

## Review

## Droplet digital PCR applications in the tuberculosis world

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## ABSTRACT

Droplet digital polymerase chain reaction (ddPCR) is a third generation of polymerase chain reaction (PCR) that enables the exact quantification of nucleic acid targets within a sample. The capability of ddPCR to accurately detect and quantify low abundant targets has led to its fast-growing applications in detection of different pathogens. This review summarizes the ddPCR technology and its applications in tuberculosis diagnosis. From current studies including a total of 9 publications on the applications of ddPCR in tuberculosis research, it is clear that ddPCR technology offers enormous advantages, such as unparalleled sensitivity, high precision, and absolute quantification without a standard curve, over common molecular diagnostic platforms like the real-time quantification PCR. The latest study also showed that rapid drug susceptibility test of *Mycobacterium tuberculosis* in sputa could be achieved within 4 days. However, the high cost is the main limitation for its wide applications, especially in developing countries. As we near the vision 2030 goal for sustainable development and ending the tuberculosis epidemic by 2030, ddPCR techniques may help achieve this objective and many more discussed in the UNGA-HLM-TB.

## 1. Introduction

According to the latest WHO Global Tuberculosis Report, tuberculosis (TB) still remains the top infectious disease killer worldwide; with a best estimate of 10 million people developing TB disease and an approximate 1.6 million people succumbing to the disease globally in 2017 [1]. This disease that has sparked debates worldwide is caused by the bacteria *Mycobacterium tuberculosis* (*Mtb*). Global efforts geared towards ending the TB epidemic by 2030 have been undermined by the emergence of multi-drug resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) [2,3]. This has prompted nations to come together in an effort to find solutions on how to fight the disease. An affirmative step towards the right direction includes the first ever United Nations General Assembly High-Level Meeting on Tuberculosis (UNGA-HLM-TB) held on 26 September 2018 [3,4]. The theme of this historic meeting attended by various stake holders was 'United to End TB: An Urgent Global Response to a Global Epidemic' [4]. All declarations made in this meeting affirmed the vision 2030 goal for sustainable development and ending the tuberculosis epidemic by 2030 [5]. These goals target reducing TB deaths by 90% and reducing TB incidence by 80%, with no family affected facing devastating financial costs [6].

The gold standard for tuberculosis detection and diagnosis has

always been culture based (solid and liquid culture systems). As effective as this method may be, it still suffers from challenges in rapid diagnosis of the tuberculosis disease. Such limitations have drove scientists to seek alternatives that are non-culture based to replace the old fashioned culture techniques [7,8]. Among these alternatives includes advancements in molecular diagnosis of TB that have proved to be superior to the culture-based methods. Polymerase chain reaction (PCR) is a molecular diagnostic technique extensively used to amplify a specific gene of interest. Applications of PCR in molecular biology have led to the development of quantitative PCR (qPCR) and digital PCR (dPCR) systems to detect and diagnose disease pathogens [8,9]. Both these systems have been used widely in absolute quantification of pathogens with the digital PCR system having many advantages over qPCR due to it not needing a standard curve when absolutely quantifying nucleic acid target sequences.

Digital PCR was conceptualized in the 1990s, and works by diluting a sample then partitioning it into hundreds to millions of separate reaction chambers so that each chamber may contain a single or more copies of target sequence while others contain no target sequence [10,11]. From this, positive partitions (those that contain target sequence) versus negative partitions (those with no target sequence) are counted. This enables the exact copies of nucleic acid targets in the original sample to be determined without a standard curve [10,12].

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Due to sample partitioning challenges and sample volume constraints observed in dPCR, technological refinements have been done by various companies to achieve better instruments that can partition samples into hundreds to millions of nanoliter or picoliter-sized partitions [10,13]. Such technological advancements include the recently developed droplet digital PCR. Droplet digital PCR is a third generation of qPCR where reaction chambers are not separated by walls of a well, but by water-in-oil emulsions of microfluidic channels to generate thousands to millions of droplets that can be counted and used to quantify nucleic acid targets [10,12,13]. Many clinical applications of ddPCR have been conducted to detect and quantify many pathogens due to its advantages in absolute quantitation without a calibration curve, unparalleled sensitivity, and high precision [12,13]. This review will focus on droplet digital PCR (ddPCR) technology and how it can be used in tuberculosis diagnosis, the advantages it offers over qPCR and normal PCR, and also review various studies done using ddPCR in diagnosis of various pathogens including tuberculosis.

## 2. Summary of the droplet digital PCR technology

Accurate quantification of nucleic acids with high precision plays an important role in a variety of research fields including molecular diagnostics. Real time PCR or qPCR have been widely used for this purpose with a few limitations. The real time PCR systems are quite analogous as they require one to construct a standard curve and monitor amplifications after numerous cycles to finally quantify nucleic acids. Many factors can affect qPCR efficiency which may influence the overall results making the technique sometimes unreliable.

### 2.1. Development

The technological concepts of ddPCR are based on the working principles of digital PCR which were first explained in the 1990s [10,13,14]. These principles show that dPCR is an end-point method for absolutely quantifying nucleic acids without the need of a standard curve [12].

This is achieved through randomly distributing samples into discrete chambers ensuring some chambers have one or more nucleic acids, while some have no nucleic acid. From this partitioning the nucleic acids are amplified to end-point through thermal cycling and

finally read by a machine that can calculate concentrations based on the fractions of negative to positive partitions as shown in Fig. 1. This calculation is based on the equation;

$$M = - \ln \left( 1 - \left( \frac{P}{R} \right) \right) \text{Copy per droplet}$$

Where  $M$  equals the average number of target molecules per partition;  $P$  is the number of partitions having amplified products; and  $R$  is the number of reactions or partitions analyzed [15,16]. Digital PCR reliability can however be influenced by the amount of reactions analyzed ( $R$ ) and template amount in a certain molecule [17]. This calls for consideration in adding partition volumes during quantification by dPCR. This has been done by the microfluidic chamber based digital PCR (cdPCR) system that is commercially available [12]. This system however suffers limitations in terms of costs and the amounts of replicates that can be analyzed. Droplet digital PCR offers an advantage over cdPCR in that it can partition droplets up to approximately 20 000 or more, a number far higher than the 765 droplet partitioned by cdPCR in a single reaction well [12]. In 2007, the first report of ddPCR was recorded whereby Taqman hydrolysis probes were used to demonstrate single-copy detection limits in 10 Pl droplets within 18 cycles [18]. Despite dPCR being conceptualized in the 1990s, it was only until 2011 that the ddPCR technology became commercially available [9,10,13]. Currently, different companies offer the ddPCR technology to hospitals and research institutes worldwide for use. These companies include Bio-Rad®, Stilla®, Life Technologies®, and RainDance® [10,13].

### 2.2. Instrumentation, work flow, and data analysis

Recent advancements in the ddPCR technology have led to the production of automated droplet generator systems that are efficient and can produce droplets for an entire 96 well PCR plate within a short time. With the increasing need of accurate diagnostic platforms, ddPCR offers many advantages over conventional PCR systems especially with absolute quantification and genetic studies [19]. In a typical ddPCR experiment, a single sample is prepared for droplet generation using real-time PCR TaqMan based hydrolysis probes that are fluorescently labelled with FAM and HEX (or VIC), or EvaGreen intercalating dyes [20]. This sample is subsequently divided into thousands of single nanoliter-sized droplets (20 000 or more based on system) in a

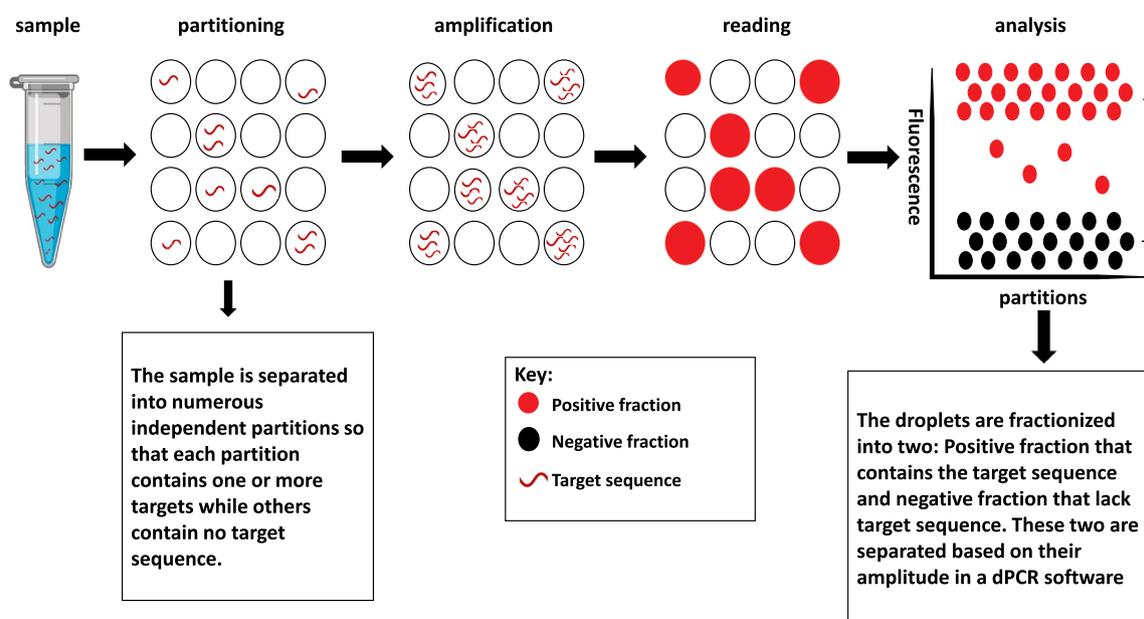


Fig. 1. Principle of dPCR showing how a single sample containing the target sequence is partitioned and amplified to generate thousands of copies that can be detected and read using a favorable system.

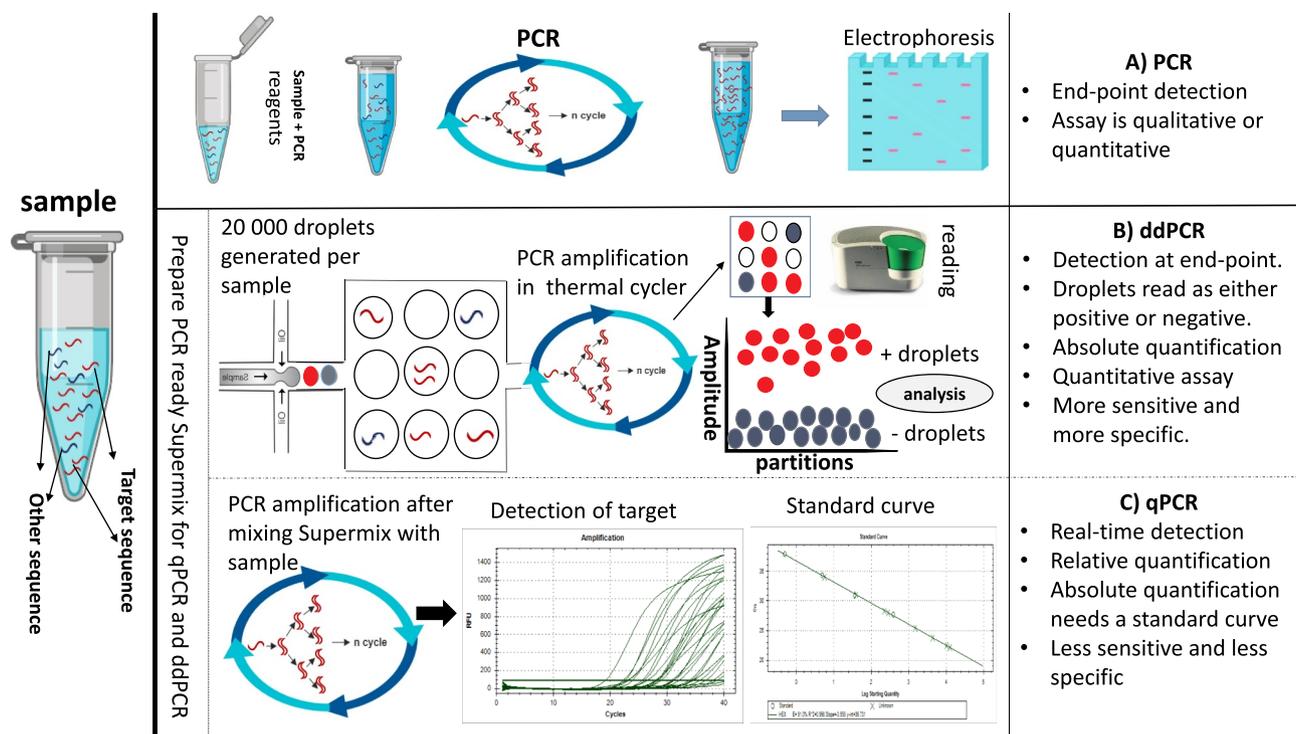


Fig. 2. The workflow of various PCR platforms. A) Detection of samples based on gel electrophoresis; B) ddPCR workflow and details of sample processing steps; C) quantification PCR workflow including details on data analysis.

combination of water-in-oil emulsion droplet technology and microfluidics [12] as shown in Fig. 2. A single droplet may contain one or more targets while others may not contain any target gene. Amplification of each droplet is carried out after droplet generation using a thermal cycler to end-point. Post amplification, droplets are placed in a droplet reader that analyzes droplets individually based on a two-color detection system depending on the fluorescent dye used. During the reading process, fluorescent signals are measured for individual droplets in various channels to differentiate positive droplets from negative droplets. Positive droplets are read as droplets that contain at least a single copy or more of the target gene exhibiting increased fluorescence, while negative droplets have no copies of the target gene and exhibit no fluorescence [12,13,21]. The reading process can be monitored and read in real-time using an appropriate Software (example Quantasoft) provided by the various companies in a personal computer. Data analysis using the software can only be done after droplet reading is finished, where one can explore various options provided by the software to visualize, analyze, and export data based on the experimental design.

### 3. Applications of droplet digital PCR

Most of the applications related to the ddPCR technology originate from its data analysis mechanisms. Data read by the ddPCRs droplet reader can be analyzed based on 4 experimental types: absolute quantification (ABS), copy number variation (CNV), rare event detection (RED), and gene expression (GEX). Based on these possibilities, numerous applications have been tailored to achieve various functions since the development of the technology. Some of these applications are summarized below.

#### 3.1. Absolute quantification

Absolute quantification (ABS) is an experimental design that can be used for the quantification of nucleic acids in copies per microliter for a given sample without the need of a standard curve. Various pathogens

including bacteria, parasites, and viruses have been detected using this application [13] with other papers showing multiplexing options and how to optimize ABS procedures [22,23]. Majority of the research that have been done in tuberculosis detection using ddPCR use this application. Target sequences for detection range from *IS6110* gene to mutation conferring gene detection like the *gyrB* gene [24]. Droplet digital PCR has been used to detect MTB-specific DNA targets in human samples from both pulmonary (PTB) and extrapulmonary (EPTB) tuberculosis cases [20]. This detection presents a unique advantage especially in cases where sufficient sample amount is hard to find like in infants or children, disseminated tuberculosis, and respiratory lesions [13]. Devonshire et al. studied the reproducibility of dPCR including ddPCR QX100 in detection of *Mycobacterium tuberculosis complex (MTbC)* [23]. In this report he showed that dPCR is highly reproducible in detecting *MTbC* and also highlighted some of the factors that can affect this reproducibility [23]. ABS has also been used in TB diagnosis and studying the efficacy of BCG vaccine in rhesus challenged monkeys by detecting circulating MTB specific-DNA [25]. Most of these studies highlight the advantages of ddPCR when compared to qPCR in absolute quantification of low abundant nucleic acid targets.

#### 3.2. Copy number variation

Copy number variation (CNV) refers to alterations in the copy number of a given target locus when compared to that of a reference invariant locus. These alterations may be caused by duplications or deletions of nucleic acids in a certain locus with respect to a reference locus within a cell [26]. CNV constitute a large part of the human genome and the role they may play in gene expression, protein functions, and disease traits is still unclear [27]. Detection of CNV greatly relies upon nucleic acid amplification tests including qPCR and dPCR [27]. Two technologies have been highlighted to be frequently used in CNV analysis and this is the ddPCR and paralogue ratio test (PRT) [28]. Recently ddPCR has been used to determine CNV in pathogens, food, and even in cancer studies. Methods to perform CNV experiments and their advantages using ddPCR have also been discussed by various

papers [27,29,30]. No research has however demonstrated the use of ddPCR in analyzing tuberculosis CNV. PRT which is another PCR technology has been used to detect the susceptibility of clinically active TB in different populations with *CCL3L1/CCL4L1* copy number variation [31]. This study was based on the fact that *Mtb* isolates differ geographically hence the immune response may also differ geographically. Despite this, no associations were however developed to link *CCL3L1/CCL4L1* copy number variation to clinically active TB in different populations [31]. In a different experiment, Bio-Rad demonstrated high-resolution CNV assessment in homogeneous samples using the *CCL3L1* gene target by ddPCR which they said varies highly among individuals. In their study they were able to determine the copy numbers reliably with a 95% confidence interval [30]. Ragheb et al. in another study used whole genome sequencing approach to detect mycobacterial interspersed repetitive units (MIRUs) CNV in *Mtb* [32]. This study was epidemiological and the results suggested that CNV in the MIRUs loci could be used for future epidemiology studies including tracking the transmission of *Mtb* strains [32]. Jiang et al. demonstrated that an increase of the copy number in exon 11 of IL-23R may be a risk factor for pulmonary tuberculosis in Chinese Uyghurs [33]. TB studies based on ddPCR CNV analysis should be done due to the advantages offered by the technology including: high throughput screening than traditional qPCR, rapid analysis, high accuracy, and high precision even with minimal sample amounts [29,30].

### 3.3. Gene expression

Gene expression (GEX) studies are done to provide insights about a gene's function, and real-time quantification methods like qPCR and ddPCR can be used for this application [34]. Reverse transcription quantitative PCR (RT-qPCR) has been widely used in gene expression analysis providing a sensitive and accurate method of quantifying gene expression levels in small-to medium-scale studies [34]. This method of quantification however may lead to poor quality results in cases where the targets are lowly abundant with small expression differences of 2-fold or lower [21]. In such instances, ddPCR offers a greater advantage because it can detect low levels of DNA gene expression even in the presence of inhibitors [21,34]. Since its introduction, ddPCR has become an increasingly powerful tool in GEX analysis studies due to its advantages over qPCR when comparing sensitivity, precision, reproducibility, and quantification of low targets [34–36]. Majority of the studies done using ddPCR's GEX involve cancer research and different targets have been used to study gene expression including miRNA quantification in sputum to diagnose lung cancer [36]. For TB research a single study was done to establish the complex mechanism of anti-mycobacterial action of 5-Fluorouracil (5-FU) on *Mtb* strains [37]. The known anticancer 5-FU drug has been used for decades and its role in antimicrobial activity has also been explored. In the study, the role of this drug was compared by detecting various mutant genes using ddPCR to see the role they play in *Mtb* resistance to 5-FU [37]. Other methods have also been used to study gene expression on tuberculosis. Microarray analysis coupled with RT-qPCR was used in a study to evaluate the diagnostic value of miRNAs in childhood tuberculosis [38]. In this study a total of 29 miRNAs were altered (15 miRNAs upregulated and 14 down regulated) in the TB group when compared to the healthy control group. These results suggested that multiple miRNA targets could be used as potential biomarkers in the diagnosis of childhood TB [38]. Recent studies by Wang et al. performed a meta-analysis of human gene expression in response to latent and pulmonary TB infections from 9 published data sets [39]. Their result identified novel targets and drug-repurposing opportunities including ion-channel blockers, intravenous immunoglobulin, and cancer immune-therapeutics that could be used in combinational therapeutics with anti-mycobacterial agents [39]. Many more studies involving tuberculosis and gene expression have also been published online [40–42]. Despite ddPCR offering many advantages in GEX studies, only a single study has been done based on

the technology. More ddPCR studies should be designed to explore the functions of GEX in tuberculosis diagnosis which may provide better results than other methods used in detecting GEX.

### 3.4. Rare event detection

Rare event detection (RED) by ddPCR can broadly be classified into two sub applications; rare mutation detection (RMD) and rare sequence detection (RSD). Just like in ABS, both the two applications are forms of directly quantifying nucleic acid target sequences. According to Yang et al., RED offers extra sensitivity because it can reliably detect rare mutants as low as 0.0005% and rare sequence as low as 0.00013% [43]. Details on how to design a RED assay have been recorded [29]. Just like in CNV, no studies have been done using RED applications in TB quantification. However most studies done by this application are cancer research based studies and virus studies. Watanabe et al. developed an ultra-sensitive detection assay (sensitivity of approximately 0.001%) that could be used in the pretreatment of EGRF T790M mutations in non-small cell lung cancer patients using ddPCR [44]. In another study ddPCR and qPCR were used to measure the frequency of total human immunodeficiency virus (HIV) DNA (pol copies per million cells), and episomal 2-long terminal repeat circles in over 300 patient cells. The results showed that ddPCR could be used practically to measure accurately HIV DNA targets with more robust target sequence variation, improved accuracy and precision, reliable quantification of proviral and episomal DNA targets far below the limits of detection using qPCR [45]. Based on this, ddPCR provides enormous advantages to researchers wishing to screen rare sequences or mutations in future TB studies. Mutations have been documented to be the cause of *Mtb* drug resistance before; mutations of the *katG* gene sequence in isoniazid-resistant clinical isolates have been said to be rare [46], also mutations in the *rpoB* gene that can lead to rifampicin resistance have been documented to be rare [47].

### 3.5. Other applications

More extensive applications using ddPCR to detect and diagnose other pathogens apart from TB have been explored. Some of which include: genetically modified organism detection [19], microRNA analysis [48], next-generation sequencing library quantification [49], single-cell analysis [50], and viral load determination [51].

## 4. Synoptic overview of ddPCRs tuberculosis research

In the past decade research data have been published online regarding the application of ddPCR to detect TB pathogens. This summary reviews the data published in a chronological manner. Summary of the data is also provided in Table 1. The first demonstrated application of generating droplets to detect TB was done by Lyu et al. in 2014, where they used a droplet-based microfluidics system to quantify cells expressing BlaC in order to diagnose tuberculosis [52]. This system however didn't couple the technique with PCR and the droplets were read microscopically hence doesn't generally fit the criteria of this review [52].

The first two publications combining droplet technology and PCR were however published in 2015 by Sing et al. in January [37] and Devonshire et al. in February [23]. Using stable *Mtb* strains, Singh et al. used the QX200 ddPCR system to study the expression of mutant genes and the role they play in *Mtb* resistance to the drug 5-Fluorouracil (5-FU) [37]. In the study, replicate genes were normalized to *sigA* and fold changes compared to control samples for further analysis. *Mtb* resistance to 5-FU was found to be mediated by mutations either in *upp* or by mutations in *pyrR* that play different roles as shown by Singh et al. [37]. Many other mechanisms of inhibition were also discussed in the paper showing the abilities of ddPCR in performing complex studies involving TB. Devonshire et al. studied the high reproducible nature of

**Table 1**  
Summary of droplet digital PCR tuberculosis research published online.

Sample type	Application	Target	Function/Results	System	Ref.
Different <i>Mtb</i> strains	GEX	<i>PyrA</i> , <i>PyrB</i> , <i>PyrC</i> , <i>PyrE</i> , <i>PyrF</i> , <i>PyrR</i> , <i>RadA</i> , <i>recA</i> , <i>sigA</i>	Study the effect of GEX in <i>Mtb</i> drug resistance to 5-FU. Mutations in <i>upp</i> and <i>PyrR</i> leads to <i>Mtb</i> resistance to the drug 5-FU. Mutations in <i>upp</i> causes catalysis of 5-FU to FUMP that is the first step to resistance. <i>PyrR</i> mutations impairs the precursor function of pyrimidine regulator resulting in overexpression of UMP via de novo biosynthesis, hence rescuing cells from toxic effects of 5-FU metabolites.	QX200	[37]
Different <i>Mtb</i> strains	ABS	<i>rpoB</i> , 16sRNA	Study reproducibility of ddPCR. Results show that primer pairs and mastermix do not affect ddPCR performance while extraction methods may influence ddPCR performance across different laboratories. ddPCR is a robust reproducible method for quantification of <i>Mtb</i> DNA.	QX100	[23]
Different <i>Mtb</i> strains	ABS	<i>rpoB</i> , 16sRNA	Assessing a novel approach that can be used to develop a quality assurance assay using dPCR in quantifying specific <i>Mtb</i> DNA. ddPCR is highly reproducible across different laboratories even when using different instruments.	QX100	[53]
Patient plasma	ABS	<i>IS6110</i> , <i>gyrB</i>	Development of a ddPCR assay that can detect circulating <i>Mtb</i> DNA in PTB patient plasma. The assay can detect <i>Mtb</i> DNA in plasma even in very low copy numbers with a sensitivity and specificity greater than that of qPCR.	QX200	[24]
Whole blood (plasma)	ABS	<i>IS6110</i>	Detection of <i>Mtb</i> DNA in whole blood-derived samples from PTB and EPTB patient plasma. First report to show TB diagnosis in clinics using ddPCR. No significant differences in <i>IS6110</i> copy numbers between various TB types. ddPCR could detect <i>Mtb</i> DNA in PTB and EPTB patients with higher sensitivity and specificity compared to qPCR.	QX200	[20]
Human respiratory aerosol	ABS	RD9	Detection of <i>Mtb</i> in bio-aerosols using respiratory aerosol sampling chamber for sample collection. Results show that the peak aerosol size detectable by ddPCR are 2.0–3.5µm. <i>Mtb</i> can be readily detected in respiratory bio-aerosol samples collected from untreated TB patients.	QX200	[55]
Human and monkey blood	ABS	CFP10, Kv1768	Detection of <i>Mtb</i> DNA in human blood (PTB, EPTB, and infant TB) and BCG vaccinated and/or <i>Mtb</i> H37Rv-challenged rhesus monkey models for vaccine evaluation. Limits of detection using ddPCR were superior to qPCR, and ddPCR could also detect <i>Mtb</i> infections within 3 weeks and 2 weeks earlier than qPCR in monkey models. High detection in human samples even with low copies.	QX100	[25]
Patient plasma	ABS	<i>IS6110</i> , <i>gyrB</i>	Detecting <i>Mtb</i> derived DNA in a 63 year old severely immunocompromised patient using the assay developed by Ushio et al. [24]. Successful detection <i>Mtb</i> DNA in the case report.	QX200	[54]
Human sputum	ABS	<i>IS6110</i>	Development of a rapid direct culture-ddPCR assay for detection of <i>Mtb</i> and DST testing. <i>Mtb</i> detection could be achieved within 5 h and an extra 4 days to establish susceptibility. ddPCR was better than MGIT 960 in sensitivity and specificity.	QX200	[56]

ddPCR in absolute quantification of *Mtb* complex [23]. In the study, various factors including template type, mastermixes, primer pairs, and extraction methods were evaluated to analyze the performance of dPCR (using QX 100 ddPCR system and chip (BioMark) system). In the absence of any external calibration, mastermixes, and primer pairs, ABS results were generally consistent with a minimal difference of about 2 fold. However, DNA integrity and extraction methods were said to cause an influence in the dPCR performance if not controlled. Despite this, dPCR was still considered as a robust reproducible method for DNA quantification [23].

Devonshire *et al.* in 2016 also published an article based on the use of different dPCR platforms to improve tuberculosis diagnosis via quantitative molecular diagnostic methods [53]. In their study, they assessed a novel approach in development of quality assurance materials using three different dPCR platforms including QX100 droplet digital PCR System to quantify specific DNA sequences. Quantification was based on a range of prototype reference materials and the results from various labs evaluated for accuracy. The same materials were also used to evaluate qPCR and Xpert MTB/RIF quantitative performance in eight testing laboratories. The results of this evaluation showed that dPCR was highly reproducible between laboratories as compared to qPCR and Xpert MTB/RIF assays. They suggested that dPCR could be used to further improve reproducibility that will in turn elevate the performance of quantitative molecular measurements linked to TB diagnostics and prognostic monitoring. The paper also agreed with WHO and Cepheid on the fact that Xpert MTB/RIF assay should not be used for quantification [53]. They sighted the various shortcomings of qPCR and Xpert MTB/RIF assay in clinical diagnosis of TB. Conclusions showed that dPCR high accuracy made it a potential technology for more robust pathogen quantification for clinical purposes in future TB patient management studies [53]. In the same year, Ushio et al. [24] developed a digital droplet PCR assay based on the QX200 system to detect circulating *Mtb* DNA in plasma of patients with pulmonary tuberculosis (PTB). Samples were first confirmed for positivity using sputum smear microscopy and if positive, two genes *IS6110* or *gyrB* were detected using the assay. The assay sensitivity and detection capability were both fit enough for the study with the authors highlighting that ddPCR proved superior to qPCR when quantifying low targets of *Mtb* in plasma [24]. Later on in 2018, the same assay was used by different researchers including Ushio to detect circulating *Mtb* in a 63 year old severely immunocompromised male patient [54].

In August 11, 2017 Yang et al. published a comprehensive report which was the first to demonstrate diagnosis of TB in clinics using the QX200 ddPCR system from whole blood-derived DNA samples [20]. Using *IS6110* as a target gene, both ddPCR and qPCR were used to detect *Mtb* DNA in patients with pulmonary tuberculosis (PTB), extrapulmonary tuberculosis (EPTB), and healthy control individuals. Results were compared and analyzed for sensitivity and specificity. ddPCR had an advantage over qPCR as it could detect *Mtb* DNA even in low copy numbers [20]. *IS6110* copy number differences were analyzed between various TB types: 1) EPTB tuberculosis patients with different infected organs; and 2) new cases of TB and cases of TB involving re-treatment [20]. In both types no difference in copy number was reported. Conclusions from the study highlighted that ddPCR was a promising diagnostic tool for detection of low levels of *Mtb* DNA with a potential of being used to diagnose pulmonary and extrapulmonary TB obtained from clinical blood samples [20]. Confirmation of sensitivity, specificity, and dependability of the technique was prospected to be confirmed via multicenter studies involving a large number of clinical samples in future studies [20]. In the same year Patterson et al. used ddPCR in a novel collection system to identify and quantify *Mtb* levels in environmental bio-aerosols generated by newly diagnosed but untreated TB patients [55]. In this study involving 35 aerosol samples, 27 were positive for *Mtb* using both mycobacterial culture and ddPCR: 15/35 (42.8%) samples were positive by mycobacterial culture while 25/27 (92.96%) samples were positive by ddPCR [55]. The findings

suggested that *Mtb* could be found indeed in respiratory bio-aerosols. The study also demonstrated that coupling non-invasive breath sampling with rapid molecular detection could generate a point-of-care system that has got high sensitivity even if the system is prone to false positives from non-variable organisms from previously treated *Mtb* patients as observed in Xpert MTB/RIF assays [55]. Suggestions showed that this test may be effective in mass screening to establish sub-clinical cases within the community, or screening in clinical setups to measure infectivity rates in hospital settings [55].

In 2018, Song et al. were the first to demonstrate the effectiveness and sensitivity of ddPCR in quantifying circulating CFP10 and Rv1768 DNA for in vivo bacterial load evaluation, early TB (including PTB, EPTB, and infant TB) diagnosis, and vaccine development [25]. The two targets (CFP10 and Rv1768) DNA copy numbers were determined in blood samples of patients and BCG vaccinated and/or virulent *Mtb H37Rv*-challenged rhesus monkeys using qPCR and QX100 ddPCR system. From the study, the specificity, sensitivity, and detection rate of ddPCR was 100% as compared to qPCR (65% sensitivity, 100% specificity, and 65% detection rate), sputum acid-fast staining, and sputum culture (sensitivity 23%, and 58% respectively) [25]. Detection limits of ddPCR was also superior to qPCR in detecting CFP10 (ddPCR detection limit of 1.2 copies/ $\mu$ l versus qPCR 15.8 copies/ $\mu$ l). Another advantage highlighted was that ddPCR was capable of detecting both CFP10 and Rv1768 three weeks after infection and at least 2 weeks earlier than qPCR when detecting *Mtb* in H37Rv-challenged rhesus monkey models [25]. Just as shown by Yang et al. [20], here ddPCR could also detect CFP10 and Rv1768 DNA copy numbers in blood samples of clinical TB patients with PTB, EPTB, and infant TB [25]. This paper clearly demonstrated the advantages of ddPCR over qPCR in absolute quantification and sensitivity, with the application of the technology in bacterial load determination for vaccine evaluation in vivo and timely TB diagnosis. As stated before, ddPCR was also used to detect circulating *Mtb* DNA in a patient by Yamamoto et al. [54]. This study adopted the already published assay by Ushio et al. [24] in 2016 to diagnose this patient case that was reported in 2018 [54].

The latest paper online was published in February 2019 by our group [56]. This article summarized a novel approach in rapid *Mtb* diagnosis by combining both culture techniques and ddPCR technology for detection of *Mtb*, and drug susceptibility testing (DST). In the study, sputum from 102 patients were detected using ddPCRs QX200 automated system, Xpert, qPCR, and MGIT 960. For DST, samples that tested positive were subjected to three drugs and cultured in liquid medium for 4 days. Fold changes were analyzed based on a formula explained in the study to determine whether a sample was resistant or susceptible to a certain drug [56]. Susceptibility results were compared to MGIT 960 agar proportion method. From the comparison, ddPCRs sensitivity and specificity was superior to that of MGIT 960 (95.7% and 88.9% respectively) [56]. The time for detection of *Mtb* was approximated to be 5 h and an extra 4 days to determine DST using sputum samples. This research provided a novel rapid diagnostic technique that would greatly reduce the time needed for tuberculosis diagnosis both from detection to DST [56].

## 5. Advantages and disadvantages of ddPCR

The various application platforms offered by ddPCR give advantages in multiplexing, and leave room for researchers to come up with novel techniques some of which have been documented in this review. Despite the many advantages offered by the technology, less data available online on TB research may be due to the disadvantages shown in Table 2. These have led to less research being done worldwide using the technology especially in developing countries.

## 6. Perspectives

Droplet digital PCR is a fascinating technology that offers many advantages when compared to qPCR. From various studies it has been

proven to be more accurate in absolute quantification of target nucleic acids, especially when handling low abundant targets. Currently, multiple ddPCR applications have been designed to study different disease-causing pathogens successfully. The fact that we were able to show the application of the technology to rapidly detect (5 h), and determine tuberculosis drug susceptibility (4 days) in clinical samples speaks volumes to the advantages offered by this technology in future diagnostic studies [56]. This means that cases can be detected and tested for DST in a more speedily manner as compared to the slow culture methods. The technology has also made it possible to detect complicated and low template containing TB samples like in the cases of EPTB, disseminated tuberculosis, respiratory lesions, and infant TB with high accuracy [25]. Other researchers using the technology to diagnose TB have also proposed many future applications that we agree with including: using the technology's high accuracy to perform more robust pathogen quantification in clinical samples for future patient management studies [53]; creating assays to detect *Mtb* genes which cause drug resistance, or assays capable of monitoring bacterial burden in immunocompromised patients [24]; timely TB diagnosis and assessment of bacterial loads for in vivo vaccination [25]; and the use of this technique in hospital settings to measure infectivity, and/or mass screening in high burden communities to identify sub-clinical cases. Despite all this suggestions and publications, the research done in TB studies using the technology is still little with majority of the studies aimed at absolute quantification and only one in gene expression. This underscores the full potential of ddPCR applications as it has been shown to perform multiple functions with far better advantages over qPCR and other molecular platforms. The scarcity of TB research and publications online can greatly be attributed to the limitations of the technology especially in terms of cost. Very few laboratories, hospitals, or research institutions have this technology in the world especially in developing countries, greatly reducing the number of research done by various institutions. It may take a while before the price of ddPCR machines and consumables become commercially affordable, but when this happens, more applications in laboratories, research institutions, and hospitals will lead to novel techniques in tuberculosis diagnosis and possible inclusion of ddPCR as a standard test for future tuberculosis diagnosis. Researchers and scientists worldwide should take this as a challenge and explore the ever-growing applications of ddPCR.

## 7. Conclusion

Droplet digital PCR is slowly gaining attention as the best method of choice when it comes to diagnosis of pathogens including tuberculosis. From this review it is clear that the technology offers an enormous advantage over common molecular diagnostic platforms like the real-time quantification PCR. A total of 9 publications exist online on the applications of ddPCR in tuberculosis research. These reports range from gene expression studies to absolute quantification of various targets highlighting that ddPCR offers and edge over many other molecular diagnosis techniques when it comes to sensitivity, specificity, reproducibility, and limits of detection. When quantifying targets that are low in terms of copy numbers, ddPCR is preferable as it can detect targets far below the detection limits of other common molecular diagnostic platforms. More studies should be designed to fully explore the other applications of ddPCR so as to add more data to the less materials available online. As we near the vision 2030 goal for sustainable development and ending the tuberculosis epidemic by 2030, such techniques may help achieve this objective and many more discussed in the UNGA-HLM-TB. The future of tuberculosis diagnosis is still navigable, and technological developments/applications will provide answers to problems that were deemed unsolvable.

## Conflicts of interest

All the authors declare no conflict of interest.

**Table 2**  
Advantages and disadvantages of ddPCR when compared to qPCR.

Advantages	Disadvantages
Absolute quantification without a standard curve	Expensive: cost of instruments, consumables and reagents are higher
High accuracy, sensitivity, specificity, and precision	Less availability worldwide especially in developing countries
High reproducibility between laboratories	Reaction volume mixtures are limited
Accurate detection of low copy number targets.	Higher chances of sample contamination
Robust application possibilities	
Less affected by inhibitors	

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.tube.2019.07.001>.

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