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## Digital PCR: from early developments to its future application in clinics

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Digital PCR (dPCR) is the third generation of PCR technology, after conventional PCR and real-time quantitative PCR. It is based on the partitioning of a PCR mixture supplemented with the sample to analyse into a large number of parallel reactions, so that each partition contains either 0, 1 or a few nucleic acid targets, according to a Poisson distribution. Following PCR amplification, the fraction of positive partitions is extracted from an end-point measurement, allowing the computation of the target concentration. This calibration-free technology presents powerful advantages including high sensitivity, absolute quantification, high accuracy and reproducibility as well as rapid turnaround time and has therefore rapidly spread. Digital PCR offers a wide range of applications in research, clinical diagnostics, and biotechnology. Among the first clinically relevant applications of dPCR was its ability to detect rare genetic mutations within a background of wild-type genes. This breakthrough paved the way to tumour heterogeneity analysis in oncology and enabled liquid biopsy applications, such as the monitoring of treatment response. The scope of dPCR applications has since rapidly extended to include prenatal diagnosis through the detection of aneuploidy or inherited mutations, as well as pathogen identification *via* the detection of virus-specific genes or antibiotic-resistance genes in bacteria. This review focuses on the clinical applications of dPCR, highlighting its advantages over existing technologies and providing an outlook on future developments.

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### I. Introduction to dPCR

Modern medicine requires precise and sensitive techniques for disease diagnosis and patient follow-up. The pathologies should be detected and identified at the earliest to increase the chances for finding a cure. Historically, infectious diseases were diagnosed with serological tests for antibody or antigen detection, or with sample culture for bacteria identification. Although they are easy to perform, widely standardised and inexpensive, these tests can be time consuming and exhibit low sensitivity. The COVID-19 pandemic has emphasised the urgent need for highly sensitive and accurate detection methods.<sup>1</sup>

#### 1. History and principle of dPCR

In 1986, Kary Mullis invented the polymerase chain reaction (PCR), a technique that would become the gold standard for

nucleic acid detection.<sup>2</sup> This molecular biology method enables the exponential replication of specific DNA sequences, through a mix of – at least – two synthetic target-specific oligonucleotides (primers), a thermostable DNA-replicative enzyme (DNA polymerase) and deoxyribonucleotide triphosphate monomers (dNTP).<sup>2</sup> In its initial development, the product of the amplification reaction was analysed by gel electrophoresis, providing semi-quantitative information based on band intensity. In 1992, Russel Higuchi developed the second-generation PCR, the quantitative PCR (qPCR, also known as real-time PCR), where the amplification reaction is monitored in real-time using for example a fluorescent DNA-intercalating dye or specific fluorescent probes (TaqMan probes or molecular beacons).<sup>3</sup> From the fluorescence signal, the amplification time (*i.e.* the cycle at which the fluorescence crosses a given threshold) is extracted and compared to standard samples of known concentration, allowing for a relative quantification.

In a precursor work from 1989, Peter Simmonds used limiting dilution PCR to detect single copies of HIV provirus in infected cells and concluded that the disease stage correlates with the proportion of infected Peripheral Blood Mononuclear Cells (PBMC, ranging from 1 per 5000 to 80 000 cells for asymptomatic patients to 1 per 700 to 3300 cells for late stage/stage IV patients).<sup>4</sup> Three years later, Morley and Sykes combined limiting dilution PCR with Poisson statistics

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to isolate, detect and quantify single nucleic acid molecules, laying the foundations of digital PCR:<sup>5</sup> in their study, sample dilutions were replicated, PCR-amplified and analysed by gel electrophoresis, enabling an accurate count of target molecules based on the fraction of negative partitions. The authors successfully detected, within bone marrow samples of leukemia patients, mutated *IgH* rearranged heavy chain gene as low as 2 targets in 160 000 wild-type sequences. In 1999, the term digital PCR, the third and latest PCR generation, was coined by Bert Vogelstein and collaborators (see Fig. 1), who developed a workflow involving limiting dilution distributed on 96-well plates combined with a fluorescence readout to detect mutations of *RAS* oncogene in the stools of patients with colorectal cancer.<sup>6</sup>

The technology of dPCR was born, but the need for microtiter plates limited its practicability and some improvements were therefore needed. In 1997, Olga Kalinina and collaborators introduced volume miniaturisation by using microcapillaries (~10 nL) for the partition process, which reduced the cost of reagents and improved the amplification efficiency.<sup>7</sup> In 2003, Bert Vogelstein *et al.* reported the BEAMing (beads, emulsion, amplification and magnetics) technology,<sup>8–10</sup> further simplifying the compartmentalisation process by utilising water-in-oil droplets parallelising PCR. The method involved encapsulating individual DNA molecules with magnetic beads coated with primers, permitting PCR amplification within the droplet. The amplified products were then recovered magnetically and analysed by flow cytometry using DNA probes and/or immunostaining. Some derived protocols of BEAMing replaced the flow cytometry analysis by the imaging of planar arrays of hydrogel beads.<sup>11</sup> This adaptation has been used to detect early-stage colorectal cancer by assessing oncogene expression in tissue and stool samples.<sup>12</sup>

Modern dPCR protocols are built upon those foundational principles and generally follow four key steps: i) partitioning the PCR mixture that contains the sample into thousands to millions of compartments. This step implies the random distribution of the targets among the partitions; ii) amplifying individual target-containing partitions; iii) performing end-point fluorescence analysis of the partitions; iv) computing the target concentration using Poisson statistics, based on the fraction of positive and negative partitions (see Fig. 2). This provides PCR with high sensitivity and calibration-free absolute quantification<sup>13</sup> owing to the single-molecule detection attribute.<sup>13</sup> For the past decades, two major types of partitioning methods have emerged: water-in-oil droplet emulsification and microchambers.

In droplet digital PCR (ddPCR), the sample is dispersed into tiny (pL to nL) droplets within an immiscible oil phase. Monodisperse droplets can be generated at high speed (typically 1–100 kHz) using a microfluidic chip leveraging passive forces or actively breaking the aqueous/oil interface (for an exhaustive review on microfluidic designs for droplet generation, see Xu *et al.*<sup>14</sup>). It is to be noted that water-in-oil droplets are prone to coalescence (especially during the harsh temperature variation of the PCR protocol) and their stabilisation with an appropriate surfactant is of prime importance.<sup>15</sup>

Microchamber-based dPCR uses an array of thousands of microscopic wells or chambers embedded in a solid chip. While ddPCR offers greater scalability and cost-effectiveness, it requires precise emulsification and droplet stability. On the other hand, microchamber dPCR provides higher reproducibility and ease of automation but is limited by the fixed number of partitions and typically higher costs.

As for the droplet-signal reading technology, again, two primary readout methods are available: in-line detection and

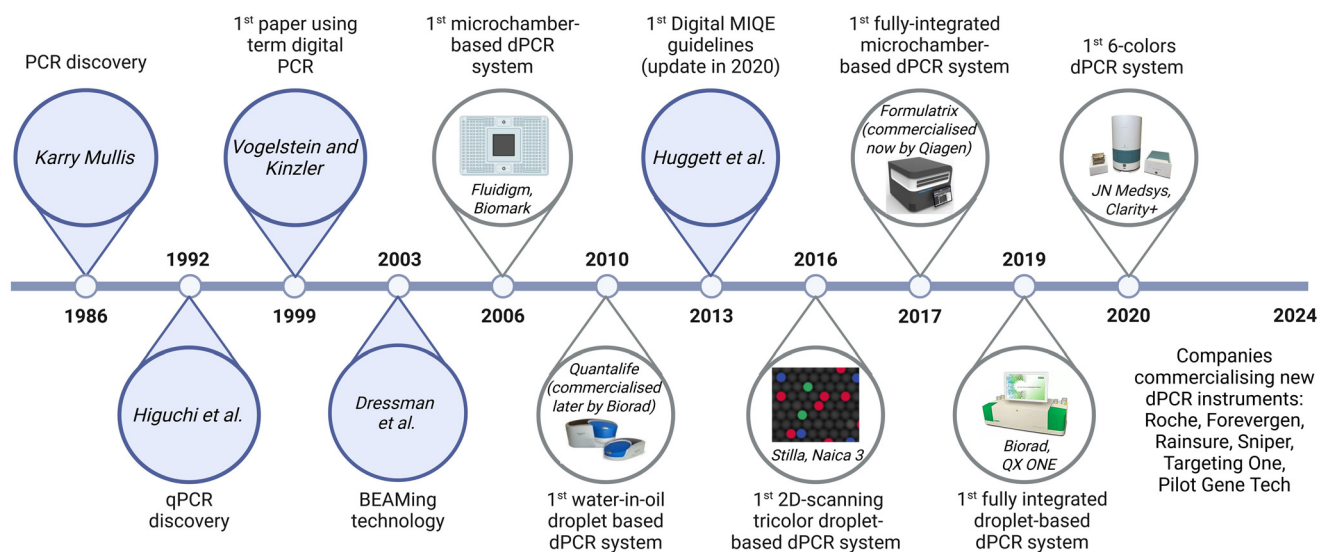


Fig. 1 Schematic chronology of dPCR focused on historical works and commercial developments. Created with <https://Biorender.com>. References cited are Mullis *et al.*,<sup>2</sup> Higuchi *et al.*,<sup>3</sup> Vogelstein *et al.*,<sup>6</sup> Dressman *et al.*,<sup>8</sup> Huggett *et al.*<sup>390</sup>



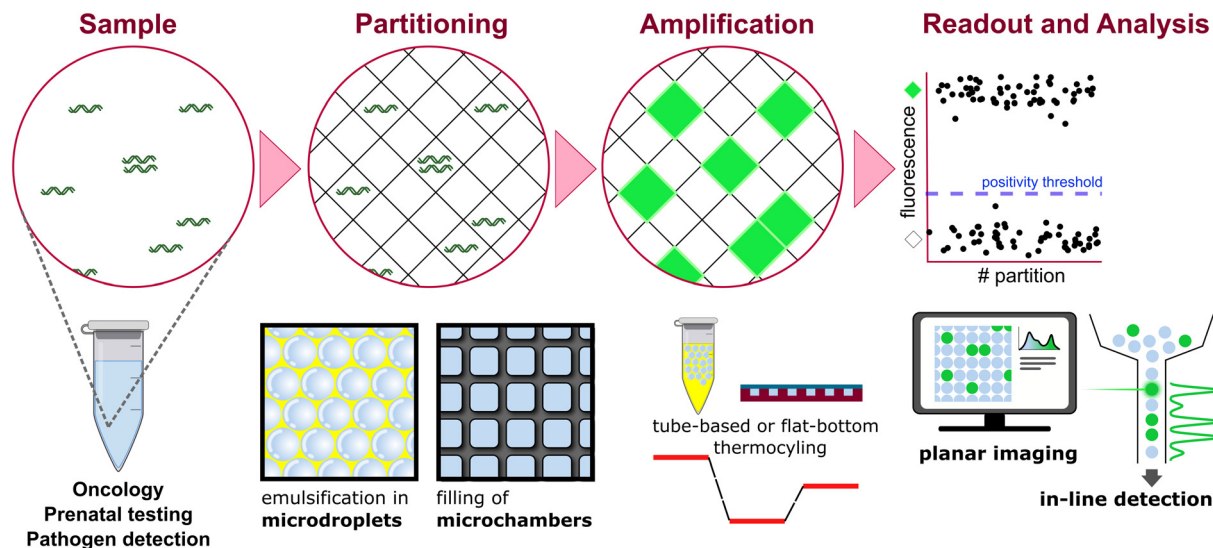


Fig. 2 Principle of dPCR based on limited dilution, distribution in partitions, amplification, fluorescence detection and data analysis.

planar imaging. In in-line detection, commonly used in ddPCR, the droplets are flowed through a microfluidic channel or capillary and their fluorescence is measured one by one using a light source coupled to detectors. This allows the analysis of a large number of droplets but requires precise control of the flow. In contrast, planar arrays of microchambers or microdroplets can be imaged using a fluorescence microscope or scanner and provide a static snapshot of the partitions. Note that 3D imaging<sup>16</sup> and analysis<sup>17</sup> techniques have been developed to assay in a shorter time a larger number of droplets.

## 2. A path towards commercialisation of dPCR platforms

The rise of dPCR has been driven by significant advances in microfabrication and microfluidics, expanding the possibilities for volume miniaturisation.<sup>18,19</sup> This progress has led to the development of various dPCR techniques, ranging from 96-well plate-compatible protocols<sup>20</sup> to sophisticated lab-on-chip prototypes, potentially suitable for commercialisation.

One notable example is Slip Chip, a microfabricated chip composed of a bottom plate with microchambers filled with PCR solution. This chip slides under a top plate which contains the samples, enabling interaction and amplification with an end-point analysis under a fluorescence microscope.<sup>21</sup> Another innovative system, the spinning disk, uses centrifugation to separate the sample into nanoliter wells for an end-point fluorescent analysis.<sup>22</sup> Although these systems are technologically advanced, they remain mainly used as laboratory prototypes.

In contrast, the first compartment-based dPCR nanofluidic platform was commercialised by Fluidigm in 2006. It is composed of an integrated fluidic controller (IFC) that loads the samples automatically into microchambers using on-chip valves; a fluorescence analyser with or without

an integrated thermocycler – that allows real-time PCR (Biomark) or endpoint (EP1) analysis respectively. Although no longer commercially available, this platform was proven to be efficient for the detection of bacterial signatures,<sup>23</sup> for the measurement of gene expression in tissues,<sup>24</sup> or gene copy numbers in breast cancer samples.<sup>25</sup> The next significant commercial dPCR system was the Quantstudio 3D (QS3D), marketed by Applied Biosystem in 2013. Originally developed as the Open Array Platform by BioTrove, it was acquired by Life Technologies in 2009 and replaced by Absolute Q in 2022. In 2013, Formulatrix introduced its Constellation dPCR instrument. The company was bought by Qiagen in 2019 and the same instrument was renamed QIAcuity in 2020. The system developed by Roche (Digital LightCycler) followed in 2022 (see Table 1).

In droplet-based dPCR, laboratory prototypes tend towards fully integrated on chip systems. They usually contain microfluidic valves<sup>26</sup> and/or electrodes and magnets<sup>27</sup> to generate the droplets that are thermocycled in either a chamber<sup>28</sup> or in a microchannel that traverses alternating temperatures areas.<sup>27,29</sup> However, at the present time, the use of a separated 3-step protocol for on-chip droplet generation, off-chip in-tube thermocycling and on-chip droplet fluorescence analysis still presents clear advantages such as reliability and flexibility. Moreover, it permits compliance with clinical constraints which could imply separate rooms for pre-PCR, PCR and post-PCR with the aim of avoiding cross-contaminations. It is thus central to most commercial systems and has been used in research and clinical laboratories, for example, to analyse rare mutations of the *KRAS* gene.<sup>13</sup> An optimised multiplex with fluorescence intensity encoding (using different green and red probe concentrations, see also section 2.a) led to a 5-plex assay capable of the simultaneous identification of the c815A>G mutation and copy number variation of genes implicated in spinal muscular atrophy.<sup>30</sup> In the same article, the authors





Table 1 Characteristics of main digital PCR commercial instruments

| INSTRUMENT characteristics |   |                             |  | CHIP/ARRAY characteristics |  |   |  |                    |                        |                  |
|----------------------------|---|-----------------------------|--|----------------------------|--|---|--|--------------------|------------------------|------------------|
| Brand                      | Instrument  | Launch Date                 | Integration/number of machines   | Type of analysis           | Nb of chips or plates per run  | Nb. of analysis channels and dyes associated  | Chip, samples and nb. of partitions  | Type of partitions | Volume of partition    | Real-time option |
| ThermoFisher Scientific    | Quantstudio Absolute Q  | 2022                        | 1 instrument for partitioning, thermocycling and data acquisition  | Planar                     | 1 plate: up to 16 samples (4, 8, 12 or 16) 90 min per run (depend on thermocycling conditions) | 5 channels 4 for sample analysis: FAM, HEX/VIC, TAMRA/Atto550, Cy5<br>1 used as a reference/QC: ROX/Atto590 (589 nm/625 nm)   | Microfluidic array plate (MAP), MAP16 plate for 16 samples, each divided into 20 480 partitions  | Micro-chambers     | ~0.4 nL                | No               |
|                            | Applied Biosystems QuantStudio 12K Flex with the OpenArray platform | 2009                        | 2 instruments for:<br>- Partitioning: manually (+ a sealing device) or Accu Fill system for automated partitioning of 4 arrays<br>- Thermocycling integrated with data acquisition   | Planar                     | Up to 4 chips in 4 hours   | 6 channels: FAM, HEX/VIC, TAMRA, ROX, Cy5, Cy5.5  | 1 chip Open Array for 1 sample, divided into 3072 partitions   | Micro-chambers     | ~3.3 nL                | Yes              |
|                            | Quantstudio 3D <sup>o</sup> (QS3D)                                  | 2013 (discontinued in 2023) | 3 instruments for:<br>- Partitioning: Chip loader for 1 chip<br>- Thermocycling: need for the additional thermocycler Dual Flat block PCR system with adapters<br>- Data acquisition | Planar                     | Up to 24 chips per run of thermocycling (2.5 h)<br>1 chip per run of data acquisition (30 s)   | 3 channels: FAM, HEX/VIC, ROX   | 1 chip for 1 sample, divided into 20 000 partitions  | Micro-chambers     | ~0.8 nL                | No               |
| Qiagen                     | QIAcuity One  | 2020                        | 1 instrument for partitioning, thermocycling and data acquisition  | Planar                     | 1 nanoplate per run  | QIacuity one 2 plex: 2<br>QIacuity one 5 plex: up to 8 (6 plus 2 hybrid (e.g. for long Stokes shift (LSS) probes)<br>8 channels: 6 plus 2 hybrid (e.g. for long Stokes shift (LSS) probes)<br>8 channels: 6 plus 2 hybrid (e.g. for long Stokes shift (LSS) probes) | Nanoplates: 24 samples in 8500 partitions 96 samples in 85 000 partitions 24 samples in 26 000 partitions 8 samples in 26 000 partitions | Micro-chambers     | ~1.5 nL                | No               |
|                            | QIAcuity Four   |                             |  |                            | up to 4 nanoplate per run  |   |  |                    |                        |                  |
|                            | QIAcuity Eight  |                             |  |                            | up to 8 nanoplate per run  |   |  |                    |                        |                  |
| Roche                      | Digital LightCycler system  | 2022                        | 2 instruments for:<br>- Partitioning: partitioning engine for 1 plate<br>- Thermocycling: integrated with data acquisition   | Planar                     | Up to 12 plates per run (8–96 samples)   | 6 channels: Cyan500/Atto425, FAM, HEX/VIC, LC610/Texas Red, CY5/LC640, Cy5.5  | 1 plate for 8 samples 3 types of plates: high resolution plate: 100 000 partitions Universal plate: 28 000 partitions High               | Micro-chambers     | ~0.15 nL ~1 nL ~2.5 nL | No               |





Table 1 (continued)

| INSTRUMENT characteristics |                            |             |   | CHIP/ARRAY characteristics |  |  |   |                    |                     |                  |
|----------------------------|----------------------------|-------------|---|----------------------------|--|--|---|--------------------|---------------------|------------------|
| Brand                      | Instrument                 | Launch Date | Integration/number of machines  | Type of analysis           | Nb of chips or plates per run  | Nb. of analysis channels and dyes associated   | Chip, samples and nb. of partitions   | Type of partitions | Volume of partition | Real-time option |
| Bio-Rad                    | QX100 <sup>a,d</sup>       | 2011        | 3 instruments for:<br>- Partitioning: QX Droplet Generator (1-8 samples per partitioning) or AutoDG for automatic generation (up to 96 samples per partitioning) + plate sealer<br>- Thermocycling: need for the additional thermocycler BioC1000 Touch or PTC tempo<br>- Data acquisition: QX Droplet reader | In-line <sup>b</sup>       | Up to 96 samples in a 96-well plate  | 2 channels: FAM, HEX/VIC<br>2 channels: FAM, HEX/VIC<br>6 channels: FAM, HEX/VIC, Cy5, Cy5.5, ROX, and Atto590 | sensitivity plate: 20 000 partitions<br>DG8 cartridge for 8 samples, each divided into 20 000 partitions<br>DG32 Automated Droplet Generator Cartridges: 4 × 8 samples, each divided into 20 000 partitions | Droplets           | ~nL                 | No               |
|                            | QX200                      | 2013        |   |                            |  |  |   |                    |                     |                  |
|                            | QX600                      | 2022        |   |                            |  |  |   |                    |                     |                  |
| Stilla Technologies        | QX ONE                     | 2019        | 1 instrument for partitioning, thermocycling and data acquisition   | Planar                     | 5 plates = 480 samples   | 4 channels: FAM, HEX/VIC, Cy5, Cy5.5   | GCR96 cartridges: 96 samples, each divided into 20 000 partitions   | Droplets           | ~nL                 | No               |
|                            | Raindrop dPCR <sup>a</sup> | 2012        | 3 instruments for:<br>- Partitioning: Raindrop Source, 1 chip for 8 samples<br>- Thermocycling: need for an additional thermocycler<br>- Data acquisition: Raindrop Sense   | In-line <sup>b</sup>       | 1 chip per run (8 samples)   | 2 channels: FAM, HEX/VIC   | 1 chip for 8 samples, each divided into 1 million to 10 million partitions  | Droplets           | ~pL                 | No               |
| Fluidigm                   | NAICA 3                    | 2016        | 2 instruments for:<br>- Partitioning and thermocycling: Geode   | Planar                     | 3 chips per run (for both instruments)   | Prism 3 with 3 channels: FAM, HEX/VIC, Cy5 Prism 6 with 6 channels: FAM, YY, Atto550, ROX, Cy5, Cy5.5          | Sapphire Chip: 4 samples per chip, each divided into 30 000 partitions<br>Ruby chip: 16 samples per chip, each divided into 17 000 partitions   | droplets           | ~0.8 nL<br>~0.3 nL  | No               |
|                            | NAICA 6                    | 2020        | - Data acquisition: PRISM3 or 6   |                            |  |  |   |                    |                     |                  |
|                            | Nio <sup>TM</sup> E        | 2023        | 1 instrument for partitioning, thermocycling and data acquisition<br>Nio e: 3 PCR programs per run<br>Nio+: 2 integrated thermocyclers = 24 PCR programs per run  | Planar                     | 3 chips per run (48 samples)<br>12 chips per run (192 samples)<br>24 chips per run (384 samples) | 7 channels: FAM, YY, Atto550, ROX, Cy5, Cy5.5, DY-521-XL   |   | Droplets           | ~0.3 nL             | No               |
| JN MedSys                  | Nio <sup>TM</sup>          |             |   |                            |  |  |   |                    |                     |                  |
|                            | Nio <sup>TM</sup> +        |             |   |                            |  |  |   |                    |                     |                  |
| Fluidigm                   | Biomark HD <sup>a</sup>    | 2006        | 2 instruments for:<br>- Partitioning: 3 devices (MX, RX or Juno)<br>- Thermocycling integrated with data acquisition  | Planar                     | 1  | 2 channels: FAM, HEX/VIC   | Integrated microfluidic circuits (IFC) allowing the partitioning of 12 to 192 samples into a range of partition numbers from 12 to 770 partitions   | Micro-chambers     | ~nL                 | Yes              |
|                            | Clarity <sup>a</sup>       | 2016        | 3 instruments for:<br>- Partitioning in tube: Clarity   | Planar                     | 32 samples per   | 2 channels: FAM, HEX/VIC   | 1 sample per tube, divided into 10 000 partitions   | Micro-chambers     | ~1.5 nL             | No               |



Table 1 (continued)

| INSTRUMENT characteristics |                     |             |  | CHIP/ARRAY characteristics |  |  |   |                    |                     |                  |
|----------------------------|---------------------|-------------|--|----------------------------|--|--|---|--------------------|---------------------|------------------|
| Brand                      | Instrument          | Launch Date | Integration/number of machines   | Type of analysis           | Nb of chips or plates per run                                | Nb. of analysis channels and dyes associated             | Chip, samples and nb. of partitions               | Type of partitions | Volume of partition | Real-time option |
| Clarity+                   |                     | 2020        | Auto-Loader + Clarity Sealing Enhancer, 8 tubes/partitioning run<br>- Thermocycling: need for an additional thermocycler with adjustable ramp and 0.2 mL tube<br>- Data acquisition: Clarity Plus Reader | Planar                     | Up to 96 samples per thermocycling and data acquisition runs | 6 channels: FAM, HEX/VIC, Atto550, Texas Red, Cy5, Cy5.5 | 1 sample per tube, divided into 40 000 partitions | Micro-chambers     | ~0.3 nL             | No               |
| Optolane                   | Genotizer™/Dr. PCR™ | 2019        | 2 instruments for:<br>- Partitioning: POSTMAN (sample loader) for 1 chip<br>- Thermocycling and data acquisition: LOAA analyzer  | Planar                     | 1  | 2 channels: FAM, HEX/VIC                                 | 1 sample per chip, divided into 20 163 partitions | Micro-chambers     | ~33 nL              | Yes              |

Abbreviations: nb.: number, <sup>a</sup> not commercialized anymore, <sup>b</sup> In-line: droplets flow in front of a detector; planar: partitions are analyzed by 2D-scanning, <sup>c</sup> OpenArray was initially commercialized by BioTrove; QX100 was developed following Quantalife acquisition; QIacuity suite was developed based on the Formulatrix Constellation system

mentioned the achievement of a 10-plex assay. Concurrent developments focused on using a 96-well plate for droplet collection to parallelise sample thermocycling. It allowed processing of 8 samples simultaneously and led to the first droplet-based dPCR commercialised instrument by Quantalife.<sup>31</sup> The American company Bio-Rad bought Quantalife in 2011 and its other competitor Raindance in 2017, making it the global leader in droplet dPCR.

Since 2011, Bio-Rad has commercialised a 2-color system based on the 3-step workflow, namely the QX100™ Droplet Digital™ PCR System and its next version the QX200™ followed by the QX600 6-color version, and the all-in-one fully automated QX1 system (see Table 1 for characteristics). In 2016, the French company Stilla Technologies commercialised a 3-color system (NAICA 3), replaced by a 6-color system (NAICA 6) and the brand-new all in one Nio+ system series (7 colors ddPCR). In 2025, Bio-Rad entered the process of acquiring Stilla Technologies. From 2019, other competitors emerged including Rainsure, Targeting One, Forevergen, Sniper or Pilot Gene Tech, diversifying the market of ddPCR platforms. Important studies that compared these droplet dPCR platforms confirmed a high degree of consistency, as shown in the case of SARS-CoV-2 gene-associated detection.<sup>32,33</sup> The calibration of droplet volume remains recommended to maintain the consistency between platforms.<sup>33–35</sup>

Many studies have compared dPCR instruments based on microchambers *versus* droplets with the ultimate goal of calibrating differences (see Table 1) and standardising dPCR for clinical use. The evaluation of the QX100/QX200 and QS3D instruments, with different types of partition and readout, was conducted to assess their ability to detect mutations in samples in various situations including prenatal non-invasive testing,<sup>36</sup> lung cancer follow-up<sup>37</sup> and HIV follow-up.<sup>38</sup> It was demonstrated that both platforms achieved comparable results with similar sensitivity. The QX200 platform was also compared to the Absolute Q for the detection of early-stage breast cancer. These platforms displayed >90% concordance in ctDNA positivity within 46 plasma samples.<sup>39</sup> Further work also compared QX200 and QIacuity platforms for the detection of specific mutations,<sup>40,41</sup> and both allowed the detection of DNA quantities as low as 9 picograms,<sup>40</sup> although a moderate agreement was found due to the sampling effect and threshold settings.<sup>41</sup> To assess the impact of partition number, the QX200, QS3D and Raindrop (a system with 250-fold more partitions, from Raindance Technologies) systems were compared for the detection of the *BCR-ABL1* fusion gene (leukemia biomarker) and were found to have a common 4 log dynamic range and to correlate only for frequency >0.1%.<sup>42</sup> These platforms were also compared to the QX100, the Constellation, and the Biomark systems for the detection of mutated *KRAS* oncogene, taking plasma mass spectrometry as a reference. It showed a variability in concentration values less than 1.3-fold.<sup>43</sup> The QX100, the

Biomark and the Raindrop systems have also been compared to the Quantstudio 12K Flex. The analysis required partition volume correction and indicated comparable effectiveness for the quantification of a certified plasmid reference material.<sup>44</sup>

The numerous advantages of dPCR (high sensitivity<sup>45</sup> and reproducibility, absolute quantification, less competition between DNA targets and so less bias due to PCR efficiency differences, less sensitivity to PCR inhibitors,<sup>46</sup> easy analysis,<sup>47</sup> lower volumes and turnaround times) explain its rapid expansion, in the last years (see Fig. 3a), as a powerful tool for potential clinical applications. This is, all the more true, knowing that the clinical implementation of liquid biopsies is becoming a standard of care. Liquid biopsy is the act of sampling biological fluids for the analysis of nucleic acids, circulating cells or subcellular structures as exosomes. It is mostly known in the field of oncology, but it is also used for other disease diagnoses. Despite the minimally invasive character of liquid biopsy, the minute amount of material available is still a real challenge.

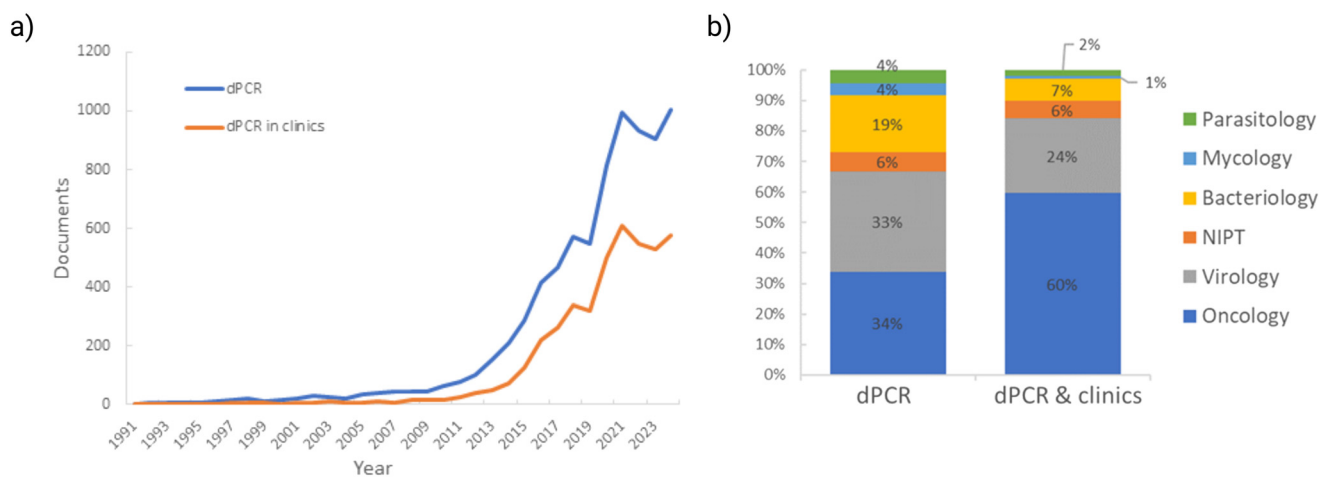
However, very few applications are, at the present time, FDA-approved in clinics. Indeed, the FDA validated the use of dPCR in 3 particular cases: SARS-CoV-2 detection,<sup>48</sup> BCR::ABL1 detection to follow up patients with chronic myelogenous leukemia<sup>49</sup> and residual host cell DNA detection in biologic drugs produced in *E. coli*.<sup>50</sup> It's worth noting that the FDA has so far decided not to regulate laboratory-developed tests, such as non-invasive prenatal testing.<sup>51</sup> In order to pave the way of dPCR for patient diagnosis and follow up, a large range of clinical trials are comparing its performance to the gold standard methods currently used in clinics, particularly in oncology (see Fig. 3b). This review will present chosen examples of these studies, referencing advantages and drawbacks of dPCR. Finally, an overview of promising improvements will be proposed, although the list is not exhaustive.<sup>14</sup>

## II. Applications of dPCR in oncology

In 2022, cancer was responsible for approximately 9.7 million deaths worldwide and remains the second leading cause of mortality.<sup>52</sup> Among the estimated 20 million new cases per year, the most common are breast cancer (BC), colorectal cancer (CRC) and lung cancer (LC). According to the National Cancer Institute, cancer is defined as a disease “in which some of the body's cells grow uncontrollably and spread to other parts of the body”.<sup>53</sup> This abnormal cell proliferation is triggered by the accumulation of alterations within cells implying changes at different key levels including genomic, transcriptional or epigenomic. All these alterations constitute potential cancer biomarkers<sup>54</sup> useful for cancer detection, disease prognosis, treatment selection or analysis of response to treatment.<sup>55</sup> At the genomic level, somatic molecular alterations can be fusion gene, point mutations or copy number variation (CNV) of specific oncogenes or tumour suppressor genes such as *HER2*, *PIK3CA*, *KRAS*, *BRAF*, *EGFR*, and *TP53* (ref. 56) (see Table 2).

### 1. Solid tissue analysis

Tissue biopsies are used by the pathologist to confirm the diagnosis of cancer by direct observation of the cells and tissues' morphologic features. This observation is combined with other analyses such as immunohistochemistry (IHC) and/or fluorescent *in situ* hybridisation (FISH) and/or traditional molecular analysis. These methods are both expensive and time-consuming and could introduce a subjective dimension into the diagnosis.<sup>54</sup> Complementary analysis by dPCR can deliver interesting information on DNA extracted from solid tumour tissue. As an example, it is possible to analyse the CNV of genes of interest (using a gene of reference) to discriminate the tumour from the normal tissue. For instance, the matrix metalloproteinase-9 gene (*MMP-9*) CNV was found only in tumour tissue and not in adjacent tissues. Coupled to mRNA expression



**Fig. 3** a) Number of yearly publications on digital PCR and digital PCR in clinics. b) Applications of dPCR in different fields in general and in clinics. Source: Scopus [data assessed: 27/11/2024].



**Table 2** Most prevalent cancer types and their main biomarkers. Abbreviations: CRC: colorectal cancer, NSCLC: non-small cell lung cancer

| Gene of interest | Type of alteration  | Biomarker type     | Type of cancer  | References   |
|------------------|---------------------|--------------------|---|--|
| HER2             | CNV                 | Tissue, ctDNA      | Breast cancer, gastric cancer, NSCLC  | 12, 61, 79, 96, 97   |
| RAS              | Point mutation      | Tissue, ctDNA, CTC | CRC, pancreatic cancer, melanoma  | 41, 55, 72–75, 82, 86, 100, 102, 109, 157, 341–343, 347, 348, 392–394            |
| BRAF             | Point mutation      | Tissue, ctDNA      | CRC, melanoma   | 55, 68, 73, 75, 85, 86, 95, 99, 101, 102, 341, 347, 393, 394                     |
| EGFR             | Point mutation      | Tissue, ctDNA, CTC | NSCLC   | 37, 41, 55, 68, 70, 71, 78, 84, 88, 90–94, 106, 125, 150, 151, 153, 158, 395–397 |
| TP53             | Point mutation      | Tissue, ctDNA      | Ovarian cancer, breast cancer, NSCLC, pancreatic cancer, hepatocellular carcinoma, melanoma | 39, 65, 80, 106, 109, 122, 398   |
| PIK3CA           | Point mutation      | Tissue, ctDNA, CTC | Breast cancer, CRC, NSCLC   | 39, 75, 81, 100, 102, 106, 110, 147, 148, 347, 399, 400                          |
| TERT             | CNV, point mutation | Tissue, ctDNA      | Lung cancer, bladder cancer, hepatocellular carcinoma                                       | 58, 120, 122–124, 398, 401   |
| MYC              | CNV                 | Tissue, ctDNA      | Lung cancer   | 58, 402  |
| ESR1             | Point mutation      | ctDNA, CTC, EV     | Breast cancer   | 147, 149, 346  |
| BCR::ABL1        | Fusion gene         | ctDNA              | Leukemia  | 34, 42, 49, 87, 113–117  |
| ALK              | Fusion gene         | Tissue, ctDNA, CTC | NSCLC   | 63, 119, 165, 166, 169   |
| MET              | CNV, point mutation | Tissue, ctDNA, CTC | Lung cancer, ovarian cancer   | 55, 59, 62, 150, 169   |
| PD-L1            | Transcript          | CTC                | Head and neck squamous cell carcinoma   | 154  |
| WIF1             | Hypermethylation    | ctDNA              | CRC   | 127  |
| NPY              | Hypermethylation    | ctDNA              | CRC   | 127  |

analysis, it showed a potential value as a diagnostic biomarker in hepatocellular carcinoma (HCC) samples ( $P < 0.0001$ , AUC = 0.76).<sup>57</sup> Similarly, the CNV analysis of tumour and non-tumour tissues has enabled the diagnosis of lung cancers with a pre-defined specificity of 99% and a sensitivity of 41% for *MYC* and 51% for *TERT* individually, whereas the combination of both genes gave an improved sensitivity of 60%,<sup>58</sup> highlighting that targeting several genes can improve clinical sensitivity. The *MET* polysomy detection by dPCR indicated a 100% concordance with FISH in non-small cell lung cancer (NSCLC) samples.<sup>59</sup> The determination of *HER2* CNV is particularly interesting: if upregulated, *HER2* is a treatment target in several solid tumours including breast and gastric cancers. The traditional analysis of *HER2* CNV by FISH uses a reference gene, often *CEP17* (centromere of chromosome 17). Up to 20% of false negative results have been reported with FISH, especially in the case of polysomy of chromosome 17.<sup>60</sup> In BC,<sup>61</sup> a strategy based on a 3 duplex dPCR with 3 different reference genes has been developed, resulting in the same *HER2* CNV detected as with IHC and FISH combined, without the use of *CEP17*. Thereby using several reference genes can significantly improve the sensitivity, making it competitive with standard methods. The CNV determination by sequencing analysis is also feasible, but dPCR has been suggested as a pertinent validation for unambiguous results.<sup>62</sup>

For the analysis of point mutations, dPCR is often challenged against sequencing methods (Sanger or next-generation sequencing).<sup>63–67</sup> Indeed, dPCR had a similar

sensitivity and a faster turnaround time than NGS for testing DNA and RNA biomarker panels in samples from patients with NSCLC<sup>68</sup> or acute myeloid leukemia (AML).<sup>69</sup> In NSCLC, the treatment with *EGFR* (epidermal growth factor receptor) tyrosine kinase inhibitor (TKI) is inefficient in patients with *EGFR* resistance mutations. It has been shown that the presence of T790M *EGFR* mutation correlates with a faster rate of disease progression in the first five months.<sup>70</sup> In contrast, dPCR offers quantitative analysis of the *EGFR* mutation and revealed that low-level T790M does not impact the treatment response or the survival, indicating that a threshold is needed to determine who will benefit from *EGFR*-TKI.<sup>70</sup> Thus, the impact of mutations on treatment resistance can be studied by dPCR.<sup>71</sup> In the case of CRC, resistance to anti-*EGFR* therapies arises from mutations of the *RAS* gene and *BRAF*. As for NSCLC, highly sensitive dPCR revealed the need for a threshold in the mutant allele frequency (MAF) detection for prognosis. The clinically relevant threshold analysed by extended pathway genotyping of *RAS* and *BRAF* in large patient populations seemed comprised between 1% (ref. 72–74) and 5% of *RAS/BRAF* mutant.<sup>75</sup> Unfortunately, solid biopsies are invasive and therefore not suitable for regular patient monitoring. Moreover, solid biopsies only provide information from a limited area of the tumour in a disease known to be highly heterogeneous.

## 2. Liquid biopsy analysis

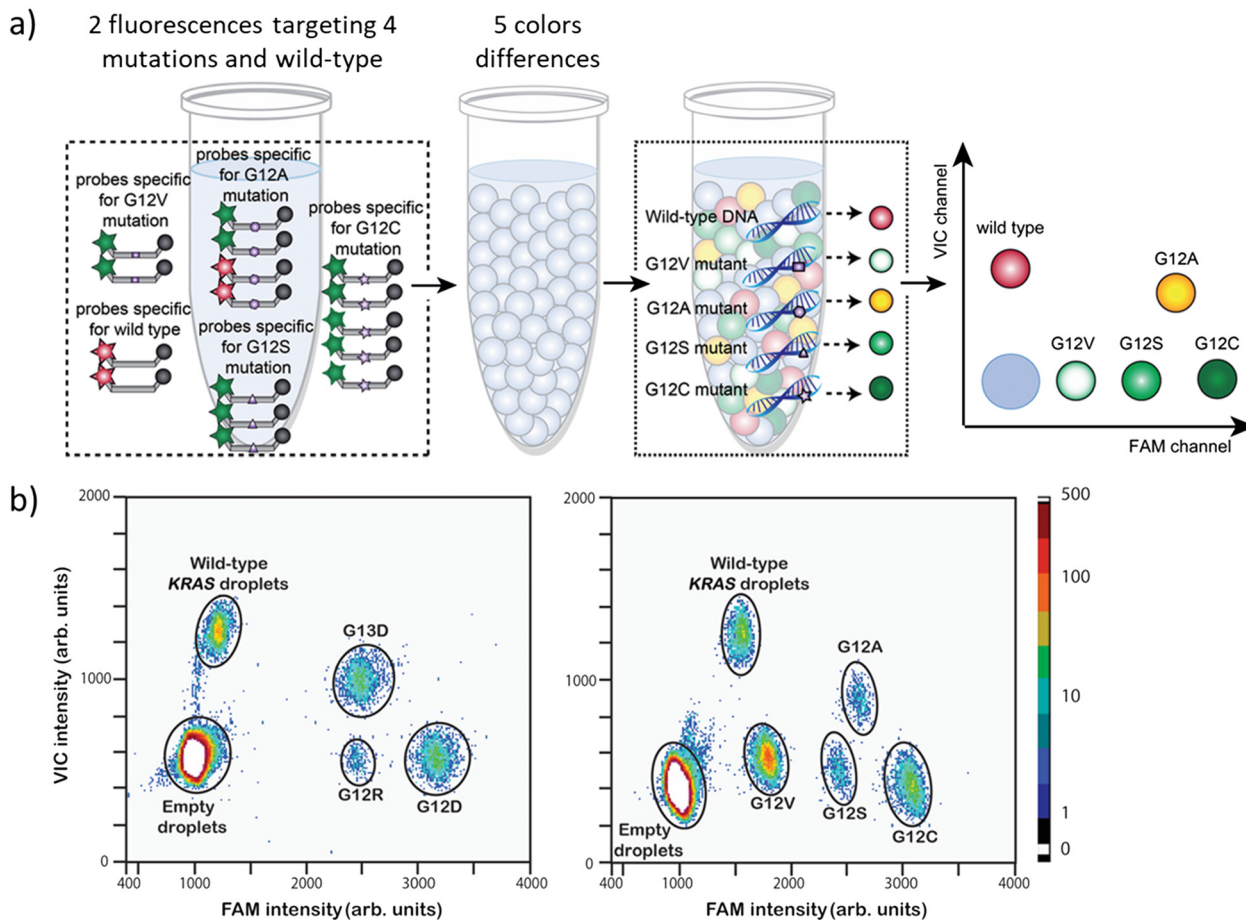
In recent years, liquid biopsy in oncology, consisting of the analysis of tumour-specific components released in bodily





fluids such as blood, urine, saliva, pleural, peritoneal or cerebrospinal fluids, has shown to be pertinent to overcome the limitations of tissue biopsy. The concept of liquid biopsy was first coined by Catherine Alix-Panabières and Klaus Pantel by detecting, in blood, circulating tumour cells (CTCs) – intact cells that detached from a primary tumour and entered the bloodstream.<sup>76</sup> Liquid biopsies are non or minimally invasive and their analysis offers several benefits in comparison with solid biopsies, including real-time analysis and reflection of tumour heterogeneity. Moreover, liquid biopsy analyses present applications for detection of early cancer, cancer progression and minimal residual disease (MRD) as well as for the real-time monitoring of treatment response. The FDA approved the Cell Search CTC system, for monitoring breast cancer *via* CTC isolation/enumeration in 2004, metastatic CRC in 2007 and metastatic prostate cancer in 2008. Many other biomarkers can be analysed in liquid biopsies including exosomes, microRNAs (miRNAs), and cell-free circulating tumour DNA (ctDNA). ctDNA consists of fragments of DNA released into bloodstream by tumour cells, allowing the search for tumour specific molecular changes in real-time.

**a) dPCR for circulating tumour DNA detection and monitoring.** In cancer diagnosis, ctDNA is particularly interesting. Many studies have challenged the efficiency of dPCR for mutation detection in paired tissue and ctDNA samples from patients with metastatic or advanced cancer. DNA extracted from tissues and plasma presents low correlation in early-stage cancer,<sup>77,78</sup> probably related to the low tumour DNA level, proven to be associated to tumour burden.<sup>79</sup> However, in the metastatic setting, good concordance has been found, for example: 62.5% for *TP53* mutations in patients with ovarian cancer,<sup>80</sup> 83.1% for *PIK3CA* mutations detected in 89 patients with BC,<sup>81</sup> 74% in multiplex dPCR (see Fig. 4, multiplexing strategy) or 84% in multiple duplex dPCR for *KRAS* mutations in 50 patients with CRC<sup>82</sup> and 86.3% in the case of *EGFR* mutations detected in 106 patients with NSCLC.<sup>78</sup> The analysis of *HER2* CNV in NSCLC patients revealed a concordance between tissue and blood oscillating between 66.7% (low CNV) to 98.9% (high CNV).<sup>83</sup> When compared to other detection methods, dPCR has proven to be more sensitive for ctDNA *EGFR* p.T790M mutation detection than ADx-ARMS PCR with a sensitivity improved from 30.77% to 53.85% for a specificity over 90% on paired samples in NSCLC.<sup>84</sup> Performances of dPCR have



**Fig. 4** Multiplex assay with two fluorescent probes labelling wild-type and mutations of *KRAS* gene for ctDNA analysis in CRC patients. a) Multiplexing strategy for a 5-plex assay and b) representative data from 2 available panels including the 7 frequently seen mutations in *KRAS* codon 12 and 13. Reproduced from ref. 82 with permission from Oxford University Press, copyright 2013.



also been assessed for *HER2* CNV detection<sup>79,85</sup> in a mix of various stages of BC, and *HER2* amplification was detected in plasma samples with an overall concordance of 66.96% with a sensitivity of 43.75% and a specificity of 84.38%, when compared to tissue analysis by FISH/IHC. Interestingly, the subgroup analysis of samples from stage IV and recurrent patients indicated a decreasing concordance between blood and tissue of 69.47% and 59.32% respectively. These findings correlate with the tumour burden and the intra/inter-tumoral heterogeneity rising.<sup>79</sup>

These comparative studies encourage considering the use of molecular analysis in liquid biopsy as companion diagnostic for routine analysis.

As already discussed, the performances of various commercially available dPCR platforms are regularly analysed. A conclusion would be that dPCR is generally more sensitive than the fully automated qPCR-based method.<sup>86,87</sup> Nevertheless, some levels of standardisation have become mandatory, particularly when so many dPCR platforms are available. In the case of NSCLC, the ctDNA detection of *EGFR* mutations was first FDA-approved in 2016 with a qPCR assay for clinical use (COBAS assay).<sup>88,89</sup> Indeed, the efficacy of the *EGFR* inhibitor depends on the *EGFR*-signalling activation with *EGFR*-sensitising mutations (exon 19 and 21) and *EGFR*-resistant mutation (T790M substitution in exon 20).<sup>90,91</sup> Provencio *et al.* described that patients with sensitising mutations detected by dPCR at a mutant allele frequency (MAF) <7% had lower risk of death (60%). During the follow-up, plasma samples, taken while under *EGFR* inhibition treatment, revealed the emergence of T790M in 52.8% of patients subjected to disease progression.<sup>92</sup> Another study that used dPCR correlates the absence of mutated *EGFR*, at baseline and/or at 4 weeks of icotinib therapy (TKI), with longer progression-free survival (PFS).<sup>93</sup> In a different study, undetectable levels of sensitising mutation, during osimertinib therapy (TKI), were associated with higher PFS, whereas its re-emergence alone or together with p.T790M was associated with shorter PFS. Surprisingly, patients with the triplet molecular pattern (sensitising+/T790M+/C797S+) had 12.3 months of median time to progression compared to 4.9 months for patients with sensitising mutation only and 2.17 months for patients also presenting p.T790M mutation.<sup>94</sup> Similarly, the FIRE-4.5 study concluded that liquid biopsy evaluating ctDNA is informative and relevant to guide treatment choices in patients with *BRAF* V600E-mutated metastatic CRC,<sup>95</sup> in which case a clear superiority of FOLFOXIRI plus bevacizumab was demonstrated. During the follow-up of patients with *HER2*+ BC, a decrease of *HER2* CNV (15% (ref. 96)) in plasma was correlated with better prognostics and could predict clinical benefit.<sup>96,97</sup> In AML, dPCR has proven to be suitable for *FLT3*-TKD mutation detection, but more clinical studies are needed to conclude about a clinical value.<sup>98</sup> These discoveries highlight the importance of treatment response monitoring and the pertinence of the use of liquid biopsy for this purpose. Moreover, the rapid turnaround time and the quantitative

character of dPCR associated with the low invasiveness of liquid biopsy make it an outstanding tool for therapy monitoring for daily clinical practice.

Apart from the metastatic setting, dPCR has also been evaluated in the perioperative period, particularly for MRD detection. After surgery of patients with CRC, the ctDNA monitoring for BRAFV600E mutation by dPCR revealed a correlation of the detected MAF with tumour diameter but not with tumour recurrence.<sup>99</sup> In contrast, after liver resection in patients with CRC liver metastases, *KRAS* and *PIK3CA* mutations were associated with a shorter overall survival (OS).<sup>100</sup> Similarly, in patients with resected cutaneous melanoma under therapy, the detection of *BRAF*-mutated ctDNA was associated with significantly worse OS.<sup>101</sup>

Furthermore, tumour-informed strategies are studied using the combination of NGS analysis of primary tumour and ctDNA monitoring by dPCR for NGS-identified mutations after surgery and during chemotherapy. They allowed the prediction of early relapse, 3 to 6 months ahead of conventional imaging examinations in the case of CRC<sup>102,103</sup> or ahead of the serum biomarker (cancer antigen 125) rise in the case of gynaecological cancers.<sup>104</sup> This combination of NGS for mutation identification followed by dPCR enables custom adjustments during long-term treatment response monitoring.<sup>105,106</sup> It can also be called personalised dPCR,<sup>107</sup> a first step into personalised medicine (see Fig. 5). Many studies using such tumour-informed approaches have shown a good early prediction of patient relapse, after surgery or chemotherapy, in various cancers and settings.<sup>66,77,107–112</sup>

Fusion gene also represents a highly interesting cancer biomarker, particularly in the case of hematopoietic and lymphoid malignancies. Although the current gold standard method for treatment monitoring is reverse transcriptase qPCR (RT-qPCR) and flow cytometry, it is actively challenged by dPCR, particularly for MRD detection that requires high sensitivity.<sup>113</sup> Indeed, in 2019, the FDA approved the dPCR assay, from Bio-Rad, aiming to detect BCR::ABL1 in samples of patients with chronic myeloid leukemia.<sup>49</sup> Since then, it exhibited good performance: the fusion gene was detected in 63% (ref. 114) and 68% (ref. 115) of patient samples initially negative by RT-qPCR. These results suggest that dPCR could help the early selection of patients admissible for treatment discontinuation.<sup>116,117</sup> Other fusion genes were targeted for MRD detection, such as Ig::TCR gene in acute lymphoblastic leukemia (ALL), which was detected by dPCR in 83% (29/35) of the ambiguous qPCR cases.<sup>118</sup> Similarly, dPCR has proved its ability to identify accurately patients with high relapse risk *via* NPM::ALK gene detection in anaplastic large cell lymphoma (ALCL).<sup>119</sup>

Even though liquid biopsy on blood samples is widespread, other biofluids could be used. For example, in the case of urothelial bladder cancer, the liquid in continuous contact with the tumour surface is urine. The high diversity of genetic alterations that can be found in this cancer (including mutations in genes *TERT*, *FGFR3*, *PIK3CA*, *ERBB2*, *HRAS* and *GPR126*)<sup>120</sup> requires the use of tumour-



## Workflow: Customized Longitudinal Analysis of Circulating Tumor DNA Using Single-Color ddPCR

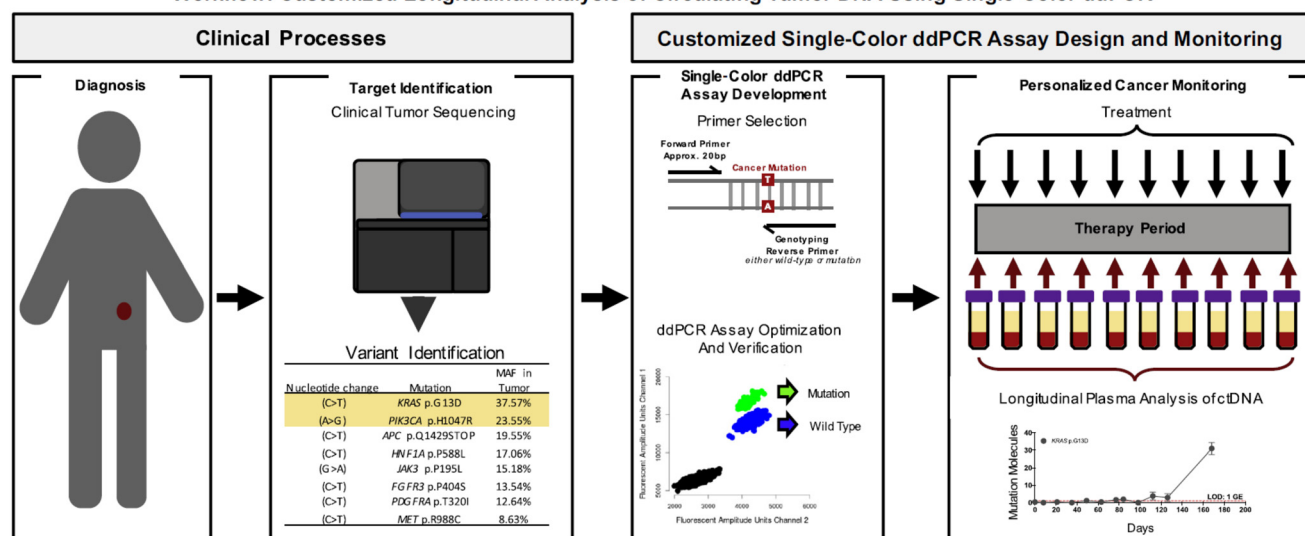


Fig. 5 Workflow of generating customised dual-color digital PCR assays for routine and extended longitudinal monitoring of circulating tumour DNA throughout treatment. Reproduced from ref. 107 with permission from Elsevier, copyright 2020.

informed dPCR (combined to NGS analysis of tumour tissues) for each patient.<sup>121,122</sup> These studies revealed the potential of urinary ctDNA variant allele frequency as a molecular biomarker of recurrence after surgery. In particular, the detection of *TERT* mutations in urine was proven to be of particular interest: studies of bladder cancer urine samples have highlighted a superior sensitivity of dPCR (79.7%) compared to cytology (59.5%) and uromonitor (56.8%)<sup>123</sup> or similar performances to qPCR.<sup>124</sup> On the other hand, in lung adenocarcinoma, a comparison between blood and other biofluid samples (pleural effusion, cerebrospinal fluid, ascites and pericardial effusion) from EGFR-positive patients concluded that cell-free DNA (cfDNA) was less abundant in blood and that sensitising mutations were detected in 16 vs. 21 samples respectively.<sup>125</sup> In the case of central nervous system tumours, dPCR successfully detected the H3K27 variant in cerebrospinal fluids and was in accordance with tissue analysis.<sup>40</sup>

Recently, a large body of literature has shown that ctDNA could be detected thanks to the detection of tumour specific methylation dysregulation.<sup>126</sup> Indeed, the search for such tumour-specific methylation markers has shown them to act as universal markers of cancer that do not require previous analysis of tumour tissue. Generally, dPCR is associated with the bisulfite conversion to detect and quantify cancer specific markers such as the hypermethylated genes *WIF1* and *NPY* in CRC,<sup>127</sup> *RASSF1A* and *GSTP1* in prostate cancer,<sup>128,129</sup> *HOXD8* and *POU4F1* in metastatic pancreatic cancer,<sup>130</sup> *SEPT9* in gastrointestinal tumours<sup>131</sup> and the hypermethylated promoters of *SOX17*, *CDO1*, *TAC1* and *HOXA7* in NSCLC<sup>132,133</sup> and of *OXT/ZSCQN12* in endometrial carcinoma.<sup>134</sup> Similarly, this strategy has been used to target biomarkers in genes *EMX1*, *Chr5q14.1* and *NXP1* for multi-cancer detection (AUC = 0.948).<sup>135</sup> These studies highlighted

that hypermethylated ctDNA was highly correlated to ctDNA and thus to tumour burden, making it a good tool for patient monitoring, treatment management and even timing of intervention.<sup>136</sup> The study of methylations by dPCR revealed the possibility to differentiate liver metastases originated from colorectal or pancreatic ductal adenocarcinoma cancers, and liver cancer types, such as liver adenocarcinoma.<sup>137</sup> Indeed, some methylations remain unaltered between primary tumours and liver metastases, whereas some others change and could potentially be drivers of the metastatic cascade.<sup>137,138</sup> Another study developed the Methyl-BEAMing technology, combining bisulfite conversion and BEAMing: the bisulfite conversion is followed by a first round of amplification of methylated DNA and reference DNA, then a second round of emulsion PCR on magnetic beads enables the analysis by flow cytometry thanks to fluorescent methylation-specific probes. This method showed a higher sensitivity than dPCR for low DNA quantities.<sup>139</sup>

Dysregulated methylation could also be assessed by using methylation sensitive restriction enzyme (MSRE) prior to dPCR analysis.<sup>140</sup> Based on this technology, the study of hypo- and hypermethylation of promoters demonstrated their value as potential biomarkers for detection of oral cavity cancer.<sup>141</sup> Moreover, the team of Takahiro Yamasaki included MSRE-dPCR in predictive models for cancer diagnosis. For example, measuring methylated somatostatin (SST) coupled to fecal immunochemical test and age (FAMS) allowed for the efficient detection of CRC and advanced colorectal adenocarcinoma (AUC = 0.90).<sup>142</sup> Evaluation of *hTERT* and methylated *RUNX3* coupled to age and sex (ASTEm-R3) allowed the detection of early gastric cancer (AUC = 0.93, sensitivity 79.7%, specificity 91.1%).<sup>143</sup> Assessment of methylated *HOXA1* coupled to classical markers (AFP, DCP)





as well as age and sex (ASDAm-H1) permitted the accurate detection of hepatocellular carcinoma (AUC = 0.96, sensitivity 86.2%, specificity 93.9%).<sup>144</sup> However, in another study, this strategy has shown some discordance with the gold standard, the OSNA method (one-step nucleic acid amplification), to detect *RASSF1A* methylated.<sup>145</sup>

**b) dPCR for the detection and the monitoring of other liquid biopsy components.** CTCs are another promising biomarker of liquid biopsy, as they are released from the primary tumour and/or metastasis into blood and lymphatic vessels and as they have the potential to induce metastasis or relapse.<sup>146</sup> Unfortunately, their scarcity in plasma samples<sup>147</sup> implies an enrichment or isolation step that is not simple. For example, the FDA-approved Cell Search system is based on an immunomagnetic selection on the epithelial cell adhesion molecule, EpCAM, discarding the EpCAM<sup>-</sup> CTC. Coupled to dPCR, it has been possible to detect mutated *PIK3CA*<sup>148</sup> and *ESR1* (ref. 149) in both ctDNA and CTC genomic DNA from patients with metastatic BC. Interestingly, for both genes, the mutations were not identical in ctDNA and CTC, suggesting that they give complementary information. However, an unbiased CTC selection strategy is still wanted, and a lot of methods are in development to overcome this enrichment step difficulty,<sup>150,151</sup> such as large volume liquid biopsy<sup>152</sup> or microfluidic devices based on cellular size filtration<sup>153,154</sup> or inertial<sup>155</sup> selection. The enrichment step coupled to dPCR permitted the quantification of important biomarkers *via* their transcripts,<sup>131</sup> such as *PD-L1* in head and neck squamous cell carcinoma.<sup>154</sup> Indeed PD-L1 is an immune checkpoint protein allowing T cell inactivation, and it is the target of immunotherapy anti-PD1/anti-PDL1. Similarly, the genes *PSMA* or *AR-V7* are novel therapeutic targets in castration-resistant prostate cancer and the detection of their transcripts was demonstrated to be feasible by a multiplex reverse transcriptase ddPCR (RT-ddPCR), enabling therapy response monitoring.<sup>156</sup> Other studies showed the possibility of detecting, by dPCR, mutated *KRAS*<sup>157</sup> or *EGFR*<sup>151,153,158</sup> in CTC genomic DNA. At the moment, dPCR applied to CTCs has already proven to be more sensitive than qPCR-based methods.<sup>154</sup>

Studies on non-coding RNA biomarkers have also been published.<sup>159</sup> For example, miRNA 320a expression levels were able to differentiate patients with ovarian cancer from healthy donors by RT-dPCR more reliably than by RT-qPCR.<sup>160</sup> Similarly, miRNA 181a appeared as a promising biomarker (ROC = 0.849) in cerebrospinal fluid for detection of central nervous system leukemia and for identification of therapy-admissible patients.<sup>161</sup> Also, the prognosis value of long non-coding RNA (lncRNA) *MYU* in prostate cancer was demonstrated by RT-dPCR on urine samples.<sup>162</sup> However, a study compared the biomarker value of several sorts of RNAs in urine samples from prostate cancer patients, and it concluded that miRNAs (miR-27b-3p, miR-574-3p and miR-125b-5p) are more efficient biomarkers than lncRNAs or mRNAs (*PCA3*, *PCAT18* and *KLK13*).<sup>163</sup> Moreover,

observations of expression changes of miR-205-5p, miR-222-3p and SNORD48 in a cohort of patients with endometrial cancer suggested their implication in cancer development.<sup>164</sup>

On the other hand, RNAs can be carried in extracellular vesicles (EV), where they are protected from degradation by RNases. It has been demonstrated that non-coding RNAs contained in extracellular vesicles (EV) are involved in regulation of transcription and post-transcription and thus are an efficient biomarker to monitor cancer progression.<sup>165</sup> Mutated tumoral RNA from EVs has been detected in small amounts by RT-dPCR in the case of ovarian cancer<sup>45</sup> and of NSCLC.<sup>166,167</sup> Furthermore, the combination of circulating and vesicle-associated miRNAs showed potential clinical significance for the identification of pancreatic cancer patients.<sup>168</sup> The EV study remains for now limited by the efficiency of the step of EV isolation/enrichment,<sup>169</sup> as for CTCs.

### 3. Other applications of dPCR in oncology

In addition to potential applications in diagnostic of solid and liquid biopsies, dPCR is a powerful tool for analysing bone marrow aspirates and/or peripheral blood, where the entire peripheral blood DNA is studied without distinguishing between cell-associated and cfDNA.

Such analyses are conducted to detect mixed chimerism (MC) following allogeneic stem cell transplantation (HSCT). In patients with haematological disorders, surgery is not an option as they do not present any solid tumour. However, HSCT offers a curative treatment, along with cellular therapies like virus-specific T cells.<sup>170</sup> HSCT involves replacing the patient stem cells with haematopoietic stem cells from a compatible donor, which can lead to MC. Prolonged MC is undesirable, as it is often linked to disease recurrence.<sup>171</sup> The detection by dPCR of MRD and MC after transplantation has shown to be competitive compared to the gold standard methods, the short tandem repeat amplification by PCR (STR-PCR) with a good correlation and a shorter turnaround time,<sup>170,172</sup> allowing for a more effective monitoring of remission and adjustment of treatment.<sup>170,171</sup> When combined to multiparameter flow cytometry, dPCR has also permitted the precise identification of patients with high risk of relapse from bone marrow aspirates after HSCT.<sup>173</sup>

dPCR can also be used for quality control of biotherapies. For instance, in the chimeric antigen receptor (CAR) T-cell therapy, an emerging and highly personalised immunotherapy: it consists in genetically modifying *ex vivo* the T cells of the patient. The transduced T cells will express CAR on their surface enabling the specific recognition of tumour cells by the immune system. This genetic modification approach has been declared as potentially oncogenic and toxic necessitating quality and safety controls. Indeed, the FDA requires a maximum of 5 vector copies per transduced cell, which is enough to be efficient while minimising the oncogenic risk.<sup>174</sup> dPCR allowed for the precise quantification of vector copy number in CAR T-cells





expressing both anti-CD19 and anti-CD22 receptors, called AUTO3.<sup>175</sup> Similarly, a triplex dPCR was demonstrated to be as efficient as two duplex dPCR, to quantify 3 targets in AUTO6NG T-cells, an improvement of AUTO6 (anti-GD2 and anti-RQR8 CARs) against neuroblastoma.<sup>176</sup> Furthermore, clinical reports have testified that the continuous proliferation of CAR-T cells *in vivo* is a key factor to ensure the therapeutic effects.<sup>177,178</sup> Thus CAR-T cell monitoring became crucial to follow treatment response. dPCR has shown stable results in quantifying the *CAR* transgene after CAR-T cell infusion in peripheral blood samples<sup>179,180</sup> and other sample types such as bone marrow and lymph node material.<sup>181</sup> The limit of detection (LoD) was 20 copies per  $\mu\text{g}$  DNA.<sup>181</sup>

In conclusion, these studies suggest that dPCR is a powerful tool for clinical applications in cancer medicine. Its performances in terms of sensitivity and specificity are mostly similar to the current gold standard methods, such as qPCR. Its high reproducibility to detect oncogene mutations, CNV or fusion gene is due to its ability to perform absolute quantification not relying on standards. It makes it reliable and suitable for patient monitoring during the perioperative period, during and after treatment, for therapy response study or minimal residual disease detection. Also, in the cases of cancers where solid biopsy is not an option (haematological disorders), the detection of biomarkers in liquid biopsy by dPCR allows diagnostic and patient follow up. However, the small quantity of ctDNA or CTCs shed into bloodstream still represents a real technological challenge for dPCR to be used in clinics, particularly for early-stage diagnostic. From a non-clinical, fundamental research perspective, dPCR has facilitated the study of complex cancer mechanisms, notably enabling biomarker discovery.<sup>182–185</sup>

### III. Prenatal testing

The emergence of cfDNA as a tool in cancer medicine has inspired researchers in the field of prenatal testing. Indeed, in 1997, a simple PCR targeting *DYS14* gene on the Y chromosome highlighted the presence of cell-free fetal DNA (cffDNA) in plasma of pregnant women bearing male fetuses.<sup>186</sup> Since this discovery, invasive procedures such as amniocentesis and chorionic villus sampling (CVS), generally associated with up to 1% risk of miscarriage,<sup>187</sup> could be avoided. Non-invasive prenatal testing (NIPT) has become a clinical reality to evaluate numerous genetic disorders. Although rising during gestation, cffDNA represents a low fraction of cfDNA in maternal plasma ranging from 0.5 to  $\approx 30\%$ .<sup>188,189</sup> For this reason, dPCR seems a more suitable method for NIPT than the current gold standard methods, namely qPCR or NGS in the case of genetic disorders. Moreover, the targeted nature of dPCR avoids ethical questions rising from NGS screening of an unborn child genome and from pregnancy choices. For inherited diseases, when the presence of an allele variant cannot conclude on the affected foetal status, genotyping is needed and dPCR

can be coupled with the digital relative mutation dosage (RMD).<sup>189,190</sup> Indeed, dPCR permits precise allele quantification and RMD determines if the dosages of the mutant and wild-type alleles of a disease-causing gene are balanced or unbalanced in maternal plasma.<sup>191</sup> Knowing the parental genotypes, it enables to deduce the foetus status (see Fig. 6b).

Evaluation of the cffDNA fraction is the first step of NIPT, playing a crucial role in determining sample quality and test reliability. For a male foetus, dPCR has shown to be efficient and reliable by targeting the *SRY* gene on the Y chromosome as early as 7 weeks of pregnancy.<sup>192</sup> For a female foetus, a positive test is preferred to the assessment of the absence of result for the *SRY* gene. Thereby, it is possible to examine the paternal X-chromosomal alleles for multiple insertion/deletion polymorphisms by dPCR, and it allowed the detection of 42/63 patients bearing a female foetus.<sup>193</sup> In addition to evaluating the cffDNA fraction, these methods enable sex determination, which can lead to further analysis, for example in the case of X-linked inherited human disorder such as haemophilia, adrenal hypoplasia or muscular dystrophy. In haemophilia, only a male foetus will suffer from bleeding disorders, caused by mutations in the coagulation factor genes *F8* and *F9*. With the study of 15 male cases, dPCR has proved to be an affordable method to directly detect these variants in samples with cffDNA ranging from 3% to 33%,<sup>194</sup> enabling an adaptive intervention, like a caesarean to reduce the risk of intracranial haemorrhage during birth.<sup>195</sup> Other strategies based on MSRE and dPCR successfully estimated the cffDNA fraction *via* seven fetal-specific differentially methylated regions.<sup>196</sup>

Although autosomal monogenic diseases are well understood due to their simple inheritance patterns (dominant or recessive), their detection through NIPT has only recently begun. In the case of dominant allele inheritance, the presence of paternal mutation in cffDNA will directly conclude an affected foetus,<sup>197</sup> whereas a maternal mutation will need RMD to determine the foetal genotype.<sup>191</sup> For example, achondroplasia is an autosomal dominant genetic disease caused by mutations in the *FGFR3* gene, leading to dwarfism or skeletal dysplasia. It is usually detected during routine ultrasound in the 3rd trimester of pregnancy and confirmed by molecular testing on foetal genomic DNA obtained by an invasive procedure. In a study on 25 women carrying a foetus at risk of achondroplasia according to ultrasound results, dPCR was compared to mini-sequencing on plasmas and to conventional Sanger sequencing on foetal DNA obtained by amniocentesis. dPCR and mini-sequencing were both concordant with traditional testing, detecting 4/4 cases of achondroplasia.<sup>198</sup> Likewise, a case study of a man affected by an autosomal dominant disease (*MEN1*) used NGS analysis to identify and reclassify the *MEN1* c.654G>T mutation as a pathogenic variant. In this study, dPCR has been performed as a personalised medicine service with a specific design of primers and probe, on the cffDNA of his pregnant wife. It resulted in the absence of the



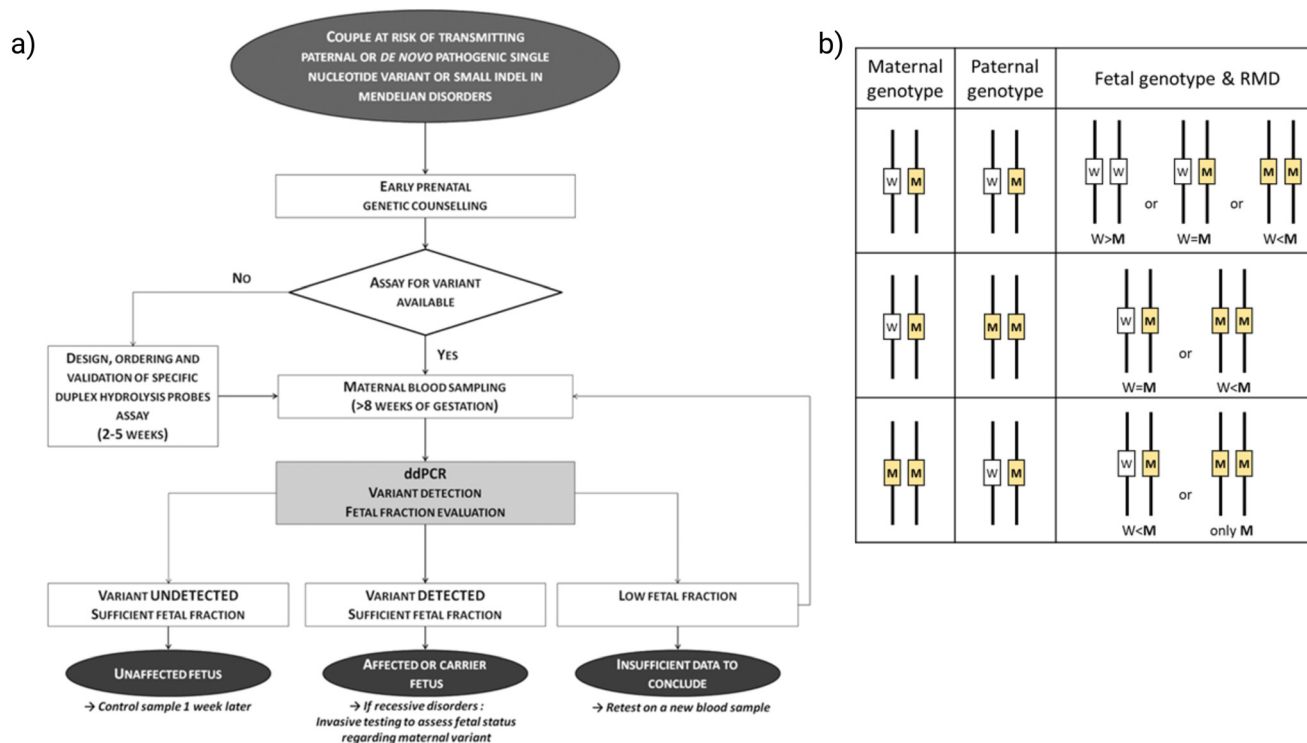


Fig. 6 a) Example of a workflow for implementation of NIPT in the case of paternally inherited monogenic disorder or in the case of *de novo* mutations in clinical practice as a first step into personalised medicine. Reproduced from ref. 211 with permission from John Wiley and Sons, copyright 2022; b) the principle of digital relative mutation dosage. It allows one to deduce the foetus status from the parental genotypes and from the amount of mutant allele (M) and wild-type allele (W) in maternal plasma. For instance, if both parents are heterozygous,  $M = W$  if the foetus is heterozygous, whereas  $W > M$  or  $W < M$ , if the foetus is homozygous for the wild-type or the mutant allele, respectively. When the mother is heterozygous and the father is homozygous and mutated,  $M = W$  if the foetus is heterozygous and  $W < M$  if the foetus is homozygous. When the mother is homozygous and mutated and the father is heterozygous,  $W < M$  if the foetus is heterozygous and without wild-type allele if the foetus is homozygous.

mutated variant, excluding the risk of disease for the foetus.<sup>199</sup> dPCR also enabled the detection of neurofibromatosis, another autosomal dominant disease, due to mutations in the *NF1* gene, at the early late trimester by targeting the paternal *NF1* variant in 3 out of 4 couples and thus correlating with the results from foetal genotyping by invasive sampling.<sup>200</sup> In parallel, this study investigated the *CFTR* mutations causing an autosomal recessive disease namely cystic fibrosis, but necessitated invasive testing to conclude.<sup>200,201</sup> Indeed, in the case of autosomal recessive disease, the presence of the variant alone is inconclusive with regards to the affected status. The use of RMD associated with the highly sensitive allelic quantification of dPCR allowed the foetal genotyping in the case of phenylketonuria due to mutations in the *PAH* gene,<sup>202</sup> in the case of spinal muscular atrophy due to deletion of *SMN1* gene<sup>203</sup> or in the case of diabetes associated with *GCK* or *HNF4A* variants.<sup>204</sup> Thalassemia is another autosomal-recessive inherited disease, resulting from abnormal haemoglobin chain synthesis and leading to blood disorders. The thalassemia type, called  $\alpha$ -thalassaemia, is caused by the deletion of the  $\alpha$ -globin gene, and is seen mainly in Southeast Asia. This variant CNV was detected accurately by dPCR in at least 90% of

cases,<sup>205,206</sup> but the detection of the second variant,  $\beta$ -thalassaemia, by dPCR was not conclusive.<sup>206</sup> Indeed,  $\beta$ -thalassaemia is caused by many mutations in the  $\beta$ -globin *HBB* genes. The most frequent mutations in the Mediterranean area are  $\beta^{\text{IVSI-110 G>A}}$ <sup>207</sup> and  $\beta^{\text{039,208}}$  whereas in Asia it's a 4-base pair deletion (-CTTT) at codon 41/42.<sup>206</sup> dPCR coupled to RMD and Z-score analysis has permitted the identification of almost all homozygous mutated cases, which correspond to the real case in which the foetus could become a  $\beta$ -thalassaemia patient independently of the mutation origin,<sup>208,209</sup> avoiding the need for invasive obstetrical procedures. Moreover, allelic ratios of the heterozygous and wild-type homozygous foetuses were clearly distinguishable without overlapping, permitting correct genotyping as early as the seventh week of gestation.<sup>208</sup> However, inconclusive or misclassified cases may occur from either an insufficient foetal fraction or excessively fragmented cfDNA,<sup>208</sup> highlighting the importance of quality control in cfDNA studies. Thanks to dPCR, it is now possible to screen for multiple disorders with reasonable quantity of maternal blood.<sup>210</sup> Moreover, it is progressively leading to personalised analysis, with the target mutations deduced directly from the parent genotyping<sup>211</sup> (see Fig. 6a).



Other haemoglobinopathies can benefit from dPCR advantages, such as alloimmunisation disorders. Indeed, a pregnant woman presenting an antibody for a blood group antigen requires intensive monitoring to prevent risks of haemolytic disease of the foetus or newborn (HDFN). In the case of Rh blood group antigen D, *RHD* genotyping done in parallel of sex determination by dPCR was found to be much more sensitive than qPCR (sensitivity of 100% for dPCR vs. 83% for qPCR), allowing the RHD-negative women to be administrated prophylactic anti-D treatment.<sup>212</sup> In late first semester samples, dPCR has been demonstrated to be highly reliable in the genotyping of other blood groups, such as in the Kell and Duffy systems, by detecting single nucleotide variants (SNVs) in the D or Fy<sup>a</sup> and Fy<sup>b</sup> antigens respectively.<sup>36,213,214</sup> This contrasts with *Rhd* genotyping, which relies on detecting a gene deletion rather than SNVs. Alloimmunised antibodies can also recognise human platelet antigens (HPA) and generate foetal and neonatal alloimmune thrombocytopenia (FNAIT). The most common antigens are HPA-1a, HPA-5b, HPA-3a and HPA-15b detectable *via* mono or biallelic polymorphisms classified in the Immuno Polymorphism Database. Here again, dPCR has shown to be efficient for the early identification of pregnancy at high risk of FNAIT,<sup>188</sup> with an LoD as low as 0.05% for HPA-1a and non-ambiguous results on the 13 pregnant women tested.<sup>213</sup>

Historically, one of the first targets of prenatal diagnosis was chromosomal aneuploidies, as trisomy diseases originate from copy number aberrations of chromosomes 13, 18 and 21 (for example). The test employed in clinical practice is FISH, a labor-intensive, long (overnight hybridisation is generally needed) and costly technique, or qPCR. In 2019, a combination of duplex dPCRs helped to identify cases of CNV of the chromosomes 13, 18, 21 and Y or X by targeting respectively the genes *MBNL2*, *EHZF*, *PRDM15* and *SRY*, and non-coding region on chromosome X, in a cohort of 133 prenatal CVS samples.<sup>215</sup> It has proved the rapidity, the simplicity and the cost-effectiveness of dPCR as a tool for NIPT. Moreover, in the development of less invasive procedures targeting cfDNA in maternal plasma, the real challenge comes from the low cfDNA concentration. In order to meet the challenge, a proof-of-concept study on trisomy 21 increased the number of targets in a two color 8-plex ddPCR, with 4 FAM-probes targeting genes on chromosome 21 (*BRWD1*, *LTN1*, *NCAM2*, *RUNX1*) and 4 VIC-probes targeting genes on chromosome 18 (*CTIF*, *RIT2*, *SMAD4*, *TCF4*) as a reference, with the aim of increasing positive droplets. This test succeeded to detect trisomic DNA content with a sensitivity of 94% and a specificity of 98% and revealed 16/21 cases of trisomy 21 on a large cohort of 213 pregnant women already screened with an invasive procedure to have foetal karyotype.<sup>47</sup> A study added an enrichment step of cfDNA by size selection to a ddPCR targeting 4 genes on chromosome 21 with FAM-probes (*SETD4*, *CRB1*, *UBE2G2*, *CLDN14*) with references (VIC probes) on chromosome 1 and 2. This method showed an improved sensitivity of 100% for the 50 positive samples and 3 false positive results for the 827 negative samples, giving an overall

accuracy of 99.66% on 877 pregnant women plasma samples.<sup>216</sup> Another study identified cases of trisomy 21, 18 and 13 in 283 clinical samples with a sensitivity of 100% and a specificity of 95.12%.<sup>217</sup>

Although not yet in NIPT, *de novo* mutations are another process for disease apparition in newborns. However, it has been reported that such mutations can actually come from parental mosaicism,<sup>218,219</sup> a condition in which cells within the same person possess more than one genetic line. Indeed, in a study on alternating hemiplegia of childhood, dPCR results revealed that 7.5% (6/80) of cases classified by sequencing as *de novo* were actually linked to parental mosaicism<sup>220</sup> and they correlated the MAF of mosaicism with phenotype severity. Many other dPCR-based studies revealed mosaicism from the mother<sup>221,222</sup> or father,<sup>223,224</sup> whereas newborns were initially classified as presenting *de novo* mutations with asymptomatic parents. Such studies highlight the importance of mosaicism identification in both parents and newborns, to provide supportive genetic counselling and guidance on fertility choices. Moreover, aneuploidy has been shown to be detectable by dPCR even with high maternal mosaic contamination.<sup>225</sup>

In conclusion, the high sensitivity of dPCR enables the analysis of the foetal DNA fraction in the maternal blood sample, making non-invasive prenatal diagnosis a reality. Genetic aberrations such as monogenic disorders, alloimmunisation, aneuploidy and even parental mosaicism can be efficiently identified. Moreover, the targeted nature of dPCR is an advantage in NIPT, compared to non-targeted NGS methods, as it decreases the costs, and it avoids rising questions on ethics from accessing the constitutive genomic sequences of an unborn child.<sup>47</sup>

## IV. Pathogen detection

### 1. Viral infection

Viruses are found in almost every ecosystem on Earth and are the most abundant type of biological entities. They need a host living cell of other organisms to enter and replicate in. For an early detection, very sensitive assays are needed. The gold standard method for detecting viruses is qPCR after an RNA/DNA extraction. But the high sensitivity of dPCR makes it very attractive for an earlier diagnosis.

A recent example of a disease that welcomed dPCR for a more accurate diagnosis is the coronavirus disease 2019 (COVID-19). The COVID-19 outbreak, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), triggered a worldwide public health problem, declared as a pandemic by the World Health Organisation (WHO). Despite the development of antigen and antibody testing kits for rapid diagnosis, the World Health Organisation (WHO) recommends the use of a nucleic acid test as a standard method of confirmation of SARS-CoV-2 infection.<sup>1</sup> Detection protocols by RT-qPCR usually target at least 2 independent genes of the virus genome among parts of the open frame reading gene (ORF1), the spike gene (S), the envelope gene



(E) or the nucleocapsid gene (N).<sup>1,226,227</sup> Although RT-qPCR is high-throughput, compatible with automation and sensitive,<sup>228,229</sup> in some cases the clinical symptoms were not in correlation with the nucleic acid test results (false negatives),<sup>229,230</sup> leading to time and material consuming repeated swab tests. These false negative results could be explained by an insufficient viral load, by experimental errors or by the presence of inhibitors in the swabs that are known to reduce RT-qPCR efficiency. Unfortunately, they could lead to a delay in infection confirmation, an incorrect diagnosis of treated patients in recovery and a relapse after discharge, leading to disease spread. In contrast, dPCR improves sensitivity and accuracy of the diagnosis in clinical samples, particularly in low viral samples.<sup>231</sup> Tao Suo and collaborators demonstrated that, with an LoD of 2.1 and 1.8 copies per reaction for ORF1ab and N, respectively, ddPCR is 500 times more sensitive than qPCR (LoD of 1039 and 873.2 copies per reaction for the same genes).<sup>232</sup> From qPCR to dPCR, the sensitivity rose from 40% to 96% and 26/77 patients were detected negative by qPCR but positive by dPCR. Similarly, Paolo Poggio and collaborators found that 11 (61%) out of 18 qPCR negative patients were positive by dPCR in a cohort of 64 patients, increasing the sensitivity to 89% compared to qPCR (72%).<sup>233</sup> Finally, Chong Liu *et al.* studied only recovering hospitalised patients (43) and determined a cut-off value of 0.6 copy per reaction. On the 9 discharged patients by qPCR, 8 turned out to be positive by dPCR.<sup>234</sup> These results clearly indicate that dPCR drastically reduced the number of false negatives, which makes it especially suited to study asymptomatic and suspected patients or close contacts. Moreover, the reproducibility of dPCR is much better than that of qPCR. Indeed, where qPCR requires calibration curves for quantification, dPCR allows an absolute quantification of RNA by counting the positive reactions. It shows a high degree of consistency by avoiding the variations coming from experimental conditions (analytical protocols, instruments, operators or laboratories) and from the references needed to produce calibration curves.<sup>32,33,235,236</sup> A study recently reported the successful use of RT-dPCR, compared to RT-qPCR, as a reference measurement procedure to perform external quality assessment for molecular diagnostic testing of SARS-CoV-2. While, among three institutes, 61 laboratories observed a good agreement of median values between both technologies, only a <2-fold difference between laboratories was demonstrated for RT-dPCR, whereas RT-qPCR differences were generally between 10 and 50-fold.<sup>237</sup> The superior accuracy and reproducibility of dPCR make it suitable for long-time monitoring of viral load in convalescent patients but also for monitoring the influence of treatment or vaccination.<sup>238</sup> Indeed, the promising drug azvudine (FNC) has been tested on a 281-patient cohort, and the results indicated that it permits a faster virus elimination and a reduced time of treatment.<sup>239</sup> Also, as dPCR is highly resistant to inhibitors, it enables the detection of viral RNA in complex body fluids such as blood. It has also been shown

that the quantitative detection of SARS-CoV-2 (RNAemia) in blood is highly correlated to disease severity.<sup>228,240</sup> This prognostic biomarker could be a crucial asset to predict clinical deteriorations. The inhibitor resistance also led to the development of more direct quantification by shortening the protocols typically with a 1-step RT-dPCR. But the efficiency of this method had questionable sensitivity compared to RNA extracted and analysed by dPCR in 2 steps.<sup>228,241,242</sup> Finally, the potential drawback of diagnosis by acid nucleic testing is the impossibility to distinguish infectious viral particles from non-infectious RNA.<sup>242</sup> However, in the epidemiological context of COVID-19, dPCR presents several advantages, such as rapidity and safety, over the classical culture-based method, which is a labor-intensive and time-consuming (3–4 days) protocol, potentially risky due to required manipulations in high biosafety level settings (BSL3 out of 4) and prone to significant variability from non-standardised protocols and operator errors.

The use of dPCR technology has also shown great interest for the detection and quantification of human immunodeficiency virus (HIV). Although HIV appeared in the 80s, it is still a major issue for global health.<sup>243</sup> It causes the acquired immunodeficiency syndrome (AIDS), which induces a progressive failure of the immune system through the infection of macrophages, dendritic cells and helper T cells (particularly the CD4+ T cells).<sup>244</sup> During the primary infection, the symptoms are not worse than the ones of a general influenza, but in time, the immune system becomes vulnerable to life-threatening opportunistic infections and cancers. The current treatment of HIV consists in the use of antiretroviral therapies (ART) that block different steps of the HIV transcriptional cycle.<sup>245</sup> Despite an effective suppression of plasma viremia by ART,<sup>246–248</sup> the virus remains present in the so-called latent reservoir of infected cells<sup>249</sup> harboring replication competent proviral HIV DNA in their genome, allowing its persistence and rebirth as soon as ART is stopped. It has been reported that HIV DNA, as well as HIV RNA, before and during treatment, has prognostic significance and can predict treatment efficacy.<sup>249,250</sup> As early as 2012, dPCR was used to monitor levels of total HIV DNA in patients on ART.<sup>246</sup> Compared to the gold standard qPCR methods, the dPCR superiority in terms of sensitivity has been questionable. Semi-nested qPCR was shown to be more sensitive than ddPCR in samples from patients on ART, particularly for low viral charge samples.<sup>251,252</sup> In contrast, similar sensitivity between these methods has been demonstrated by others.<sup>38,253</sup> False positive signals were also described to affect the detection power of dPCR,<sup>38,246,251,254,255</sup> and the threshold between positive and negative partitions is a real challenge to determine.<sup>254,255</sup> On the other hand, dPCR exhibited a better accuracy and reproducibility.<sup>252,256,257</sup> Moreover, dPCR absolute quantification enabled one to highlight the progressive loss during culture of HIV from 8E5 cells, the cell line used as a classic standard for qPCR calibration. A deviation of the number of HIV DNA contained per 8E5 cell from 1 DNA copy





initially to 0.73–0.43 copy per cell depending on sources has been demonstrated.<sup>252,256</sup> Such results imply an overestimation of the DNA copy number detected by qPCR and then of the latent viral reservoir. It could thus lead to incorrect patient monitoring that would have consequences on patient health. Follow up of treatment response by dPCR is also possible.<sup>258</sup> For example, studies revealed how important are the timing of treatment initiation and the treatment itself (regimen and exposure) to affect the HIV reservoir.<sup>259,260</sup> Furthermore, dPCR multiplexing and robustness to target sequence variations turned out to be an important feature in the detection and study of HIV. Indeed, the HIV genome often contains defects such as hypermutations or deletions and might not be efficiently transcribed after latency reversal.<sup>251,261</sup> Therefore, the study of intact proviruses is crucial. Some dPCR methods such as Rainbow 5-plex dPCR<sup>262</sup> or an intact proviral DNA assay (IPDA)<sup>234,236</sup> differentiated and quantified intact proviruses (<10% of the total proviruses) from replication-defective ones and thus studied their dynamics. For these studies, dPCR presents the advantage of being faster, more accurate and less time and reagent consuming than culture methods.<sup>263</sup> Also, this sequence tolerance allowed the development of two HIV assays by ddPCR for the detection of the worldwide most HIV prevalent subtypes.<sup>250,264</sup> Adaptation of dPCR to RNA detection has also been useful to study HIV transcription mechanisms.<sup>245,265,266</sup> Not only for patients on ART, dPCR has been used for the monitoring of patients, who underwent allogeneic stem-cell transplantation, with genetically modified cells, in remission at 18 months after ART interruption.<sup>267</sup>

The high sensitivity of dPCR was also demonstrated to be pertinent for the detection of the hepatitis B virus (HBV). Indeed, similar to HIV, HBV DNA is inserted into the nucleus of infected cells, in a more stable converted form, a covalently closed circular DNA (cccDNA).<sup>268</sup> The persistence of cccDNA in infected hepatocytes is a major obstacle to curing chronic hepatitis B. Thus, dPCR methods to detect HBV and monitor patients under treatment have been developed.<sup>269</sup> dPCR has been massively compared for cccDNA detection to classical serological tests<sup>270–272</sup> or to more sensitive qPCR assays.<sup>273,274</sup> Over these routine tests, dPCR demonstrated superior sensitivity and accuracy. Indeed, dPCR's LoD was evaluated at 8 copies per mL in plasma samples,<sup>273</sup> 100 copies per mL in serum samples<sup>271</sup> and 1 copy/20 ng in liver tissue samples.<sup>274</sup> Moreover, the correlation between the tumour stage of HCC and HBV was demonstrated by dPCR, whereas serological tests presented 18.3% of false negative results for HBV DNA detection.<sup>270</sup> Similarly, the integration rate has been correlated by dPCR to the natural clearance of chronic HBV infection.<sup>275</sup> dPCR also allowed the study of occult hepatitis B infection, that is transmitted usually during liver transplantation or blood transfusion. Indeed, as it is characterised by very low concentrations of serum HBV DNA, dPCR provides an added value in the optimisation of its diagnosis<sup>276</sup> but also in the

improvement of the patient therapeutic management before or after a liver transplantation.<sup>277</sup>

The multiplexing capacity of dPCR is highly attractive for the identification of other viruses. For example, it is useful for detecting the four serotypes of the dengue virus<sup>278</sup> or for identifying high-risk human papillomavirus (HPV) serotypes, such as HPV16/18/11/45.<sup>279,280</sup> Considering that HPV infections can increase the risk of developing cancer, it has been shown that ctHPV-DNA is highly correlated to tumour viral load in HPV-associated cancers, such as oropharyngeal squamous cell carcinoma,<sup>281</sup> cervical cancer<sup>282</sup> or anal cancer.<sup>283</sup> These studies demonstrated the potential value of ctHPV-DNA as a biomarker at baseline and during and/or after treatment, highlighting its value for treatment response monitoring,<sup>284–286</sup> as well as for diagnosis.<sup>287</sup>

The use of dPCR was also described as a quality control of adeno-associated virus vectors for HIV immunisation by neutralising antibodies,<sup>288</sup> but also for other viruses like dengue<sup>289,290</sup> or Ebola<sup>291</sup> viruses to determine the ratio of particles to infectious units requested by the WHO for vaccine manufacturing.

In conclusion, dPCR offers exceptional sensitivity and accuracy for viral diagnostics, especially in cases of low viral loads or complex infections. Its success in COVID-19 detection has paved the way for its use in monitoring chronic infections like HIV and hepatitis B, and its multiplexing ability enhances detection of various viruses. Despite some challenges, dPCR shows great potential for improving early diagnosis, treatment monitoring, and standardising viral testing globally.<sup>292</sup>

## 2. Non-viral infection

Non-viral infections can be caused by bacteria, fungi or parasites. The methods usually used for their detection are culture-based. But inevitably, they are time-consuming and labour-intensive, which inflates the costs, making them unaffordable in some countries. Nucleic acid testing such as dPCR can provide a solution to these problems. Here we present specific examples of diseases that benefited from dPCR's advantages.

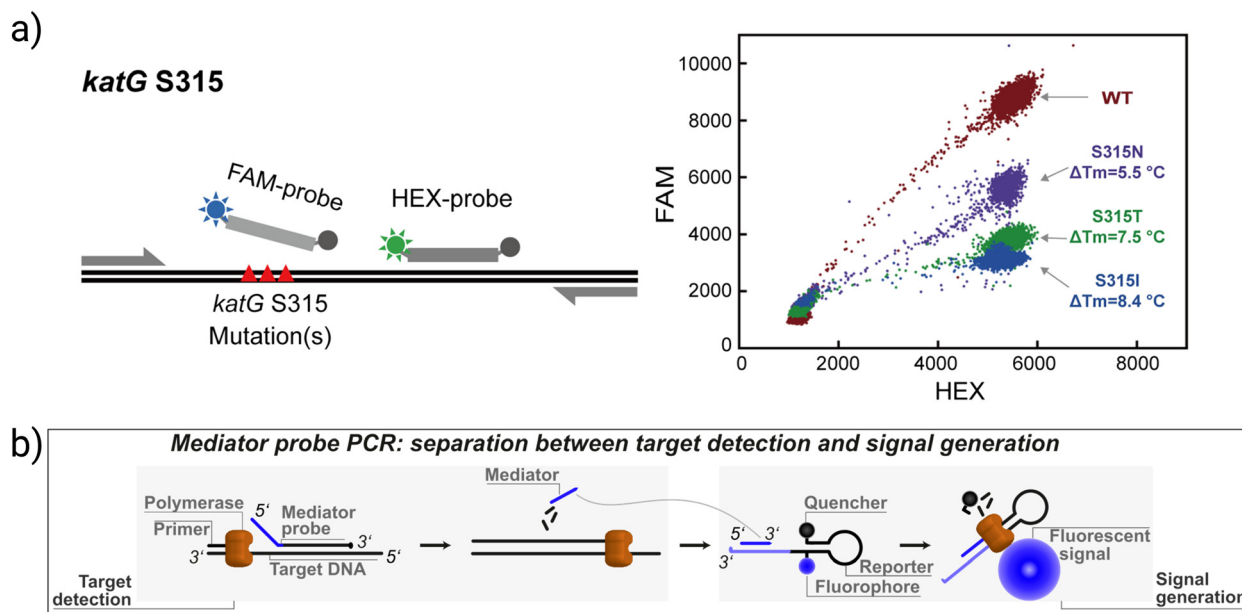
With 10.6 million infected people and 1.3 million deaths, tuberculosis, caused by *Mycobacterium tuberculosis* (MTB), was the second highest killer worldwide in 2022, after COVID-19.<sup>293</sup> MTB usually infects the lungs, but it can also disseminate in extrapulmonary organs,<sup>286</sup> complicating its detection: indeed, collecting samples from these distant sites often requires invasive procedures like surgery. It has been demonstrated by dPCR that MTB can be detected through circulating DNA in plasma.<sup>294,295</sup> Particularly in comorbidity situations, like the case reported by the team of Yamamoto, the non-invasiveness of dPCR is crucial: dPCR successfully detected MTB in plasma, where urine, sputum and blood samples all tested negative using non-dPCR based commercial tests, except in liver tissues after autopsy.<sup>296</sup> Moreover, for an earlier diagnosis of MTB, it has been shown



that the detection rate in low concentration samples is higher when using exosomal DNA than cfDNA.<sup>297</sup> The clinical sensitivity was estimated to 75% and 61.4%, respectively. Otherwise, most of the time, tuberculosis is latent and cannot be easily detected. Thanks to the multiplexing character of RT-dPCR, differentiation between MTB, latent MTB and other diseases has been proven feasible by targeting different transcriptional signatures.<sup>298</sup> Furthermore, a dPCR-based study in CD34-positive PBMCs has reported that MTB DNA is a good biomarker of latent MTB.<sup>299</sup> On the other hand, MTB is usually treated with antibiotics, but multi-drug resistance emerges when inappropriate health care is provided. Thus, precise strain identification and drug susceptibility testing (DST) are needed. Unfortunately, usual culture assays are not suitable as their turnaround times are too long (5–24 days for identification and DST), the results are not reliable, and they require a lot of material which increases the costs.<sup>300</sup> For these reasons, new methods have been developed for DST. For example, the combination of culture and ddPCR enabled the detection in 5 hours and the DST within 4 days directly from sputum.<sup>301</sup> Also, a drop-off triplex ddPCR assay targeting all the mutations on the major resistant genes for isoniazid has been optimised allowing patient monitoring during treatment (see Fig. 7a). It revealed a correlation between bacterial load and symptoms, an interference of hyperglycaemia with drug efficacy and a slower decrease of bacterial load in the case of multi-drug resistance.<sup>302</sup>

Bloodstream infections (BSI) are another important public health threat worldwide with high mortality and morbidity,

particularly those leading to sepsis.<sup>303</sup> The definition of sepsis was adjusted in 2016 as “a life-threatening organ dysfunction caused by a dysregulated host response to infection”.<sup>304</sup> 25–30% of sepsis cases are due to bloodstream infections.<sup>305</sup> These diseases can be due to diverse pathogens as fungi or bacteria, but the key ones are *Klebsiella* spp. (species), *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus* spp., *Streptococcus* spp. and coagulase-negative staphylococci.<sup>306</sup> They are treated with antibiotics, and it has been shown that the sooner the antimicrobial therapy starts the better the chances of survival. Indeed, each hour of delay between hospital registration and antibiotics administration is associated with a 9% increase in the odds of mortality and the median time is 2.1 h.<sup>307</sup> Thereby, patients are rapidly treated with broad-spectrum antibiotic, which can be inadequate and can result in drug toxicity, antimicrobial drug resistance and an increase in hospital readmissions and health costs. Indeed, the current testing assays are culture-based, but their turnaround time is too long.<sup>308,309</sup> It is thus urgent to develop fast and accurate assays for identification and DST. An infection is classically detected *via* the host immune response, *i.e.* through the white blood cell count, C-reactive protein or procalcitonin (PCT) levels for example. It has been shown that dPCR targeting HLA-DRA RNA (coding for the alpha protein of the MHCII complex) used in combination with PCT had a better predictive ability than PCT only to detect sepsis.<sup>310</sup> Also, the IgM response detected at a transcriptomic level by dPCR has been proved to be a promising approach for an early diagnosis.<sup>311</sup> On the other hand, the direct detection of species-specific signature by dPCR



**Fig. 7** Schematic illustration of the drop off dPCR strategy to target all mutations in antimicrobial resistance (AMR) genes: the reaction contains a FAM-labelled drop-off probe targeting *katG* 315 and a HEX-labelled reference probe spanning an adjacent invariable region. Mutations in *katG* 315 were detected as FAM<sup>low</sup>/HEX<sup>high</sup> with a vertical shift, which could be distinguished from the FAM<sup>high</sup>/HEX<sup>high</sup> double positive droplets of the wild-type sequence. Reproduced from ref. 302 with permission from the American Society for Microbiology, copyright 2023; b) schematic overview of the mediator probe PCR principle, showing the separation between the target detection and fluorescence signal generation steps. Reproduced from ref. 355 with permission from MDPI, copyright 2024.



has been demonstrated to detect rapidly in approximately 4 hours,<sup>312–314</sup> with a high sensitivity and specificity, the key bacteria responsible for BSI in blood,<sup>312,313,315</sup> enabling an early detection of sepsis.<sup>316</sup> Indeed, the multiplexing capability of dPCR has been a major asset for targeting both specific signatures and antibiotic resistance gene,<sup>317–319</sup> allowing guidance for antimicrobial therapy. To enlarge the BSI cause identification, fungi-specific genes can be added as targets in addition or not to bacterial genes and antimicrobial resistance (AMR) genes in blood samples,<sup>320–322</sup> but also in fecal samples<sup>323</sup> or pleural and peritoneal fluids.<sup>324</sup> One of these studies highlighted that dPCR is more efficient to detect polymicrobial infections than culture-based assays.<sup>321</sup> The highly conserved bacterial 16S rRNA and fungal 28S rRNA can also be targeted by duplex dPCR, for the differentiation between bacterial and fungal BSI.<sup>325</sup> It's worth noting that reagents used for bacterial lysis are generally incompatible with dPCR buffers or droplets,<sup>326</sup> implying an indirect detection of DNA released by bacteria in bloodstream or other biofluids. Isothermal amplification-based technologies, less prone to detergent inhibition, could be investigated for direct detection.<sup>327,328</sup> However, because of their low multiplexing and specificity, they require further development in order to be considered for the clinic.

Finally, in a non-clinical application but non negligible topic, dPCR is very useful for sensitive detection of foodborne pathogens such as Shiga toxin-producing *Escherichia coli*<sup>329,330</sup> or of biothreat bacteria such as *Bacillus anthracis* or *Yersinia pestis*.<sup>331</sup>

In conclusion, dPCR is highly efficient for the precise identification of pathogens without the need for enrichment steps, considerably reducing turnaround time to approximately 4 hours. This enables timely patient management, reduces morbidity and mortality and helps prevent the escalation of antimicrobial resistance.

## V. Perspectives of dPCR

### 1. Integration of dPCR with other methods of detection

Besides nucleic acids, proteins are another important class of biomarkers closely linked to each individual phenotype. Traditional protein detection methods, such as western blot or ELISA bioassays, often lack sensitivity and accuracy. The nascent field of digital protein detection has nonetheless rapidly expanded, offering the same benefits as digital PCR over qPCR. We will focus on the technologies that bridges these two fields (for a comprehensive review on digital detection of proteins, the reader may refer to D. Duffy's article<sup>332</sup>). In fact, PCR has for a long time been employed to enhance the sensitivity of protein detection. Notable examples include heterogeneous formats like immuno-PCR and other adaptations of the microplate ELISA protocol with isothermal nucleic acid amplification methods such as immuno-RCA or immuno-LAMP. Additionally, homogenous assays like proximity extension assays (PEA) or proximity ligation assays (PLA) have been developed. In these

approaches, two DNA-labelled monoclonal antibodies bind to separate epitopes of the same target protein, enabling the DNA tags to come into close proximity and be either extended or ligated. The resulting duplex can then be quantified by qPCR. These assays have since been adapted to a digital format to answer the need for accurate quantification.

Schröder *et al.* modified the standard microplate immuno-PCR protocol to release the DNA-tagged antibody following the formation of the complex with the target protein (IL-2 or IL-6), allowing for its subsequent quantification by ddPCR.<sup>333</sup> Another approach involves grafting the capture antibody on nano/microparticles instead of on a microplate. At high particle concentration, the formation of the sandwich [capture antibody/target/DNA-tagged detection antibody] is governed by the Poisson law, which allows for digital readout after isolating the particles followed by PCR amplification of single DNA tags. Zhang *et al.* employed magnetic nanoparticles combined with droplet PCR for the quantification of  $\alpha$ -synuclein in serum;<sup>334</sup> Vanness *et al.* used fluorescently-encoded microparticles isolated in microwells for the multiplex and multimodal detection of miRNA let-7a and cytokine IL-6.<sup>335</sup> Li *et al.* introduced another multimodal approach, termed digital simultaneous cross-dimensional output and unified tracking (dSCOUT), which integrates CTC enrichment and DNA-antibody conjugate tagging of surface markers. This approach enabled the simultaneous analysis of three proteins (including the tumour specific-marker EpCAM) and three mRNAs in CTCs, demonstrating its diagnostic potential for HCC.<sup>336</sup>

Extracellular vesicles and exosomes can similarly benefit from ddPCR quantification by being labelled with DNA-tagged antibodies. Ko *et al.* applied direct labelling of EVs with orthogonal antibody-DNA conjugates, which allows single EV phenotyping for EGFR and EpCAM markers.<sup>337</sup> Lin *et al.* adapted the PLA approach to analyse the PD-L1 status of tumour-derived exosomes;<sup>405</sup> in their method, two aptamers recognised EpCAM and PD-L1, producing a ligated product only if the exosome displays both markers, eliminating the interference of non-tumour-derived exosomes and soluble proteins.

Abasıyanık *et al.* adapted the PLA protocol to the detection of both nucleic acids (bacterial DNA) and proteins (IL-6 and TNA- $\alpha$ ) for the prediction of septic shock outcome in patients.<sup>338</sup> Byrnes *et al.* developed a simplified protocol, which does not require washing the excess of antibody and uses a polydisperse emulsion.<sup>339</sup>

In summary, these new technologies are paving the way to diversify the field of application of dPCR, beyond genomic or transcriptomic, to proteomic.

### 2. Remaining challenges of dPCR & potential ways of improvement

Despite its undeniable strengths (calibration-free, high sensitivity, absolute quantification), some technical points



remain to be optimised in order to reach better performances (Table 3).

**a) Multiplexing.** For clinical applications, the multiplexing capacity is defined as the number of markers detectable by dPCR, independently of the fluorescent channel considered. It is tightly linked to probe specificity and sensitivity. Indeed, the simultaneous detection of highly homologous targets such as single nucleotide polymorphisms calls for highly specific probes. It's worth noting that although TaqMan probes are the most widespread in the world of real-time PCR, other fluorophore-labelled molecules exist (for an exhaustive review on probes, see Navarro *et al.*<sup>340</sup>).

The use of the locked nucleic acid (LNA) in the detection probes has been considerably used and reported in dPCR. Thanks to a 2'-O,4'-C methylene bridge in the ribose moiety, these optimised probes have a higher affinity to the complementary DNA, which increases both duplex stability and mismatch discrimination. Their use has been suggested to reduce the rain droplets.<sup>341</sup> The high stability and specificity of LNA-modified probes allowed the detection of driver mutations at early stage<sup>342</sup> or in metastatic<sup>343</sup> pancreatic cancers and to target more mutations, up to 40 biomarkers, in the case of foetal aneuploidy for trisomy 21 detection.<sup>344,345</sup> Moreover, Hashimoto *et al.* developed an LNA-clamp ddPCR strategy, where LNA-modified oligonucleotides bind to the wild-type sequence in order to inhibit its amplification. This study highlighted the presence of minor mutated clones of *ESR1* with a MAF of <0.1% in fresh frozen tissues of patients with BC.<sup>346</sup>

Another strategy to increase the number of biomarkers targeted is the drop-off dPCR (see Fig. 7a), where a reference

probe targets an invariable region in the vicinity of the mutational hotspot, whereas a drop off probe targets a wild-type sequence, leading to an absence of a double positive signal in the case of a mutated allele. It has been successful in the detection of drug-resistance mutations in MTB<sup>302</sup> and of cancer mutations<sup>81,347</sup> improving the number of targets up to 69 hotspot mutations, but not permitting the identification of these mutations.

From a technological point of view, multiplexing is the ability to detect and identify simultaneously multiple targets using orthogonal signals. dPCR displayed a relatively low multiplexing capability, often limited to less than a dozen targets, as opposed to microarrays that can measure hundreds to thousands of targets simultaneously. This limitation arises primarily from the restricted number of available fluorescence channels, and the spectral overlap that occurs as more fluorophores are added. In addition, increasing the numbers of primer pairs and probes may affect the amplification efficiency and raises the risk of artefactual reactions such as primer dimers.

Multiplex dPCR typically relies on three main strategies: spectral-encoding, intensity encoding or combinatorial-encoding, or a combination of these. Spectral encoding utilises distinct fluorescence channels with orthogonal probes (up to 7-color for the latest Nio system). Intensity-encoding differentiates targets based on varying probe concentrations,<sup>30</sup> producing distinct fluorescence clusters. Alternatively, an intercalating dye can also achieve intensity encoding given that the amplicons are of different sizes. Combinatorial encoding expands multiplexing further by assigning unique targets to specific combinations of

**Table 3** Challenges associated with dPCR and potential strategies for improvement

| Challenge               | Technology  | Principle   | References                         |
|-------------------------|---|---|------------------------------------|
| Multiplexing            | Lock nucleic acid-modified probes                                     | LNA makes the probes thermally more stable and more specific  | 341, 343–346                       |
|                         | Drop-off probes   | Fig. 7a   | 81, 302, 347                       |
|                         | Melting curves analysis coupled to dPCR                               | Melting curve at the end of the real-time dPCR: amplicons discrimination by their different $T_m$   | 348–352                            |
|                         | Photobleaching probes<br>Mediator probes                              | See article<br>Separate the DNA detection from the fluorescent signal generation  | 353<br>354, 355                    |
| Dynamic range           | 3D analysis dPCR  | 1-Million bilayer droplet array   | 28                                 |
|                         | Multivolume droplet-dPCR  | dPCR coupled to light-sheet microscopy<br>Smaller droplets used for a better upper limit of quantification (LQ), larger ones are used to decline the lower LQ | 16, 356, 403<br>358, 361, 362, 404 |
|                         | Virtual partitioning  | In a high target concentration regime: intensity-encoding with multicolor probes  | 364                                |
| Threshold determination | Computation   | Automatic thresholding  | 364, 365                           |
|                         | Computation   | Automatic cluster labelling   | 366, 367                           |
| Portability             | Microfluidic-free partitioning  | Polydisperse emulsion with analog readout   | 370                                |
|                         |   | Particle-templated emulsification system  | 368                                |
|                         | Centrifuge-based partitioning<br>Smartphone-based POCT                | Pipette-based droplets microprinting  | 352                                |
|                         |   | Dual flow-focusing function; lab-on-a-disc<br>Integration of dPCR on a smartphone for point-of-care testing   | 371, 372 (respectively)<br>373–378 |
| Handling-free protocol  | Droplet microfluidic (DMF) system<br>DMF and centrifugal microfluidic | EWOD-controlled droplet movement  | 386, 387                           |
|                         |   | POCT dPCR (portable and all-integrated protocol)  | 388                                |





fluorophores, effectively increasing the number of detectable targets beyond the available spectral channels.

Planar array imaging integrated with a thermal control enables the recording of melting curves for individual partitions, which has been leveraged for discriminating different amplicons from single-color probes. Applied in microchambers, this strategy has shown promising results for KRAS genotyping in pancreatic cancer samples,<sup>348,349</sup> where the amplicons from different mutants display distinguishable  $T_m$ . To implement this idea to droplet-based dPCR, an algorithm has been developed to correct the droplet displacement during thermocycling.<sup>350</sup> In a study on AMR-bacterial infection, this technology was coupled to a machine learning algorithm, enabling the discrimination of two single-color probes with very similar signals from the same droplets, *via* the probe differences on the entire kinetic profile during amplification, not only their differences in  $T_m$ .<sup>351</sup>

In another study, Li *et al.* developed a sophisticated strategy to exploit the difference in the melting temperature between the target and probes with no need to record the complete temperature profile.<sup>352</sup> In this case, the non-hydrolytic probe is composed of a universal forward primer anchor domain, a signal domain modified with a fluorophore and a quencher and multiple barcode domains that are complementary to different reverse primers. In an asymmetric PCR regime (with an excess of forward primers that bear the probe binding domain), the single-stranded amplicon can anneal to the probe forming a loop with the universal forward primer binding domain and the target-specific reverse primer barcode domain that display differential melting temperature. As these complexes display differential melting temperature, the target amplicon can be identified by measuring the droplet fluorescence at different temperatures. Using four probes of different colours and two imaging temperatures, the authors successfully demonstrated an 8-plex, that includes a reference sequence and seven *EGFR* mutation variants encompassing 35 subtypes.

The differential photobleaching property of fluorochromes was also leveraged in a multiplex configuration. In this approach, two probes are used: one labeled with a photosensitive dye and the other with a photostable fluorophore. Droplet imaging is performed before and after photobleaching, causing the signal from the photosensitive dye to extinguish. This enables unambiguous indexing of the targets using a single fluorescence channel. Using the same strategy with three channels, the authors developed a 6-plex compatible with different target panels that gave promising results still to be tested on non-synthetic samples.<sup>353</sup> One may imagine that recording the entire photobleaching kinetics would allow the creation of more than two virtual channels, although this has not yet been demonstrated, to the best of our knowledge.

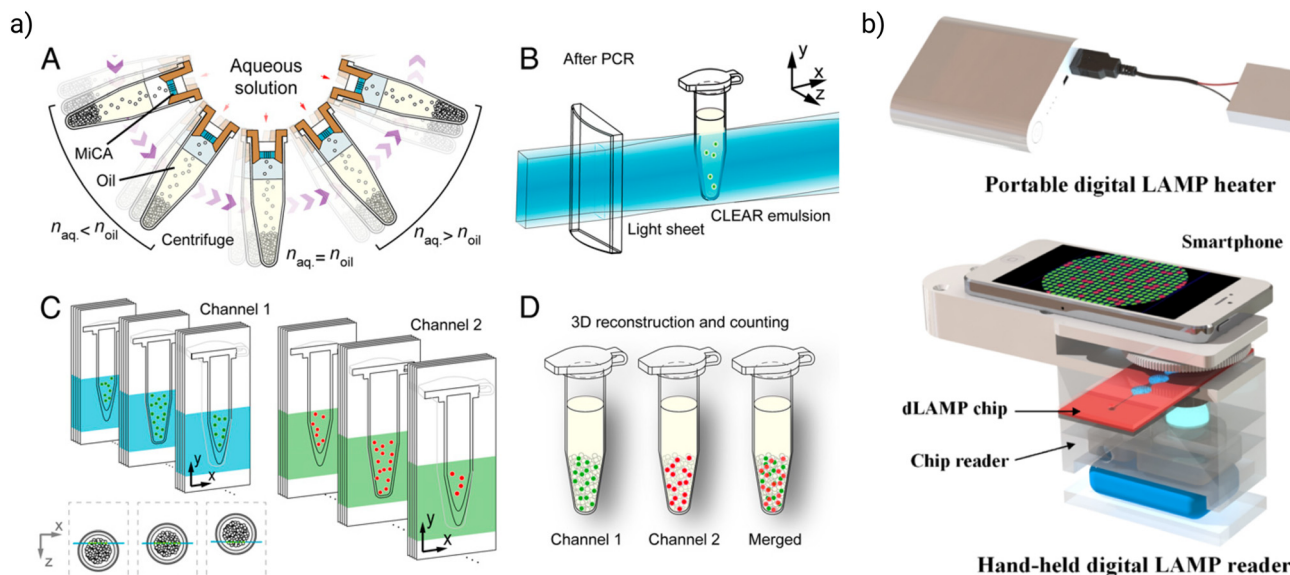
On the other hand, besides the physical limitations of fluorescence, a higher multiplexing capacity implies a cumbersome molecular optimisation coming from increasing

the numbers of primer pairs and probes. To overcome this limitation, a strategy consists in separating the DNA detection from the fluorescent signal generation, by using a mediator probe (see Fig. 7b). Compared to LNA-modified probes, this technology gave similar performances for the detection of *KRAS* and *BRAF* mutations (4-plex) on samples from patients with CRC.<sup>354</sup> Based on this technology, the same team elaborated a generic fluorogenic 6-plex reporter set compatible with different target panels that gave promising results still to be tested on non-synthetic samples.<sup>355</sup>

**b) Dynamic range.** The dynamic range of an assay represents the difference between the lower and upper limits of detection. In dPCR, this range spans 3–4 orders of magnitude (OoM), yet it still lags behind its analog qPCR counterpart by 3–4 decades. Several key factors influence the dynamic range of dPCR that include the false positive and false negative rates, which affect the lower and higher limits of detection, respectively. Additionally, partition volume plays a crucial role: larger partitions tend to shift the dynamic range toward lower concentrations, while smaller partitions favour higher concentrations. The number of partitions also impacts the dynamic range, as a greater number theoretically improves both the lower and upper limits of detection, thereby widening the range. For instance, Hatch *et al.* developed a 1-million droplet array with a wide-field planar imaging readout, achieving a 5-OoM dynamic range.<sup>28</sup> To do so, they exploited a self-assembly high-density packing of droplets in 3D, with a double layer. However, such a strategy is only viable for highly sensitive and specific amplification reactions, as an excessive number of false positives or false negatives could offset its benefits. The 3D readout of droplets has also been leveraged by coupling dPCR and light-sheet microscopy, for 3D fluorescence imaging (see Fig. 8a). A first system, namely CLEAR-dPCR, was developed by Liao *et al.*, where half a million droplets produced by centrifugation were imaged in 3D. It successfully quantified, with a 5 OoM dynamic range, CNV of chromosome for sex determination or for tuberculous sclerosis complex detection, an autosomal dominant disorder with exonal deletion.<sup>16</sup> Shum *et al.* adapted this system with 25 times smaller droplets, enabling the generation of >30 million droplets out of 50  $\mu\text{L}$  samples.<sup>356</sup> This UltraPCR protocol has been applied to aneuploidy detection, targeting 74 regions in chromosomes 13, 18 and 21 (each chromosome being detected in a separate fluorescence channel) through a 6 log dynamic range. In addition, in both CLEAR-PCR and UltraPCR, every ddPCR step happened in the same tube: compartmentalisation in droplets is done in a centrifuged tube that is then closed, thermocycled and analysed by 3D imaging. The advantage of such a process is to avoid sample loss issue and operational contamination.

The dynamic range may also be artificially enlarged simply by testing samples at different dilutions, which mathematically brings a concentrated sample that exceeds the dynamic range (by partition saturation) within the





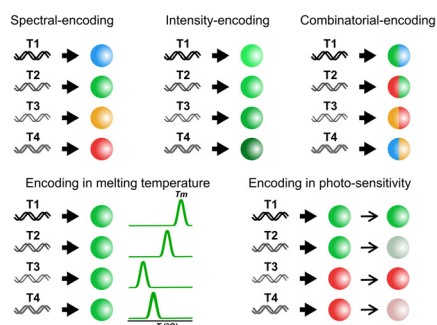
**Fig. 8** a) Schematic illustration of the CLEAR-dPCR process with (A) the droplet generation by centrifugation, (B) the high-throughput readout of bulk PCR droplets by 3D light-sheet microscopy, (C) the dual-channel light-sheet fluorescence image sequences, and (D) the volumetric reconstruction of dual-channel images. Reproduced from ref. 16 with permission from United States National Academy of Sciences, copyright 2020; b) example of a fully-integrated smartphone-based device of dPCR for point-of-care testing allowing an isothermal amplification. Reproduced from ref. 373 with permission from the American Chemical Society, copyright 2021.

quantification region.<sup>357</sup> Alternatively, the sample may be split into partitions of different sizes, effectively associating target concentrations with multiple Poisson parameters ( $\lambda$ ). This approach is compatible with multiple partitioning techniques such as surface-assisted droplet printing,<sup>358</sup> centrifugal step emulsification<sup>359</sup> or microchambers.<sup>360</sup> The Ismagilov group adapted the SlipChip design to accommodate wells ranging from 1 to 125 nL, achieving a dynamic range of 5–6 OoM for the quantification of viral RNA associated with HIV viral load.<sup>361</sup> In addition to being

cost and time-effective, polydisperse emulsions naturally generate a wide range of droplet sizes, which might be leveraged to extend the dynamic range, given a proper droplet analysis framework is provided.

To extend the upper limit of detection beyond the partition saturation point, several methods have been proposed to estimate the number of targets per partition. Luo *et al.* combined the SlipChip multivolume design with real-time partition monitoring: at partition saturation, where the digital information is lost, the  $C_q$  extraction from the

## MULTIPLEXING



**Fig. 9** Summary of challenges in dPCR, as ways of improvement of dPCR technology. Beyond spectral-encoding, intensity encoding and combinatorial-encoding can be used to index the positive partitions to their target. Other methods such as melt-curve analysis or the use of photo-sensitive/resistant dyes in PCR probes may be used in standalone or in combination with the above-mentioned strategies. The dynamic range may be extended using multi-volume partitioning. When close to saturation, strategies to infer the number of copies per partition such as switching to the analog mode of qPCR or virtual partitioning<sup>364</sup> may further increase the higher limit of quantification. Alternative partitioning strategies have been proposed to simplify the process. These include microfluidic-free techniques such as polydisperse emulsification, particle-templated emulsion or the Oscidrop technology (image adapted from ref. 391). The Slip-Chip technology has also been developed to democratize dPCR by proposing a versatile and easy-to-use microchamber-based chip.



analog PCR regime can be used to infer the average target concentration.<sup>362</sup> Similarly, Jacky *et al.* introduced virtual partition dPCR, an intensity-encoding scheme with multicolour probes, coupled with a mathematical model, known as high-definition PCR,<sup>363</sup> to estimate the number of target copies per partition.<sup>364</sup> At high target concentrations, where negative partitions are nearly absent, quantitative information can still be retrieved by analysing the target distribution across all positive droplets. However, this strategy is only applicable in a multiplex context, as it relies on detecting different target combinations within individual droplets, each yielding a unique signal (Fig. 9).

**c) Threshold determination.** In virtual partition PCR as in any dPCR data analysis, threshold determination is crucial to ensure accurate classification of positive and negative partitions, minimise errors and improve quantification precision. It has been shown that the threshold of positivity can bias significantly the results, as it represents a balance between sensitivity and specificity.<sup>103,118</sup> In addition, threshold determination may be affected by what is called “rain droplets”, which are droplets with intensity between the positive and negative clusters that may be classified in both clusters, inducing further uncertainty. Although dPCR apparatus software offers an automatic determination, it is often limited to 2 channels and based on an unverified hypothesis.<sup>365</sup> Therefore, few studies have developed automated pipelines for threshold setting, based on data-driven statistical or algorithmic functions. For example, the hypothesis of a normal distribution of the fluorescence amplitude among droplets has been avoided by modelling the fluorescence extreme values of the negative droplet population and with a baseline correction between samples, the automated method gave more accurate results than QuantaSoft (Bio-Rad).<sup>365</sup> A digital PCR cluster predictor together with an R package and a Shiny app has also been developed to automatically analyse up to 4-plex ddPCR data.<sup>366</sup> Similarly, Polytect is an automatic cluster labelling component for multiplex dPCR without limit in color.<sup>367</sup> dPCR combined with the power of computational analysis allowed one to considerably and accurately improve the multiplexing capacity of dPCR.

**d) Portability and ease-of-use towards point-of-care testing.** At the present time, commercial dPCR instruments, tested in the clinical studies and reported in this review, are not intended for point-of-care testing (POCT). They imply professional operations, expensive consumables and instruments, as well as voluminous apparatuses, which are not adapted for POCT. Indeed, POCT requires affordability, ease of operation and portability to spread in the resource-limited area.

A strategy to facilitate the accessibility to ddPCR is to develop microfluidic-free partitioning techniques. In a seminal work, the Abate group reported the use of a particle-templated emulsification system. Monodisperse hydrogel (agarose or acrylamide) particles are mixed with the PCR mix and the sample, before being vigorously agitated with the immiscible oil phase. This results in the isolation of the

particles in water-in-oil droplet with similar monodispersity.<sup>368</sup> Along the same line, Heinrich *et al.* used agarose particles covered with a chitosan layer that non-specifically binds to the DNA fragment, allowing the enrichment of the target from a large sample volume, prior to templated emulsification.<sup>369</sup> The Abate group also proposed to convert the digital information, obtained by single molecule isolation in a polydisperse emulsion, into an analog readout:<sup>370</sup> following the dPCR amplification, the emulsion is broken and the enriched amplicon population quantified by a simple qPCR protocol, eliminating the need for a complex droplet analysis technique. The author reported similar analytical performance to a standard dPCR workflow, but this system reintroduces the need for calibration and raised the question of contamination due to the breaking of the post-PCR emulsion.

Another idea was suggested to eliminate microfabricated chips by using pipette-based droplet microprinting.<sup>352</sup> This Oscidrop system allowed a multiplex dPCR to detect *EGFR* mutations or *HER2* CNV in LC or BC FFPE samples, respectively. Despite reduced costs and a rapid process, the machine remained bulky and not portable. Centrifugal microfluidics, cleared from pressure control, has emerged as a simpler and promising solution to address the portability limitation of ddPCR. The team of Shuwen Zeng has developed a lab-on-a-disc (LOAD) device enabling, on the same chip, the generation, thermocycling and analysis (by an external microscope) of droplets. Good performance was demonstrated for screening viruses in clinical samples, but the process still required an external microscope.<sup>371</sup> To fully integrate the analysis, the team of Gangyin Luo has proposed a fully automated instrument with a microfluidic chip, where a rotary valve allows the flow-focusing design (with pressure control) to both generate and analyse droplets. This in-line readout based instrument showed good results for the quantification of the *HER2:CEP17* ratio in cell lines, and tests on human samples could validate this promising technology for clinical diagnosis.<sup>372</sup> In the case of microchamber-based dPCR, smartphone-adapted dPCR devices have been designed, offering on chip compartmentalisation, thermocycling, data acquisition *via* a smartphone camera and image analysis (see Fig. 8b). A lot of research has been conducted on the optimisation of an embedded heating/cooling system, which requires both flexibility and accuracy to support various protocols, from isothermal<sup>373,374</sup> to thermal cycling amplification.<sup>374–378</sup> Another challenge of miniaturised dPCR devices is designing chips compatible with industrial scale production<sup>374</sup> and that can accommodate as many wells as possible to reach the best sensitivity possible with a smartphone camera imager. Most of the devices developed are still at the level of proof of concept with tests on plasmid DNA, but good performances have been proven with 45 cycles in 49 minutes tested for cancer, aneuploidy and COVID-19 detection<sup>377</sup> or with an LoD of 1 copy per





$\mu\text{L}$  in the range of 2–1000 copies per  $\mu\text{L}$  in the detection of EGFR L858R gene mutation in NSCLC.<sup>373</sup>

To improve the ease of operation, the next level of optimisation is the integration of all the protocol steps in a single automated apparatus. In addition to the fully integrated commercial instruments such as NIO, QX ONE, QIAcuity and Absolute Q systems, new platforms are currently under development. For example, an integrated platform based on microfluidic array partitioning has been tested to quantify EGFR T790M in NSCLC samples and BCR::ABL1 fusion gene in chronic myeloid leukemia, detecting MAF as low as 0.01%.<sup>379</sup> Although these platforms are integrated in a single apparatus, they still require a professional to handle the various protocol steps. An emerging technology, digital microfluidics (DMF), could address this problem. DMF is based on electrowetting-on-dielectric (EWOD), and it allows the precise handling of picoliter-to-nanoliter-sized droplets on an array with microelectrodes. Over the past decade, DMF has found extensive applications in molecular diagnostics.<sup>380–384</sup> The GenMark ePlex system, a DMF-based platform for syndromic pathogen detection, has even been approved by the FDA.<sup>385</sup> A DMF-Bimol system has been developed to couple PLA and RT-ddPCR for the analysis of CD147 protein and its transcript, once again revealing a poor correlation between the two.<sup>386</sup> One of the main challenges of an automatic sample preparation for dPCR arises from the wide variety of contaminants depending on the type of sample (sputum, blood, saliva...). Walter Hu's team has developed a system, using magnetic bead-based nucleic acid extraction coupled to DMF qPCR for the detection of 15 pathogens in nasopharyngeal swabs, oropharyngeal swabs, bronchoalveolar lavage fluid and sputum.<sup>387</sup> A handling time of 1 minute could be achieved, as well as a total sample-to-answer detection within 80 minutes with good sensitivity (200–628 copies per mL). The strategy of DNA extraction by magnetic beads could easily be adapted to dPCR.

Another system took advantage of both centrifugal microfluidic and DMF, to propose a sample-to-answer quantification of viral load by a fully-integrated RT-ddPCR. It uses the centrifuge force to move the sample from chambers, each associated with one step of the protocol, namely the lysis, RNA extraction, wash and elution, mixing with PCR reagents, droplet generation and finally droplet imaging.<sup>388</sup> Its applications to SARS-CoV-2 detection demonstrated an LoD of 0.1 copies per  $\mu\text{L}$ , as well as 100% accuracy compared to qPCR on 14 nasopharyngeal swab samples. This very promising system allows a handling-free workflow for sample preparation thanks to a centrifugal platform with pneumatic pumping, as well as portability with on-chip droplet generation, thermocycling and analysis thanks to an embedded heater and epi-fluorescence imaging modules.

## Conclusion

Digital PCR has the potential to become a major technology in the diagnostic field. The demonstration of its high

sensitivity and specificity compared to other techniques, along with its accuracy and reproducibility, has made dPCR a powerful tool for interrogating genetic or epigenetic information of individual patients – particularly in the context of the shift towards personalised and non-invasive medicine. The samples for dPCR can range from DNA extracted from tissue samples (FFPE, frozen) to cfDNA, ctDNA, CTCs or EVs recovered from body fluids such as blood, urine, saliva, pleural or cerebrospinal fluids. The use of dPCR has been validated for a wide range of clinical applications in many medical fields including oncology, prenatal testing, and pathogen detection. However, this technology still presents limitations that hinder its widespread clinical implementation. For example, detection of mutations in ctDNA at early cancer stages remains challenging, and complementary systems are often needed alongside dPCR. Moreover, despite the wide choice in commercialised dPCR platforms, they are still cost intensive (as compared to qPCR for example) and are not yet suitable for point-of-care applications.

Nevertheless, further developments in dPCR are being fuelled by technological advancements, including isothermal amplification,<sup>327,328,389</sup> novel imaging and scanning technologies, and algorithm optimisation for automated thresholding and analysis. These may lead to even better performance in terms of multiplexing, sensitivity and dynamic range, as well as reduced costs. In the field of personalised medicine, dPCR and NGS could represent complementary technologies. An example can be found in oncology, where tumour-informed strategies may involve initial tumour characterisation by NGS, followed by the design of patient-tailored assays for follow-up using dPCR. Furthermore, the increasing development of fully automated dPCR instruments, coupled to the decreasing costs of NGS, may accelerate the spread of personalised medicine, often described as the future of healthcare. Moreover, portable, hands-free, all-integrated processes are central to ongoing development efforts, paving the way for point-of-care applications, such as pathogen detection in resource-limited areas.

## Data availability

No primary research results, software, or code have been included and no new data were generated or analysed as part of this review.

## Author contributions

Supervision: VT; conceptualization: AT, GG, VT; investigation (analysis of >300 publications) and writing of original draft: AT; writing – review & editing: VT, GG, AT, LB.

## Conflicts of interest

There are no conflicts to declare.





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