER2-targeted therapy [43] (Table 3).

**HR-positive breast cancer**

Currently, two-thirds of invasive breast cancers can be classified as Luminal A (or B) breast cancer that is HR-positive. Given the absence of independent subtype-based research, we just reviewed the use of ddPCR in HR-positive breast cancer. ddPCR has mainly been used to evaluate the drug resistance in endocrine therapy (ET) for this disease. The cancer can become resistant to estrogenic agents repeatedly used in ET. In recent years, estrogen receptor 1 (ESR1) activating mutations have slipped into the research focus. NGS has found that more than 80% of ESR1 mutations are enriched on its tyrosine 537 and aspartic acid 538 (ESR1 Y537S, Y537N, Y537C, D538G) and associated with ET resistance [44-46]. When using these sites as markers of ET resistance, ESR1 should be first genotyped. cfDNA analysis can overcome the disadvantages of core needle biopsy. In cfDNA analysis, mutation frequency can be detected in an extremely low
amount of cfDNA. In 10-30% of ET-resistant and HR-positive invasive breast cancer, NGS has detected the enrichment of ESR1-LBD gene [47, 48] (Table 3).

ESR1 mutation frequency is low in primary breast tumor (PBT). Based on cfDNA, ddPCR can be used to detect the CNVs of HER2 gene. Using ddPCR and a cutoff of 1.25, Gevensleben et al. screened out 7 HER2-positive samples in 11 FISH+IHC verified HER2-positive samples (64% detection rate) and 44 HER2-negative samples in 11 FISH+IHC verified HER2-negative samples (94% detection rate). The samples were collected from PBT. The three false positive results might be caused by the loss of some reference genes or the amplification of HER2 after tumor metastasis. The three false negative results might indicate the changed status of HER2, since all the false negative patients had received anti-HER2 therapy in which the tumor became heterogeneous and HER2 were not amplified [49].

Using a cutoff of 2.0, Garcia-Murillas et al. filtered out 18 HER2-positive samples in 18 FISH+IHC verified HER2-positive samples (100% detection rate) and 57 HER2-negative samples in 58 FISH+IHC verified HER2-negative samples (98% detection rate, 100% sensitivity, 98% specificity) [50]. It has been theoretically proven that ddPCR can be used to precisely detect the CNVs of HER2, with a great prospect in clinical use. A recent study detected the status of HER2 in the cfDNA of gastric cancer (preoperative, postoperative, and recurrent) [51]. The effectiveness of trastuzumab therapy showed temporal and spatial heterogeneity. During the treatment, genetic differentiation or chemotherapeutics use may change the profile of HER2, which may trouble the making of treatment strategy. In this case, ddPCR that has shown its usefulness in real-time detection of breast cancer HER2 may be introduced. In 2016, Takeshita et al. demonstrated that the ratio of cfDNA ESR1 mutations in estrogen receptor-positive breast cancer patients changed during treatment and that an increased frequency of cfDNA ESR1 mutations was associated with a poor treatment outcome [52] (Table 3). Accordingly, using ddPCR to monitor recurrent ESR1 mutations in cfDNA may predict estrogen therapy response. With a very high sensitivity, its detection limit could reach 0.05%, compared with 1% in multiplex allele-specific and real-time PCR [53] and 3.1% in NGS [54].

Table 3. dPCR for Gynecological tumor diseases

<table>
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<th>Roles</th>
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<td>Invasive breast cancers</td>
<td>HER2 gene</td>
<td>The concordance reaches 93.9% (31/33) in the positive results and 100% (77/77) in the negative results between ddPCR and IHC+FISH test</td>
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<tr>
<td>Triple negative breast cancer</td>
<td>PIK3CA</td>
<td>PIK3CA mutation, just next to TP53 mutation, appears in 20-40% of breast cancers, it is of great value to uncover the effect of PIK3CA mutation on TNBC prognosis and AR pathway</td>
<td>[61]</td>
</tr>
<tr>
<td>Ovarian cancer</td>
<td>MACC1 and S100A4</td>
<td>MACC1 and S100A4 transcripts were significantly elevated in serum of ovarian cancer patients, compared to healthy controls</td>
<td>[69]</td>
</tr>
</tbody>
</table>

ESR1, estrogen receptor 1; PIK3CA, phosphatidylinositol 3-kinase; MACC1, metastasis-associated in colon cancer 1; S100A4, 100 calcium-binding protein A4.
Recently, Dr. Sara Tolaney of Dana-Farber Cancer Institute and her colleagues have utilized ddPCR to figure out the benefits of drug-resistant mutation in ctDNA of estrogen receptor-positive metastatic breast cancer patients. Her team detected the mutation of two drug-resistant-related genes (PIK3CA and ESR1). A total of 334 plasma samples and 434 tissue samples was collected from 669 patients who were treated with either fulvestrant or fulvestrant combined with abemaciclib. The study found the PIK3CA and ESR1 mutations in ctDNA was associated with the patients’ responses to abemaciclib. In contrast, the association was not found in the tissue samples. They also showed that although abemaciclib worked in all patients, the patients with PIK3CA and ESR1 mutations benefited more from the therapy. These results suggest that ctDNA and fluid biopsy can identify the molecular changes [55].

**Triple negative breast cancer**

TNBCs comprise a group of highly heterogeneous cancers. The subtypes, targets and mutants in TNBC are mainly detected according to molecular pathology and gene expression profiles [56, 57]. Phosphatidylinositol 3-kinase (PIK3CA), a research focus, encodes p110 complex in PI3K signaling pathway. PIK3CA mutation, just next to TP53 mutation, appears in 20-40% of breast cancers [58]. PIK3CA mutation varies among breast cancers with different receptors [59]. A study has verified that PIK3CA mutation is of limited significance in predicting the prognosis of HR-positive breast cancer [60]. The predictive value of PIK3CA in TNBC has been rarely reported.

Androgen receptor (AR) is involved in PI3Ks pathway and PIK3CA mutation in TNBC, but the AR’s association with TNBC prognosis remains unclear. Therefore, it is of great value to uncover the effect of PIK3CA mutation on TNBC prognosis and AR pathway [61] (Table 3). Using ddPCR, Takeshita et al. analyzed the mutation frequency of PIK3CA in 49 blood samples and 42 paired cancer samples, finding not interaction in between [62]. But this finding does not underestimate the accuracy of ddPCR: the cancer shows high heterogeneity; ESR1 mutation frequency difference between cfDNA and cancer samples may arise from this heterogeneity [63-65].

Recently, Dr. Carausu M of Institut Curie et al. conducted a research on the late-stage breast cancer to evaluate the effectiveness of changing treatment when ctDNA mutations were detected in patients. This phase III clinical trial was conducted in 80 plus cancer centers in France, recruiting 1,000 HER2-negative metastatic breast cancer patients being treated with endocrinotherapy. The researchers detected drug-resistance-related EST1 mutation during endocrinotherapy using the fluid biopsy analysis based on ddPCR. Though the research is on-going, the preliminary results have shown ddPCR could rapidly detect ESR1 mutation. ddPCR is a cost-effective solution for the researchers to perform real-time tracking of ESR1 mutation in thousands of ctDNA samples [66].

**Cervical cancer**

Detecting circulating tumour cells (CTC) in cancer patients can be used to evaluate the efficacy of therapy and predict the relapse of cancer. A sensitive assay based on HPV-oncogene transcripts highly specific for cervical cancer cells was established. The Digital-Direct-RT-PCR (DD-RT-PCR) combines ficoll-separation, thinPrep-fixation and one-step RT-PCR in a low-throughput digital-PCR format enabling the direct analysis and detection of individual CTC without RNA isolation. Experimental samples demonstrated a sensitivity of one HPV-positive cell in 500,000 HPV-negative cells. Spike-in experiments with down to 5 HPV-positive cells per millilitre EDTA-blood showed positive results with those of PCR and immunocytochemistry. Each blood sample from 3 out of 10 CxCa patients contained a single HPV-oncogene transcript expressing CTC among 5 to 15*10(5) MNBC. Only 1 of 7 patients with local disease and 2 of 3 women with systemic disease found to have CTC. This highly sensitive DD-RT-PCR for CTC detection may also be applied to other tumour entities which express tumour-specific transcripts [67].

In 2017, Carow K et al. compared two quantification methods: digital PCR and standard quantitative PCR. Serial dilutions of 5 ng-5 pg RNA (± 500-0.5 cells) of the cervical cancer cell
line SiHa were prepared in 5 µg of RNA of the HPV-negative human keratinocyte cell line HaCaT. Clinical samples of 10 sentinel lymph nodes with varying HPV transcript levels were also used. Reverse transcription of total RNA (5 µg of RNA each) was performed in 100 µl and cDNA aliquots were analyzed by qPCR and dPCR. Digital PCR was run in the RainDrop® Digital PCR system using a probe-based detection of HPV E6/E7 cDNA PCR products with 11 µl of template. qPCR was performed on a Rotor Gene Q 5plex HRM (Qiagen) amplifying HPV E6/E7 cDNA in a SYBR Green format with 1 µl template. For the analysis of both clinical and serial dilution samples, dPCR and qPCR showed comparable sensitivity. With regard to reproducibility, the two methods differed considerably, especially for low template samples. Here, Katrin Carow et al. found a mean variation coefficient of 126% using qPCR whereas dPCR enabled a significantly lower mean variation coefficient of 40%. Generally, dPCR showed a substantial reduction of subsampling errors, reflecting the large amount of cDNA available for analysis [68].

**Ovarian cancer**

For the first time, T Link et al. systematically detected circulating serum levels of MACC1 and S100A4 transcripts in surgery and chemotherapy and analyzed their clinical relevance with ovarian cancer. MACC1 and S100A4 transcripts from 318 serum samples of 79 ovarian cancer patients were quantified by RT-qPCR and ddPCR, respectively. Compared to healthy controls, MACC1 and S100A4 transcripts in serum of ovarian cancer patients were significantly elevated. At primary diagnosis, high levels of MACC1 or S100A4 were correlated with advanced FIGO stage, and therefore could be used to predict the shorter progression-free survival and overall survival after suboptimal debulking surgery. This study of T Link et al. is the first to propose circulating MACC1 and S100A4 transcripts as potential liquid biopsy markers of ovarian cancer [69] (Table 3).

**Prospect**

As a single-molecule-amplification technique with high signal/noise ratio and sensitivity, dPCR can be used to detect low-level DNA [70]. But limitations still exist. The sample volume in each microunit controls the lower limit. The dynamic range becomes narrower since the number of microunits is limited. In these microunits, not all the DNA is amplified, leading to continuous signals and false low-level quantification, especially in RNA PCR in which the reverse transcription of the targets may be incomplete and not all the copies are detected.

Besides, dPCR is not suitable for large amplicons. Low throughput of extraction and high risk of sample contamination are obstacles that dPCR must overcome. The current dPCR systems can only detect two colors and cannot carry out multiplex detection for multiple targets in one sample. Therefore, the internal reference should be molecules with complex dyes of different intensities. The dPCR reaction is still designed according to the parameters of the devices, producing great difference in the assay outcomes [71]. In addition, cdPCR is challenged by high cost and low throughput. ddPCR becomes unstable in detecting low-copy-number samples and have to be repeated to get the qualified data. Furthermore, no protocols have been established to guide dPCR in detecting fetal chromosomal abnormality and monogenic disorder. Its efficiency should be validated with larger-cases analysis. In summary, dPCR is being transformed and widely used in obstetrics.

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**Disclosure of conflict of interest**

None.

**Abbreviations**

CdPCR, Chip digital PCR; qPCR, Real-time quantitative PCR; NGS, Next generation sequencing; ddPCR, Droplet digital PCR; CffDNA, Cell-free fetal DNA; NIPT, Noninvasive prenatal testing; PDMS, Polymethylsiloxane; SCD, Sickle cell disease; HR, Hormone receptor; ESR1, Estrogen receptor 1; PI3KS, Phosphatidylinositol 3 kinases; TNBC, Triple negative breast cancer; CGH, Comparative genomic hybridization; MA-
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CC1, Metastasis-associated in colon cancer 1; S100A4, 100 calcium-binding protein A4.

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