This book, "***Digital Polymerase Chain Reaction (dPCR): Advances and Applications in Contemporary Laboratory Practice***," explores the principles, fundamentals, advantages, and applications of dPCR in research and clinical practice. The book is divided into seven chapters, with the first chapter providing an introduction to dPCR and its significance in modern molecular biology. The second chapter delves into the fundamentals of dPCR, while the third chapter explores the advantages of dPCR over conventional PCR techniques. The fourth chapter discusses the diverse applications of dPCR in research and clinical practice, and the fifth chapter examines the latest advances and innovations in dPCR platforms and methodologies. The sixth chapter speculates on future directions and opportunities in dPCR research and applications, and the final chapter provides a comprehensive summary of the book's key findings, implications, and insights. The book is intended to provide researchers, clinicians, and students with a comprehensive resource that not only elucidates the principles and applications of dPCR but also inspires further exploration and innovation in this dynamic field.

The authors of "*Digital Polymerase Chain Reaction (dPCR): Advances and Applications in Contemporary Laboratory Practice*" are experts in preventive medicine, public health science, biological science, and environmental science. Until now, the authors worked as university lecturers in Indonesia.

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Digital Polymerase Chain Reaction (dPCR)

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Advancements and Applications in Contemporary Laboratory Practice

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Preface

Welcome to "**Digital Polymerase Chain Reaction (dPCR): Advances and Applications in Contemporary Laboratory Practice**." In the field of molecular biology, the advent of digital polymerase chain reaction (dPCR) represents a groundbreaking innovation that has revolutionised the quantification and analysis of nucleic acids. This book delves into the intricacies of dPCR and explores its fundamentals, advantages, applications, technological advancements, future directions, and implications in both research and clinical settings.

Chapter 1 provides a comprehensive introduction to dPCR, offering readers a foundational understanding of its principles and its significance in modern molecular biology. Chapter 2 delves into the fundamentals of dPCR and elucidates the key concepts and methodologies essential for conducting precise and reliable dPCR experiments. In Chapter 3, we explore the advantages of dPCR over conventional PCR techniques and highlight its enhanced sensitivity, accuracy, and reproducibility. This section underscores the transformative potential of dPCR in various fields from basic research to clinical diagnostics.

Chapter 4 discusses the diverse applications of dPCR in research and clinical practice. By illuminating case studies and examples, readers have gained insight into how dPCR is being utilised to address pressing scientific questions and improve patient care. Continuing the exploration of technological innovation, Chapter 5 examines the latest advances and innovations in the dPCR platforms and methodologies. This

section shows the cutting-edge technologies driving the evolution of dPCR from microfluidics to digital microfluidics.

Looking toward this horizon, Chapter 6 speculates on future directions and opportunities in dPCR research and applications. As the field continues to evolve, this chapter offers valuable insights into the emerging trends and potential areas of exploration. Finally, Chapter 7 provides a comprehensive summary of the book's key findings, implications, and insights. Serving as a culmination of the preceding chapters, this section encourages readers to reflect on the broader significance of dPCR and its implications for contemporary laboratory practices.

In compiling this volume, our aim is to provide researchers, clinicians, and students with a comprehensive resource that not only elucidates the principles and applications of dPCR, but also inspires further exploration and innovation in this dynamic field. We hope that this book will serve as a valuable guide for navigating the complexities of dPCR and harnessing its full potential to advance scientific knowledge and improve health care outcomes.

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Summary

Digital polymerase chain reaction (dPCR) is at the forefront of modern molecular biology techniques, revolutionizing the quantification and analysis of nucleic acids with unparalleled precision and sensitivity. As an advanced variant of the conventional PCR method, dPCR offers a powerful platform for the absolute quantification of nucleic acids, enabling researchers to detect and measure targets with exceptional accuracy even in samples with low abundance or complex backgrounds. This introduction delves into the fundamental principles underlying dPCR, its applications, and the technological advancements that have driven its widespread adoption in various fields of research and diagnostics.

dPCR operates on the same principles as traditional PCR, involving amplification of target DNA or RNA sequences through repeated cycles of denaturation, annealing, and extension. However, dPCR can partition PCRs into thousands of individual microscale reactions, each containing either zero or one target molecule. This partitioning is achieved through various methods, such as microfluidics, droplet generation, or emulsion-based PCR. Following partitioning, PCR amplification is carried out within each discrete compartment, leading to binary outcomes: compartments containing the target molecule exhibit amplification signals, whereas those without these signals remain negative. By counting the positive and negative partitions, dPCR allows for precise quantification of the initial target concentration without the need for standard curves or reference materials, making it inherently absolute and highly reproducible.

The versatility of dPCR has fueled its adoption across diverse applications, including basic research, clinical diagnostics, and environmental monitoring. In genetics and

genomics, dPCR plays a pivotal role in gene expression analysis, copy number variation detection, and rare allele quantification and offers insights into disease mechanisms, biomarker discovery, and personalized medicine. Its sensitivity and accuracy make it indispensable for detecting minimal residual disease in cancer patients and monitoring viral loads in patients with infectious diseases, such as HIV and hepatitis or complications with tuberculosis disease.

Moreover, dPCR is useful in environmental studies because it facilitates the detection and quantification of microbial contaminants, GMOs, and environmental pollutants with unparalleled precision, thereby aiding in environmental monitoring and ensuring food safety. Additionally, its ability to detect and quantify nucleic acids from complex matrices, such as soil, water, and air, holds promise for applications in agriculture, ecology, and bioremediation. In the realm of diagnostics, dPCR is poised to revolutionize healthcare by enabling the early and accurate detection of genetic mutations, infectious pathogens, and circulating tumor DNA in liquid biopsies. Its high sensitivity and specificity make it an ideal tool for diagnosing genetic disorders, monitoring treatment responses, and guiding therapeutic decisions with precision medicine approaches.

The rapid evolution of dPCR technology has driven significant advancements in instrument design, assay development, and data analysis algorithms, thereby enhancing its performance, throughput, and ease of use. Modern dPCR platforms offer automation capabilities, multiplexing options, and integrated data analysis software, thereby empowering researchers with streamlined workflows and faster turnaround times. Furthermore, ongoing innovations in droplet generation techniques, microfluidic chip designs, and digital detection methods continue to push the boundaries of dPCR sensitivity, enabling the detection and quantification of targets at single-molecule resolution. These advancements have expanded the scope of dPCR applications and paved the way for their integration into routine laboratory workflows and clinical diagnostic platforms.

Chapter 1

Introduction of Digital PCR (dPCR)

1.1 Brief overview of digital polymerase chain reaction (dPCR) technology

Digital polymerase chain reaction (dPCR) is a polymerase chain reaction method that has been modified to digitally enable molecular detection. This method utilizes the conventional PCR principle in which DNA fragments are grown exponentially using polymerase enzymes. However, dPCR introduces innovation by incorporating digital technology to individually detect and analyze PCR results [1]. In dPCR, the results are monitored individually using technologies such as digital microscopy or optical sensors to detect the fluorescence generated by the DNA envelope, as the target molecules are generated during PCR. Each generated DNA envelope was counted separately, allowing for the identification of the target molecule with high sensitivity [2,3]. The dPCR workflow is illustrated in **Figure 1**.

dPCR is a cutting-edge molecular biology technique that has revolutionized the quantification and analysis of nucleic acids [4,5]. Unlike traditional PCR methods, which rely on amplification curves to estimate the initial amount of target DNA or RNA, dPCR partitions a sample into thousands or millions of individual reactions, each containing a single molecule or a small number of molecules. This partitioning allows for the absolute quantification of the target without the need for standard curves, making dPCR highly precise and reproducible [6].

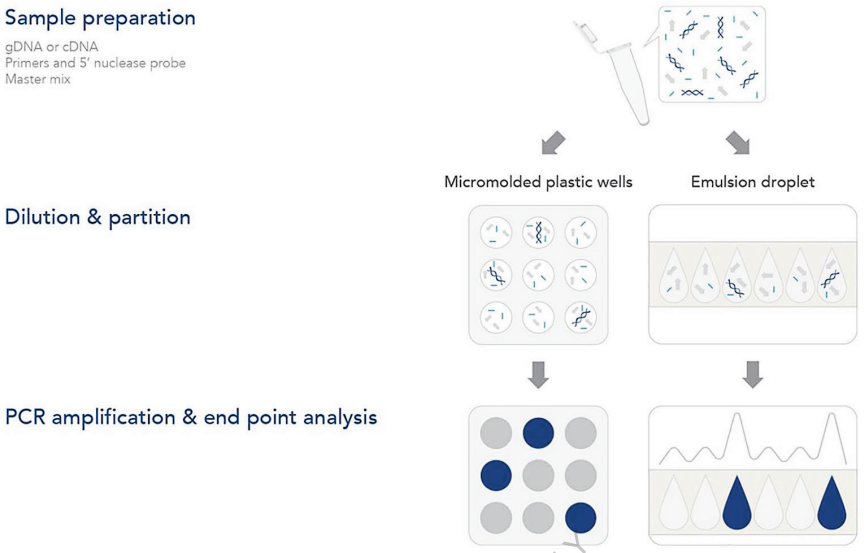


Figure 1. Digital PCR (dPCR) workflow Remarks: After successfully isolating genomic DNA (gDNA) or converting an mRNA sample into cDNA, a mixture consisting of a master mix, primers specific for the intended target, and a 5′ nuclease probe is added. The mixture was then diluted and divided into small partitions, where each partition contained one target or no target. Some dPCR platforms divide the mixture into separate compartments, whereas others form an emulsion of oil and water droplets. After the PCR amplification process was completed, the fluorescent signal for each partition was recorded. The number of partitions or droplets that show positive fluorescence provides an accurate estimate of the initial amount of target gDNA or cDNA molecules in the sample (Image courtesy of Integrated DNA Technologies [7]).

In dPCR, a sample is typically partitioned into discrete compartments, such as droplets or wells, either physically or digitally. After amplification of the target molecules within each compartment, the presence or absence of the target was determined based on endpoint fluorescence analysis. By counting the number of positive and negative partitions, the absolute concentration of the target in the original sample could be calculated with high accuracy [8–10].

This technology offers several advantages over traditional PCR, including increased sensitivity, improved detection of rare targets, and enhanced robustness to PCR

inhibitors. dPCR is widely used in research and clinical settings for applications such as the quantitative analysis of gene expression, detection of genetic mutations, monitoring of viral load, and assessment of copy number variations [11,12]. Moreover, ongoing technological advancements continue to refine dPCR platforms, making them more accessible, efficient, and cost-effective for a broad range of applications in molecular diagnostics, personalized medicine, and basic research.

1.2 History of Digital PCR (dPCR)

dPCR reflects the evolution of molecular detection and analysis technology, providing sensitive and accurate solutions for a wide range of applications in molecular biology, medical diagnostics, and scientific research. DNA and RNA research has undergone an important shift since the introduction of polymerase chain reaction (PCR) technology by Kary Mullis in 1983 [13]. Since then, dPCR has emerged as an important breakthrough in molecular detection methods that allows the precise quantification of genetic targets. Historically, conventional PCR has been the standard method for detecting DNA duplication in various laboratory applications. However, this method has limitations in terms of sensitivity, especially when detecting targets at low concentrations. In an attempt to improve the sensitivity and accuracy of molecular detection, dPCR has been developed as a promising alternative.

Initially, the basic concept of dPCR emerged in 1992, when Sykes and Colton [14] proposed dividing PCR samples into discrete partitions to count the number of target genes individually. However, the development of dPCR technology began in the early 21st century by utilizing advances in microfluidics and fluorescence detection [15]. In 2006, Ahmadian et al. [16,17], introduced the first concept of emulsion-based dPCR, in which a PCR sample is divided into small isolated droplets in an oil phase. This approach enables precise quantification of the number of target molecules in a sample by monitoring each droplet individually. This technology provides significant advantages in terms of the sensitivity and accuracy of molecular detection. In addition, in the same year, microfluidic-based dPCR platforms were also developed in which the

PCR sample was divided into discrete compartments in a microfluidic chip [18,19]. This approach enables faster and more accurate analysis by separating the sample into discrete volumes required for high-precision measurements [20,21].

In recent years, dPCR technology has continued to undergo significant developments. New methods, including gel-based microfluidics and indentation-based microfluidics, have been introduced to improve sample partitioning efficiency and detection sensitivity [22,23]. In addition, the use of new fluorescent probes and the development of sophisticated data analysis algorithms have improved the precision of calculating the number of target molecules in a sample [6,24]. The superiority of dPCR has been proven by its applications in various fields. In medical diagnostics, dPCR is used for infectious disease detection, genetic mutation analysis, and quantitative monitoring of gene therapy. dPCR is a valuable tool for gene expression studies, genetic variation mapping, and digital RT–PCR monitoring [25].

Recent developments in dPCR technology include integration with automation platforms and the use of microfluidic chips, which continue to increase throughput and analysis efficiency. As this technology continues to evolve, dPCR has great potential to become the standard for precise molecular detection in the future. With continuous improvements in sensitivity, speed, and accuracy, dPCR will continue to support advancements in a wide range of biomedical and scientific research applications.

1.3 Importance of dPCR in modern laboratory settings

dPCR plays a pivotal role in modern laboratory settings by providing highly accurate, sensitive, and quantitative analysis of nucleic acids. Its versatility, precision, and robustness make it an indispensable tool for a wide range of applications, driving advancements in research, diagnostics, and personalized medicine. dPCR has several advantages and diverse applications, including [8,11,26,27].

1. With enhanced precision and sensitivity, dPCR offers greater precision and sensitivity than traditional PCR techniques. The ability to partition samples into

thousands or millions of individual reactions enables the precise quantification of target nucleic acids, even at extremely low concentrations. Thus, dPCR is particularly valuable for detecting rare mutations, low-abundance targets, and subtle variations in gene expression.

2. Absolute quantification, unlike traditional PCR methods that rely on relative quantification using standard curves, dPCR enables absolute quantification of target nucleic acids. This eliminates the need for reference standards and improves the accuracy and reproducibility of quantitative measurements. Absolute quantification is especially critical in applications where precise determination of the target concentration is essential, such as determining the viral load in clinical samples or quantifying gene expression levels.
3. Improved detection of genetic variants and alterations, the high sensitivity and precision of dPCR make it well suited for detecting and quantifying genetic variants, including single nucleotide polymorphisms (SNPs), insertions, deletions, and chromosomal rearrangements. This capability is invaluable in research and clinical settings for the study of genetic diseases, cancer mutations, and microbial diversity.
4. Robust against PCR inhibitors, dPCR is less susceptible to PCR inhibitors than traditional PCR methods. Partitioning of the sample into individual reactions helps mitigate the effects of inhibitors present in complex sample matrices, such as blood, tissue, or environmental samples. This robustness ensures reliable and accurate results, even in challenging sample types.
5. The broad range of applications of dPCR has a broad range of applications across various fields, including molecular diagnostics, oncology, infectious disease research, environmental monitoring, and basic research. It is used to quantify nucleic acids in liquid biopsies, monitor viral loads in HIV or hepatitis infections, detect minimal residual disease in cancer patients, assess gene expression levels, and study microbial communities in environmental samples.

6. Advances in technology and automation and continuous advancements in dPCR technology have led to the development of automated platforms, microfluidic devices, and high-throughput systems. These innovations have made dPCR more accessible, user friendly, and suitable for large-scale studies, accelerating research progress and clinical translation.

1.4 The current landscape of dPCR applications and advancements in the laboratory

dPCR has gained significant traction in laboratory research owing to its ability to provide absolute quantification of nucleic acids with high precision and sensitivity. An overview of the current landscape of dPCR applications and advancements in laboratory research is presented.

1. In cancer research, dPCR is widely used to detect and quantify tumor-specific mutations and circulating tumor DNA (ctDNA) and to monitor treatment response. Advancements in dPCR technology have enabled the detection of rare mutations in heterogeneous tumor samples, offering potential insights into tumor evolution and personalized treatment strategies [28,29].
2. Infectious disease diagnosis and dPCR have been applied in the diagnosis and monitoring of infectious diseases, such as HIV, hepatitis, and COVID-19. Its high sensitivity allows for the detection of low levels of viral or bacterial nucleic acids, aiding early diagnosis, monitoring treatment efficacy, and detecting drug-resistant strains [30–32].
3. Liquid biopsy, which involves the analysis of circulating nucleic acids in body fluids such as blood, urine, and cerebrospinal fluid, has emerged as a noninvasive method for disease detection and monitoring. dPCR plays a crucial role in liquid biopsy applications by enabling the detection and quantification of circulating nucleic acids, including ctDNA, circulating tumor cells (CTCs), and cell-free RNA (cfRNA) [33].

4. Gene expression analysis and dPCR are used for accurate quantification of gene expression levels, offering advantages over traditional qPCR methods, particularly for low-abundance transcripts and highly variable expression levels. Recent advancements in dPCR technology have improved multiplexing capabilities, allowing simultaneous analysis of multiple targets in a single reaction [34,35].
5. For environmental monitoring, dPCR is utilized for the detection and quantification of microbial contaminants, pathogens, and genetically modified organisms (GMOs) in soil, water, and food samples. Its high sensitivity and precision make it suitable for monitoring environmental changes and assessing the impacts of pollutants on ecosystems [36,37].
6. Prenatal Diagnosis and Noninvasive Prenatal Testing (NIPT) and dPCR are increasingly being used for prenatal diagnosis and NIPT by detecting fetal DNA present in maternal circulation. This noninvasive approach offers an alternative to invasive procedures, such as amniocentesis and chorionic villus sampling, reducing the risk to both the mother and fetus [38].
7. For quality control in biopharmaceutical production, dPCR is employed in the biopharmaceutical industry for the quality control of recombinant protein and viral vector production. It enables the precise quantification of gene copy number, viral titer, and vector integration efficiency, ensuring product consistency and meeting regulatory requirements [39,40].

Recent advancements in dPCR technology have focused on improving assay sensitivity, throughput, and automation capabilities [41,42]. Overall, dPCR continues to be a versatile and powerful tool in laboratory research, driving advancements in various fields, including medicine, infectious disease research, molecular diagnostics, biopharmaceuticals, and environmental monitoring. Its ability to provide absolute quantification with high accuracy and reproducibility makes it indispensable for researchers seeking precise measurements of nucleic acids in diverse biological and environmental samples.

Chapter 2

Fundamentals of Digital PCR (dPCR)

2.1 Explanation of the principles underlying dPCR

dPCR operates on the same fundamental principles as traditional PCR but employs a different approach for quantification. The main principle underlying dPCR is partitioning of the sample into numerous individual reactions, each containing a fraction of the original sample. This partitioning can be achieved either physically (e.g., through microfluidics) or digitally (e.g., using droplet-based or chip-based methods) [20,43–45]. Once the sample was partitioned, PCR amplification of the target nucleic acid sequences was performed within each reaction. Importantly, the amount of target DNA or RNA present in each reaction is such that, ideally, each reaction contains either zero or one target molecule [18,46].

After amplification, the reactions were analyzed to determine which products contained the target sequence and which did not. By counting the number of positive and negative reactions, the absolute concentration of the target in the original sample could be calculated using statistical methods. dPCR provides superior sensitivity, precision, and capability to assess the amount of target DNA in a given sample. This high sensitivity facilitates the identification of rare mutations, copy number variations (CNVs), low-expression transcripts, rare microRNAs, and minimal viral load. Substantial differences between the three generations of PCR are presented in **Figure 2.**

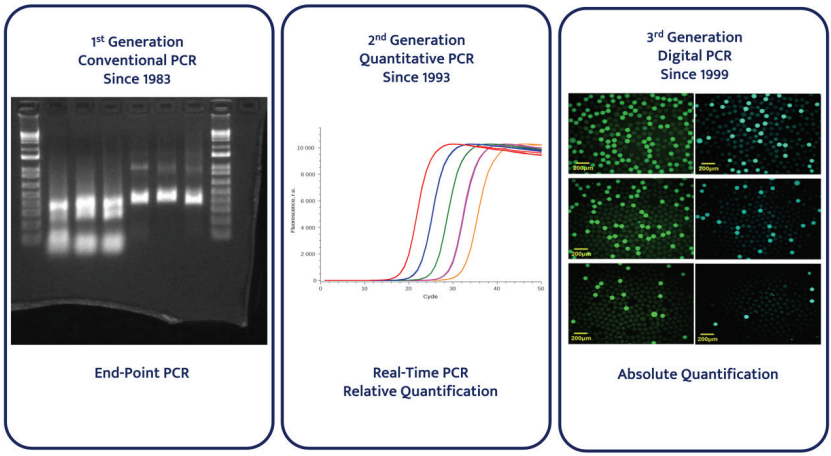


Figure 2. Basic principle of PCR for each generation.

2.2 Comparison with traditional PCR techniques

Polymerase chain reaction traditional and digital PCR (dPCR) are powerful molecular biology techniques used for the amplification and detection of specific nucleic acid sequences. Traditional PCR relies on the exponential amplification of DNA or RNA through repeated cycles of denaturation, annealing, and extension. The amplified products were then quantified using gel electrophoresis, fluorescence detection, or real-time PCR (qPCR), which provides relative quantification based on amplification curves [47–49].

In contrast, dPCR provides absolute quantification without the need for reference standards or amplification curves. By partitioning the sample and performing PCR amplification in individual reactions, dPCR can accurately determine the concentration of the target nucleic acids in the original sample. This makes dPCR particularly useful for applications requiring precise quantification, such as detecting rare mutations, quantifying gene expression levels, or measuring viral load. These techniques complement each other and are used synergistically in research and diagnostic laboratories to address diverse experimental needs [50,51]. The following is a comparison of traditional PCR techniques with dPCR [52].

1. **Principle of Amplification**
   - **Traditional PCR**: In traditional PCR, the target DNA or RNA sequence is exponentially amplified through repeated cycles of denaturation, annealing, and extension using a DNA polymerase enzyme, primers, and nucleotides.
   - **Digital PCR**: dPCR partitions a sample into thousands of individual reactions, each containing a single molecule or a few molecules of the target nucleic acid. After amplification, the presence or absence of the target gene was determined by counting the number of positive and negative reactions.
2. **Quantification accuracy**
   - **Traditional PCR**: Traditional PCR provides relative quantification, where the abundance of the target is determined based on the cycle threshold (Ct) value or relative fluorescence intensity compared with a control sample. This approach is susceptible to variability in reaction efficiency and PCR inhibitors.
   - **Digital PCR**: dPCR enables absolute quantification by directly counting the number of target molecules present in a sample. This approach provides higher accuracy and precision, particularly for low-abundance targets and samples with complex matrices.
3. **Detection sensitivity**
   - **Traditional PCR**: Traditional PCR is limited by its detection sensitivity, typically being able to detect targets present at concentrations above a certain threshold, which can vary depending on the assay and instrumentation used.
   - **Digital PCR**: dPCR offers increased sensitivity and is capable of detecting and quantifying targets present at very low concentrations, even at the single-molecule level. This makes it suitable for applications that require the detection of rare mutations or minimal residual disease.
4. **Dynamic Range**
   - **Traditional PCR**: The dynamic range of traditional PCR is limited by factors such as primer efficiency, amplification kinetics, and signal saturation. The quantification accuracy decreased at both low and high target concentrations.

- **Digital PCR**: dPCR offers a wider dynamic range, spanning several orders of magnitude, without the limitations associated with signal saturation. This allows accurate quantification across a broad range of target concentrations.

5. Multiplexing Capability

- **Traditional PCR**: Multiplex PCR allows the amplification and detection of multiple target sequences in a single reaction using multiple primer pairs and fluorescent probes with distinct emission spectra. However, multiplexing can increase the complexity of assay design and optimization.
- **Digital PCR**: dPCR supports multiplexing by partitioning the sample into distinct compartments, each containing a different primer-probe set. This enables simultaneous detection and quantification of multiple targets without cross-reactivity or interference.

6. Workflow and Hands-on Time

- **Traditional PCR**: Traditional PCR involves fewer steps, including setup, amplification, and analysis, and can be performed within a few hours. However, careful optimization of the reaction conditions and validation of the results are needed.
- **Digital PCR**: dPCR workflows can be more labor intensive and time consuming because of the need for sample partitioning, droplet generation (in-droplet dPCR), and data analysis. Automated platforms and integrated systems are available for streamlining workflows and reducing hands-on time.

2.3 Key components and workflow involved in dPCR

dPCR involves partitioning a sample into numerous individual reactions, each containing a single or few molecules of the target nucleic acid [53]. This partitioning allows for absolute quantification of the target without relying on standards or reference samples. The key components and workflow steps involved in dPCR are as follows:

1. **Sample Preparation**

The target nucleic acid must be isolated and purified from the sample of interest using the appropriate extraction methods. The workflow began with the preparation of nucleic acid samples for analysis. This may involve extraction and purification steps to isolate target DNA or RNA from the sample matrix. The extracted nucleic acids are typically quantified and checked for purity to ensure optimal performance in the dPCR assay [2,54].

2. **Primer and Probe Design**

Primers and probes specific to the target sequence of interest were designed. Probes are typically labeled with fluorophores, such as FAMs and VICs, and quenchers to enable signal detection. FAM is employed in the formulation of fluorescein-labeled oligonucleotide probes and is primarily utilized for identifying the presence of complementary nucleic acids or primers in polymerase chain reaction applications. Oligonucleotides featuring fluorescein labeling at one terminus and a quencher at the opposite terminus can function effectively as molecular beacons [25,55].

Furthermore, VIC is an asymmetric xanthene dye with fluorescence in the yellow–green region of the spectrum, and its spectral properties are similar to those of HEX and JOE. This dye is widely used to label real-time PCR probes. VIC azide, a 6-isomer, is an asymmetric xanthene dye with spectral properties similar to those of HEX and JOE and is widely used for labeling PCR probes. This derivative is an azide used for dye conjugation via click chemistry [56,57]. Similarly, VIC phosphoramidite (6-isomer) is an asymmetric xanthene dye with spectral properties similar to those of HEX and JOE. Useful labeling for qPCR probes.

3. **Partitioning the sample**

Sample partitioning was performed to distribute nucleic acid molecules into individual reactions. Each sample was partitioned into numerous individual reactions, each containing a small fraction of the original sample. Partitioning can be achieved using various methods, including droplet-based techniques (e.g.,

droplet dPCR, chip-based methods, or microfluidic devices. There are two main methods for partitioning: a) droplet-based dPCR, in which the sample is emulsified into water-in-oil droplets, with each droplet containing a single molecule or a few molecules of the target nucleic acid; and b) chip-based dPCR, in which the sample is partitioned into microfluidic chambers or wells on a chip, with each chamber/well containing a single molecule or a few molecules of the target nucleic acid (NA) [10,22].

4. PCR Amplification

PCR amplification of target sequences was performed within each reaction compartment or droplet. The amplification process typically involves denaturation of the DNA, annealing of the primers to the target sequences, and extension of the primers using a DNA polymerase enzyme is presented in **Figure 3**.

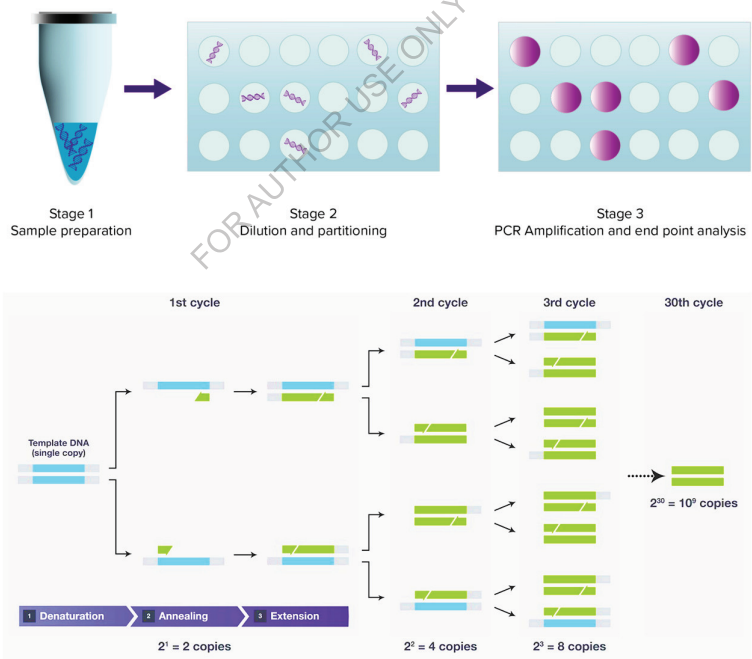


Figure 3. Three sequential stages of PCR denaturation (1), annealing (2), and extension (3) are exemplified within the initial cycle. Furthermore, it visually depicts the iterative nature of PCR cycling, resulting in exponential amplification of the desired DNA target.

The reaction mixture typically contains the DNA polymerase, primers, probes, nucleotides, and buffer components necessary for PCR amplification. In the amplification process, commonly known as PCR, which is carried out in thermal cycles, these conditions include several main steps for the replication of DNA segments [8,9,58]:

a) Denaturation, the initial step, involves the separation of double-stranded DNA into two single strands. This is achieved by heating the DNA sample to a high temperature, typically approximately 94-98°C, which breaks the hydrogen bonds between complementary base pairs. As a result, the DNA strands unwind and form two separate strands.
b) Following denaturation, the reaction temperature was lowered to allow the primers to anneal to their complementary sequences within the target DNA. The primers used were short, single-stranded DNA sequences designed to bind specifically to regions flanking the target sequence. Annealing typically occurs at a temperature of approximately 50-65°C, depending on the melting temperature (Tm) of the primers and the specific requirements of the reaction. During this step, the primers are hybridized to their complementary sequences on a single-stranded DNA template.
c) Extension (elongation): Once the primers are annealed, the temperature is increased to an optimal range for the DNA polymerase enzyme to extend the primers by synthesizing new DNA strands. The DNA polymerase enzyme catalyzes the addition of nucleotides to the 3' end of the primer using a single-stranded DNA template as a guide. This extension occurs in the 5'–3' direction, resulting in the synthesis of a new complementary DNA strand. The optimal temperature for DNA polymerase activity is typically approximately 72°C, although this temperature can vary depending on the specific enzyme used. The aforementioned procedures are iteratively conducted, termed "cycled," spanning approximately 25 to 35 repetitions. This iterative process leads to the exponential generation of precise replicas of the intended DNA target.

5. **After PCR amplification**, the products were subjected to endpoint analysis to determine which sequences contained the target sequence and which sequences did not. This can be achieved using various detection methods, such as fluorescence detection, which allows discrimination between positive and negative reactions based on the presence or absence of fluorescent signals. In droplet-based dPCR, fluorescence signals from labeled probes are detected in each droplet using a fluorescence detector, while in chip-based dPCR, fluorescence signals are detected using imaging systems or fluorescence microscopes.
6. **For the data analysis**, the results from the endpoint analysis were used to calculate the absolute concentration of the target in the original sample using statistical algorithms. This involves counting the number of positive and negative reactions and applying mathematical models to estimate the concentration of target nucleic acid molecules.
7. **Quantification and Interpretation,** the absolute concentration of the target nucleic acid was determined based on the number of positive reactions and the total number of reactions. The results are typically reported as copies per microliter or copies per nanogram of the input nucleic acid, providing precise quantification of the target.
8. **Validation and quality control of dPCR assays** involve assessing sensitivity, specificity, linearity, and reproducibility using reference materials or control samples. Quality control measures, including proper instrument calibration, assay optimization, and data normalization, ensured the accuracy and reliability of the dPCR results.

2.4 Other digital PCR methods

dPCR is reliable and can be used to precisely quantify nucleic acids; therefore, various methods have been developed to establish the use of this method in contemporary laboratories. There are various methods and platforms for performing dPCR based on the method, usefulness, and advantages described below.

1. **Chip-based Digital PCR (cdPCR)**

Chip-based digital PCR (cdPCR) involves the use of microfluidic chips to partition a sample into thousands of reaction chambers. The sample containing the target nucleic acid molecules was loaded onto the chip, and each chamber on the chip was occupied by either zero or one molecule of the target sequence. After PCR amplification, the presence or absence of the target sequence in each chamber was detected, and the cells were counted for absolute quantification [21,26,40].

The advantages of cdPCR include a) high precision and sensitivity, where the chip partitions the sample into thousands of chambers, enabling precise quantification even at very low target concentrations; b) reduced risk of contamination, where microfluidic chips minimize the risk of sample contamination because the reaction takes place in sealed chambers; and c) automation, where cdPCR platforms often come with automated workflows, reducing hands-on time and increasing throughput. cdPCR is particularly useful for applications that require high precision and sensitivity, such as rare mutation detection, copy number variation analysis, and absolute quantification of nucleic acids in clinical diagnostics and research [59,60].

2. **Microfluidic Chamber-based dPCR (mcdPCR)**

Like cdPCR, microfluidic chamber-based dPCR also utilizes microfluidic technology to partition samples into discrete reaction chambers. The principle of microfluidic chamber-based dPCR is that sample partitioning is achieved by using microfluidic channels to compartmentalize the sample into individual chambers. Each chamber was then subjected to PCR amplification, and the presence or absence of the target sequence was detected [19–21].

The advantages of microfluidic chamber-based dPCR include the following: a) precise quantification, in which microfluidic chambers ensure accurate partitioning of the sample, leading to precise quantification of target molecules; and b) scalability, in which microfluidic devices can be designed to accommodate different sample volumes and numbers of partitions, making them suitable for various

experimental needs. Microfluidic chamber-based dPCR is useful for applications that require high sensitivity and accuracy, such as rare allele detection, gene expression analysis, and microbial quantification [18].

3. Microwell Chip-based dPCR

Microwell chip-based dPCR involves the use of chips containing microwell arrays for sample partitioning. The sample was loaded onto the chip, where it was partitioned into individual microwells. Each microwell served as an independent reaction vessel for PCR amplification, allowing for absolute quantification of the target molecules [61]. The advantages of microwell chip-based dPCR include the following: a) high-throughput, microwell chips can accommodate a large number of reactions simultaneously, enabling high-throughput analysis; and b) versatility and can be adapted for various dPCR applications, including genotyping, gene expression analysis, and viral load quantification. Microwell chip-based dPCR is useful for applications requiring high throughput and scalability, such as population-scale genotyping, diagnostics, and environmental monitoring [62–64].

4. Crystal Digital PCR

Crystal dPCR utilizes emulsion droplets containing PCR reagents and target nucleic acids to partition samples into discrete compartments [65–67]. Sample partitioning was achieved by encapsulating the sample in water-in-oil emulsion droplets, with each droplet containing either zero or one target molecule. After PCR amplification, droplets containing the amplified products were counted to determine the absolute concentration of the target molecules. The advantages of crystal dPCR include the following: a) single-molecule sensitivity, crystal dPCR can detect and quantify single target molecules, making it highly sensitive; and multiple targets can be analyzed simultaneously within the same sample using different fluorescent probes [67]. Crystal dPCR is useful for applications that require high sensitivity and multiplexing, such as rare mutation detection, liquid biopsy, and viral load quantification.

5. Chip-in-a-tube Digital PCR (ctdPCR)

Chip-in-a-tube dPCR combines the advantages of microfluidic chip technology with the simplicity of tube-based reactions. Sample partitioning was achieved using microfluidic channels within a disposable tube, which was sealed for PCR amplification. After amplification, the tube was analyzed to determine the presence or absence of target molecules [68–70]. The advantages of chip-in-a-tube dPCR include a) Chip-in-a-tube systems offer the convenience of tube-based reactions while benefiting from the precision of microfluidic partitioning and b) the disposable nature of the tubes reduces the risk of cross-contamination and eliminates the need for complex instrument cleaning protocols. Chip-in-a-tube dPCR is useful for applications that require simplicity, such as point-of-care diagnostics, environmental monitoring, and food safety testing [71,72].

6. Semiconductor Chip-based dPCR

Semiconductor chip-based dPCR utilizes semiconductor technology for digital signal detection during PCR amplification. During PCR amplification, hydrogen ions released as byproducts of DNA synthesis are detected by a semiconductor chip, generating a digital signal that corresponds to the number of target molecules present in the sample [73,74]. The advantages of semiconductor chip-based dPCR include a) real-time detection, semiconductor chip-based dPCR enables real-time monitoring of PCR amplification, allowing rapid quantification of target molecules, and b) high sensitivity, which makes it suitable for applications requiring detection of low-abundance targets. Semiconductor chip-based dPCR is useful for applications that require real-time monitoring and high sensitivity, such as gene expression analysis, pathogen detection, and oncology research [75–78].

Chapter 3

Advantages of Digital PCR (dPCR)

3.1 Enhanced precision and sensitivity

dPCR offers enhanced precision and sensitivity compared with traditional PCR techniques. By partitioning the sample into numerous individual reactions, dPCR reduces the potential for stochastic effects and amplification bias, resulting in more accurate quantification of target nucleic acids. The ability to analyze each reaction independently allows for precise determination of the target concentration, even at extremely low concentrations. This heightened precision and sensitivity make dPCR particularly well suited for applications requiring the detection of rare mutations, the quantification of low-abundance targets, and the accurate measurement of subtle differences in gene expression levels [4,6].

The enhanced precision of dPCR stems from its ability to partition the sample into numerous discrete reactions, allowing for more accurate quantification of the target molecules. This high precision is particularly valuable in research settings where precise quantification is crucial for studying gene expression, detecting rare mutations, or quantifying copy number variations [29,79]. Researchers can rely confidently on dPCR to obtain precise and reproducible results, which is crucial for advancing scientific understanding in various fields.

Moreover, the heightened sensitivity of dPCR enables the detection of extremely low levels of the target DNA, making it invaluable for clinical diagnostics. Accurate and

sensitive detection of genetic mutations or pathogens is important for disease diagnosis, prognosis, and treatment monitoring in clinical laboratories. The ability of dPCR to detect rare mutations or low-abundance targets with high sensitivity enhances its utility in clinical settings, potentially leading to earlier disease detection and personalized treatment approaches [53,80].

However, although enhanced precision and sensitivity are desirable attributes of dPCR, their implications vary depending on the context of use. In research settings, the primary concern is to achieve acceptable precision to ensure reliable and reproducible data. Researchers often strive for low coefficients of variation (CVs) to minimize experimental variability and ensure the accuracy of their findings. With the superior precision of dPCR, researchers can confidently detect subtle differences in target abundance, facilitating more insightful data analysis and interpretation.

In contrast, clinical diagnostics prioritize sensitivity to accurately detect disease-related biomarkers. The ability of dPCR to detect rare mutations or trace amounts of pathogens is particularly beneficial for diagnosing diseases at early stages, when conventional methods may fail to detect them [81–83]. However, it is essential to balance sensitivity with specificity to minimize false-positive results, which can lead to unnecessary interventions or anxiety in patients. Thus, clinical laboratories must rigorously validate dPCR assays to ensure their reliability and accuracy in disease diagnosis [84].

Furthermore, the implementation of dPCR in clinical laboratories requires robust quality control measures and adherence to regulatory standards to ensure consistent and accurate results. Laboratories must validate dPCR assays according to established guidelines, such as those provided by regulatory agencies, such as the FDA or international standards organizations. Additionally, ongoing quality assurance programs and proficiency testing will help ensure the reliability and comparability of dPCR results across different laboratories. Several components contribute to enhancing the precision and sensitivity dPCR, each of which plays a crucial role in achieving accurate quantification of target DNA molecules.

1. Microfluidic partitioning, microfluidic devices, or droplet generators partition the PCR mixture into thousands or millions of discrete compartments, each containing a single DNA molecule. This partitioning minimizes competition between DNA molecules during amplification, reduces false positives, and enhances precision [85,86] .
2. High-fidelity DNA polymerases ensure accurate amplification of target DNA sequences, minimizing errors during PCR amplification. These polymerases have proofreading capabilities, reduce the likelihood of introducing mutations, and improve quantification accuracy [87–89].
3. Fluorescent probes, such as hydrolysis probes (e.g., TaqMan probes) or molecular beacons, specifically bind to the target DNA sequence and emit fluorescence upon amplification. Using fluorescent probes, dPCR achieves high specificity, reduces background noise, and improves sensitivity [82,90].
4. Advanced detection systems, high-resolution imaging systems, and sensitive photodetectors detect fluorescence signals from individual partitions, allowing the precise quantification of positive reactions. These detection systems enable accurate counting of target molecules and contribute to the enhanced sensitivity of dPCR [53,91].
5. Data Analysis Algorithms, in data analysis algorithms, sophisticated data analysis algorithms process fluorescence signals from individual partitions, distinguishing between positive and negative reactions with high confidence. These algorithms account for background noise, variations in fluorescence intensity, and partitioning efficiency, thereby improving quantification accuracy [92] .

In testing samples using dPCR, precision and sensitivity are important measures that assess the reliability and accuracy of the results obtained, as demonstrated by the various precision and sensitivity measures described below. To measure precision, three main aspects are reviewed, including the coefficient of variation (CV), which quantifies the variability of replicate measurements within a sample. A lower CV

indicates higher precision, implying that the measurements are more consistent and reproducible, and b) confidence intervals provide a range within which the true value of the target DNA concentration is likely to lie. Narrower confidence intervals indicate greater precision; and c) reproducibility: assessing the reproducibility of results across multiple experiments or runs provides insights into the precision of the dPCR assay. Consistent measurements with minimal variation indicated high precision [8,93].

Furthermore, to measure the sensitivity of testing with dPCR, several factors were confirmed, including the limit of detection (LOD), which represents the lowest concentration of target DNA that can be reliably detected with a specified level of confidence [94–96] . Lower LODs indicate greater sensitivity, implying the ability to detect smaller amounts of target DNA. b) The limit of quantification (LOQ) is defined as the lowest concentration of target DNA that can be accurately quantified with acceptable precision [97]. A lower LOQ suggests greater sensitivity in quantifying target DNA, and c) the false positive rate, which reflects the occurrence of false positives, that is, detecting the target DNA when it is not present, helps assess the sensitivity of the dPCR assay [98,99]. A lower false-positive rate indicates higher sensitivity. d) Dilution Series Analysis: Constructing a dilution series with known concentrations of target DNA allows for the determination of the assay's sensitivity across a range of concentrations. Reliable detection of the target DNA at lower dilutions signifies higher sensitivity [74,100,101] .

Based on this explanation, measures of high precision and sensitivity indicate that sample testing results obtained with dPCR are accurate and reliable. The optimization of assay conditions, including primer and probe design, cycle parameters, and data analysis algorithms, plays an important role in improving the precision and sensitivity of dPCR-based sample testing. Routine quality control measures and validation experiments are essential to ensure the robustness and performance of dPCR assays in real-world applications, especially in contemporary research laboratories. Several factors can lead to decreased sensitivity, precision, and inaccurate results in dPCR assays, thereby reducing the reliability and validity of the assay.

1. Contamination with extraneous DNA molecules, either from environmental sources or from previous experiments, can lead to false-positive results. Strict laboratory practices, including the use of separate workspaces for pre- and post-PCR activities, sterile equipment, and routine decontamination procedures, are essential to minimize contamination.
2. In primer and probe design, inaccurate primer and probe design can result in nonspecific amplification, leading to false-positive signals. Thorough bioinformatics analysis and validation of primers and probes against nontarget sequences are crucial for ensuring specificity and minimizing cross-reactivity.
3. PCR inhibition and inhibitory substances present in the sample, such as PCR inhibitors or contaminants from sample extraction procedures, can interfere with PCR amplification, leading to false-negative results. Dilution or purification of the sample, as well as optimization of PCR conditions, can help alleviate PCR inhibition and improve assay performance.
4. The partitioning efficiency, variability in the partitioning efficiency of dPCR platforms can introduce quantification inaccuracies. Monitoring and optimizing partitioning parameters such as droplet size and distribution can help improve the accuracy and precision of dPCR assays.
5. The signal-to-noise ratio, high background fluorescence, or noise levels can obscure true positive signals, leading to inaccurate quantification. The optimization of fluorescent probes and the use of appropriate signal processing algorithms can help enhance the signal-to-noise ratio and improve the reliability of dPCR results.
6. Sample integrity, degradation, or fragmentation of target DNA during sample preparation can compromise the accuracy of dPCR quantification. Quality control measures, including the assessment of DNA integrity and fragmentation, are essential to ensure the reliability of sample inputs.
7. Data analysis artifacts, improper data analysis techniques or the use of inadequate analysis software can result in inaccuracies in the quantification and interpretation of dPCR results. Standardized data analysis pipelines and thorough validation of

analysis algorithms are necessary to minimize the risk of false sensitivity and precision. By addressing these potential sources of error and implementing rigorous quality control measures, researchers and clinical laboratories can mitigate the risk of false sensitivity, precision, and inaccurate results in dPCR testing, thereby ensuring the reliability and validity of their findings.

3.2 Absolute quantification without reliance on standards

One of the key advantages of dPCR is its ability to provide absolute quantification of target nucleic acids without reference standards. Unlike traditional PCR methods, which rely on relative quantification based on standard curves, dPCR calculates the absolute concentration of the target in the original sample directly from the number of positive and negative reactions [57,102,103].

This eliminated the variability associated with standard curve generation and calibration, resulting in more reliable and reproducible quantification. Absolute quantification plays an important role in the precise and accurate determination of target concentrations and is used in several clinical diagnostics, environmental monitoring, and quantitative gene expression analysis. dPCR has its own way to achieve absolute quantification without relying on external standards and is described as follows.

1. After partitioning the sample, dPCR partitions the sample into thousands or millions of individual reactions, each containing a single DNA molecule or a small number of DNA molecules. This partitioning is typically achieved using microfluidic devices or droplet generators that distribute the sample into discrete compartments. Ideally, each reaction should contain either zero or one target molecule, allowing for discrete counting of target molecules. The process of partitioning samples using dPCR is presented in **Figure 4.**

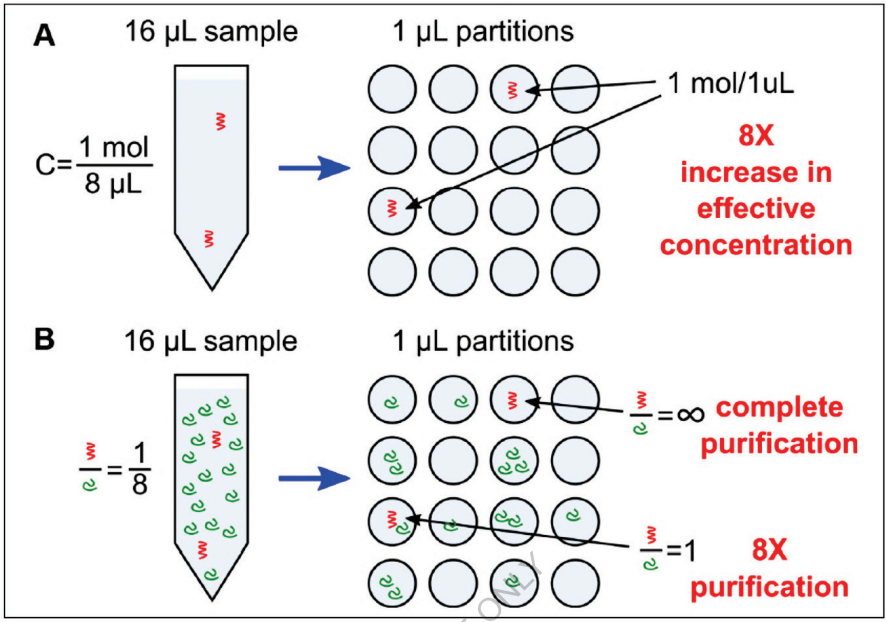


Figure 4. Processing of partitioned samples using digital PCR. **Remarks**: In row A, the sample partitioning process is performed to increase the effective concentration up to eightfold, whereas in row B, the purification process is performed by removing the concentration, compounds, and interfering substances in the original sample, thereby increasing the accuracy and precision of the sample.

2. Poisson distribution, by ensuring that each partition contains only a limited number of DNA molecules, dPCR exploits the principles of Poisson distribution. According to Poisson statistics, the probability of a partition containing more than one DNA molecule is low when the average occupancy per partition is low [104]. This allows dPCR to achieve digital amplification, where either a DNA molecule is present in a partition (resulting in a positive signal) or absent (resulting in a negative signal). The process of Poisson distribution in dPCR is presented in **Figure 5**, and the target concentration determined by dPCR is presented in **Figure 6**. In contemporary laboratories, the use of the Poisson distribution in dPCR offers several key advantages that contribute to improved accuracy and reliability of absolute quantification, which are outlined as follows:

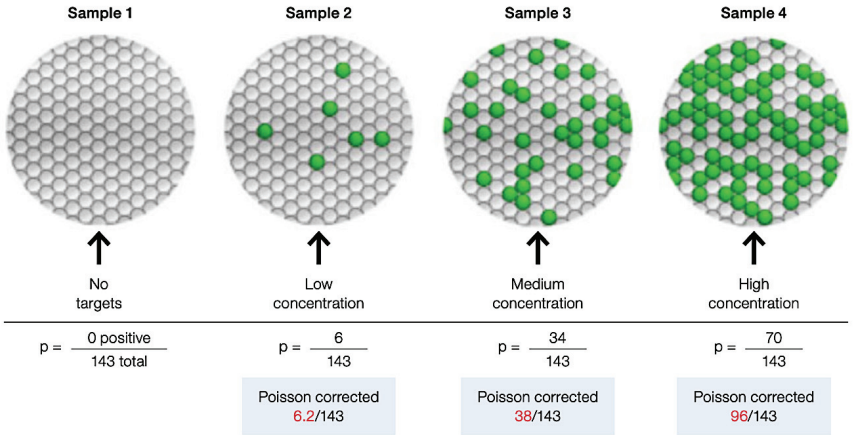


Figure 5. The process of Poisson distribution in dPCR.

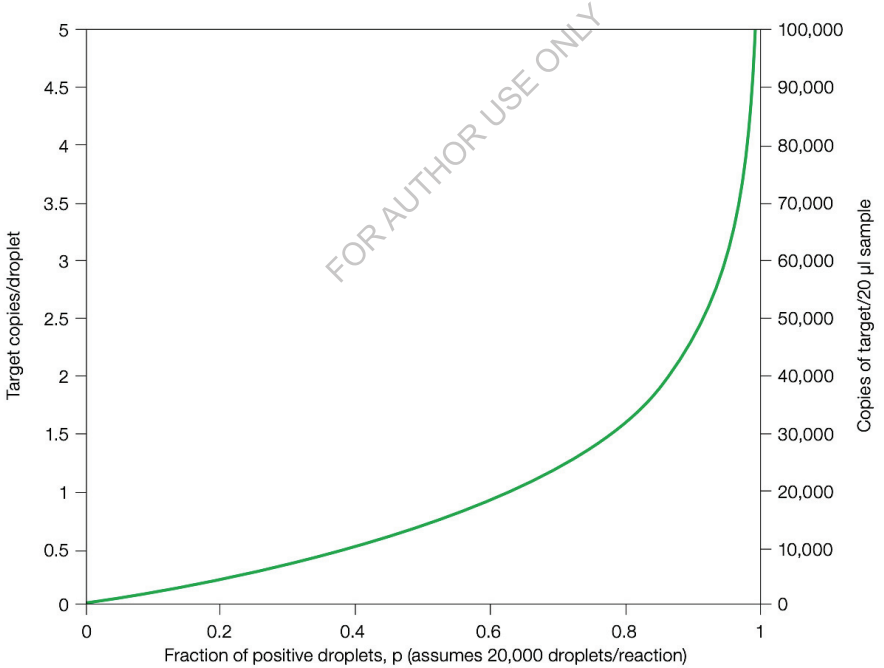


Figure 6. Target concentration by dPCR.

a. *At low target concentrations*, the Poisson distribution accurately describes the probability of observing a certain number of events (e.g., DNA molecules) in a given volume, assuming a random and independent distribution. In dPCR, where samples are partitioned into numerous discrete compartments, this distribution enables precise quantification, particularly at low target concentrations. Even with sparse occupancy per partition, the Poisson distribution provides a robust framework for estimating the true number of target molecules.
b. *Elimination of quantification bias*, unlike traditional PCR methods that rely on relative quantification using standard curves or reference samples, the reliance of dPCR on the Poisson distribution allows for absolute quantification. This eliminated potential biases introduced by variations in amplification efficiency, DNA extraction efficiency, or differences in PCR conditions. As a result, dPCR provides unbiased and accurate quantification irrespective of assay-specific factors.
c. *Digital amplification, sensitivity*, and Poisson distribution facilitate digital amplification in dPCR, in which amplification occurs at the single-molecule level within individual partitions. This digital nature of amplification enables sensitive detection and quantification of low-abundance targets, as each positive reaction unequivocally represents the presence of at least one target molecule. Consequently, dPCR exhibits enhanced sensitivity, which is particularly beneficial for detecting rare mutations, low-level gene expression, and trace amounts of pathogens.
d. *In terms of statistical confidence in the results*, the Poisson distribution provides a statistical framework for assessing the confidence and uncertainty associated with dPCR results. Confidence intervals and statistical tests derived from the Poisson distribution aid in evaluating the precision and reliability of quantification estimates. Researchers can express confidence in the accuracy of the results and determine the uncertainty surrounding the reported target concentrations, thereby enhancing the robustness of the data interpretation.

e. *In terms of flexibility and adaptability, the* Poisson distribution accommodates variations in sample volume, partitioning efficiency, and amplification conditions, offering flexibility in experimental design and data analysis. This adaptability allows dPCR assays to be tailored to diverse applications and sample types, thereby ensuring accurate quantification across a wide range of experimental conditions.

5. Endpoint PCR Amplification, in dPCR amplification is performed to the endpoint meaning that the reaction is stopped before the plateau phase is reached. This ensured that the amplification was quantitative, where each positive reaction represented the presence of at least one target molecule at the start of the reaction. An illustration of signaling in endpoint PCR amplification is shown in **Figure 7**.

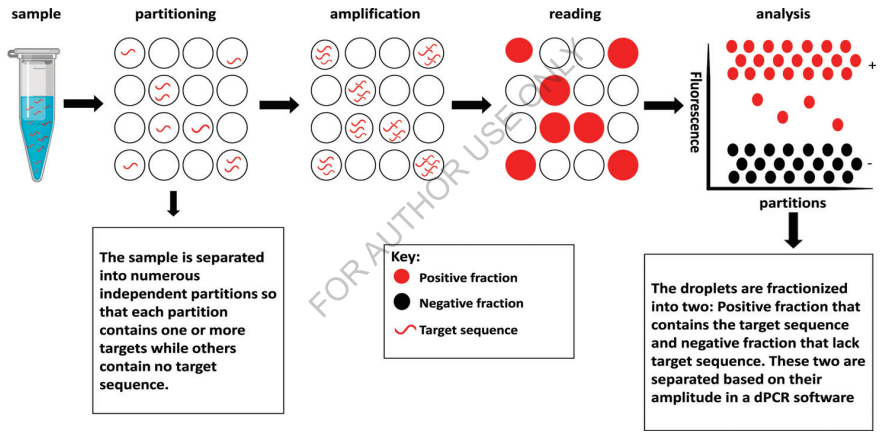


Figure 7. Signaling in endpoint PCR amplification

6. After amplification, dPCR was used to measure the fluorescence signal in each partition to determine whether amplification occurred. Partitions showing fluorescence above a predetermined threshold were considered positive, indicating the presence of a target DNA molecule. dPCR calculates the concentration of the target DNA in the original sample by counting the number of positive partitions and the total number of partitions.
7. Absolute quantification, since dPCR directly counts the number of positive reactions without relying on external standards or reference samples, achieves absolute

quantification of the target DNA. The concentration is expressed as copies per unit volume of the original sample (e.g., copies/μL), providing an absolute measure of the target's abundance.

3.3 Improved detection of rare mutations and targets

dPCR has the advantage of detecting mutations and rare targets because of its high sensitivity and ability to analyze samples independently. In the signaling mechanism of dPCR, all samples that are considered rare cases and in which mutations are present are partitioned into thousands or millions of reactions despite the low frequency of sample conditions and abundant target sequences [81,82,105]. In terms of advantages, individual amplification provides absolute quantification and detection of mutations in rare targets. This is performed in various ways, including:

1. The enhanced sensitivity of dPCR stems from its ability to partition the sample into thousands or millions of individual reactions, each containing a fraction of the original sample. This partitioning increased the likelihood of capturing rare target molecules present at low frequencies within the sample (<0.1%). Even when the target is present at very low concentrations, dPCR can reliably detect and quantify it by independently analyzing each reaction.
2. Absolute quantification, unlike traditional PCR methods that rely on relative quantification based on standard curves, dPCR provides absolute quantification of target nucleic acids. This indicates that dPCR can accurately quantify rare mutations or targets without the need for reference standards. Absolute quantification enables precise measurement of the exact number of target molecules present in a sample, making it particularly valuable for detecting rare variants and mutations.
3. Digital counting, the digital nature of digital counting and dPCR allows precise counting of target molecules in each reaction. dPCR provides a digital readout of the target molecule concentration by independently analyzing individual reactions. This digital counting approach ensures the accurate detection and quantification of even the rarest mutations or targets present in the sample.

4. With reduced amplification bias, partitioning the sample in dPCR helps minimize amplification bias compared to bulk amplification in traditional PCR. Each reaction was amplified under uniform conditions, reducing the impact of the factors that contributed to amplification bias. This reduces the risk of false negatives or false positives, thereby enhancing the reliability of mutation detection in dPCR.
5. The high precision enhanced precision of dPCR ensure the reliable detection and quantification of rare mutations or targets. With reduced variability and improved reproducibility, dPCR provides precise measurements even at low target concentrations, enabling the accurate identification of rare variants and mutations.

Another signaling mechanism applied in dPCR for target detection and rare cases, fluorescence resonance endpoint energy transfer (FRET), was investigated using FRET-based probes. These probes consisted of two fluorophores, a donor and an acceptor, with overlapping emission spectra. When the probe binds to the target sequence, the fluorophores become adjacent, allowing energy transfer from the donor to the acceptor. The change in the fluorescence signal upon digitation indicates the presence of the expected target. Furthermore, in digital droplet PCR (ddPCR), the PCR mixture is partitioned into water-in-oil emulsion droplets. Each droplet serves as an individual reaction vessel. After amplification, droplets containing the target showed fluorescence, whereas droplets without the target did not. The number of positive and negative droplets was used to calculate the absolute target concentration [70,101].

In the field, especially in clinical laboratories, dPCR is widely used to detect rare mutations associated with cancer, such as mutations in the epidermal growth factor receptor (EGFR) gene found in individuals with non-small cell lung cancer (NSCLC) or B-Rapidly Accelerated Fibrosarcoma (BRAF) mutations in melanoma [67,105,106]. By accurately measuring these mutations, dPCR aids in diagnosis, prognosis, and treatment selection to minimize the expansion of necrosis caused by cancer cells. In addition, in liquid biopsies, the use of dPCR allows the detection of circulating tumor DNA (ctDNA) or rare mutations present in body fluids, such as blood or urine.

This approach is useful for monitoring disease progression, predicting treatment response, and detecting minimal residual disease. It can also be applied to the surveillance of infectious diseases. dPCR can detect low levels of pathogenic DNA or RNA in clinical samples, facilitating the diagnosis of infectious diseases. This approach is particularly useful for detecting drug-resistant strains, monitoring the efficacy of antiviral therapy, and future drug development using nanoapproaches, such as stem cells. The better detection capability of dPCR compared to other detection methods allows the study of rare genetic variants, allelic imbalances, and clonal populations in diverse biological samples to be carried out to realize precision medicine with patient safety in mind.

3.4 Reduced susceptibility to PCR inhibitors

Compared with traditional PCR methods, dPCR shows reduced susceptibility to PCR inhibitors. This is because dPCR is an endpoint PCR, so it is less sensitive to inhibitors than quantitative PCR or real-time PCR (qPCR or RT–PCR). This resistance to inhibitors allows for more reliable and accurate results even for difficult sample types. Therefore, dPCR is well suited for applications that require the analysis of difficult samples, such as forensic DNA analysis, food safety testing, and comprehensive environmental and health monitoring.

In particular, decreased susceptibility to PCR inhibitors has been studied in depth, resulting in high sensitivity and accuracy of dPCR. This is mediated by various components of dPCR, such as individually performed sample partitions, each containing a fraction of the original sample. The use of replication in dPCR experiments can help reduce the effects of PCR inhibitors and improve the reliability of results. In addition, the use of blocking agents or modified DNA polymerase in dPCR can reduce susceptibility to PCR inhibitors and improve test accuracy [33].

In addition, comparing dPCR results with those of other PCR-based methods, such as qPCR, may provide further evidence regarding the sensitivity and accuracy of dPCR for detecting PCR inhibitors. By detecting and quantifying even low-level PCR

inhibitors, dPCR offers a more robust and reliable method for the accurate analysis and interpretation of nucleic acid samples.

In addition, dPCR can lower inhibitor concentrations. As the PCR inhibitor was distributed among multiple reactions in dPCR, the inhibitor concentration in each reaction was diluted. As a result, any inhibitory effects on PCR amplification were mitigated, allowing for more robust and reliable amplification of the target nucleic acid. Each reaction in dPCR was analyzed independently, indicating that the presence of an inhibitor in one reaction does not affect the amplification efficiency or detection sensitivity of the other reactions. This independence ensures that the inhibition of one reaction does not compromise the accuracy or reliability of the overall results.

Independent sample partitioning and reaction analysis contributed to the overall robustness of the dPCR results. Even in the presence of PCR inhibitors, dPCR can still accurately detect and quantify target nucleic acids owing to its ability to mitigate inhibitory effects through independent dilution and reaction analysis. Although dPCR is less susceptible to PCR inhibitors than traditional PCR methods, assay optimization and careful assay design are still important for minimizing the impact of inhibitors. Strategies such as using alternative DNA polymerases, optimizing the reaction conditions, and incorporating internal controls can further improve the robustness of dPCR assays in the presence of inhibitors.

Chapter 4

Applications in Research and Clinical Practice

4.1. Quantitative analysis of nucleic acids in various samples

The quantitative measurement of DNA or RNA, known as nucleic acid quantification, is often required to establish the relative concentration of DNA or RNA in a sample prior to further experimentation. Another important use is to ensure sample cleanliness, which is a crucial factor for determining the exact amount of genetic material in a sample. In this context, two commonly used optical technologies are UV–Vis measurements and fluorescence measurements. Selection of the appropriate technology according to sample characteristics and experimental needs can result in accurate quantitative estimation of RNA or DNA, as well as potentially reduce the risk of experimental failure, which can significantly reduce time and costs. The differences between the UV–Vis and fluorescence measurements for the quantitative analysis of DNA and RNA are presented in **Table 1.**

Table 1. Differences in UV–Vis and fluorescence measurements in quantitative analysis

	UV–Vis Measurement	Fluorescence Measurement
Optical signal process generated	Photometric methods for measuring the concentration of nucleic acids rely on their natural absorption properties. When an absorption spectrum is taken, nucleic acids absorb light mainly at a wavelength of 260 nm,	Fluorometric methods for measuring nucleic acids rely on the use of fluorogenic dyes that specifically bind to DNA or RNA.

	displaying a characteristic absorption peak at that value that is the result of the intrinsic absorbance of purine and pyrimidine nitrogenous bases.	
Optical signal measurement	This signal is measured using a spectrophotometer or spectrometer, where the attenuation of the light that reaches the detector after passing through the sample is measured relative to the intensity of the incoming light, and then expressed as the absorption value of the sample in solution. The wavelength separation process can occur both before and after light passes through the sample, with the optical path having either a horizontal or vertical orientation.	The signal is measured using a fluorometer, where the sample is highlighted by filtered light at a specific excitation wavelength, and the light emitted at the emission wavelength is then detected and recorded. There are several methods that can be used to perform wavelength separation, such as using filters or monochromators.
Calculating nucleic acid concentration	The concentration of nucleic acid (C), measured in molar (M), can be calculated using the Beer–Lambert equation: $$c = \frac{A}{\varepsilon . L}$$ where: A, is the UV absorbance in absorption units (AU), ε, is the wavelength-dependent molar absorptivity coefficient (or extinction coefficient), measured in M^{-1} cm^{-1}, L is the light path traversed by light in centimeters (cm). This concentration calculation is often done automatically by many spectrophotometer instruments.	The concentration of nucleic acids is often measured using the fluorescence signal from the sample, where a calibration curve is created using standard samples that have a known concentration. This calibration curve is then fit to a suitable regression model to determine the concentration of nucleic acid in the measured sample. The detection limit and linear response of the measurement are specific characteristics that depend on the test method used in each measurement.
Advantages	Measurements are simple and no sample, dye, or standard preparation is needed.	This method is specific for the measurement of DNA or RNA, demonstrating high sensitivity

	The instrument is also capable of providing direct measurements of purity ratios, such as A260/280 and A260/230. In addition, these instruments can identify nonnucleic acid contamination such as proteins, phenols, or guanidine salts in the sample, and can correct the concentration of mammalian DNA/RNA. This applies specifically to the NanoDrop One/One C/Eight instruments.	with the ability to measure at the pg/mL level. It is the recommended choice for highly diluted nucleic acid samples. In addition, even if there is contamination in the sample, including nucleic acid contaminants, this method still provides accurate results.
Disadvantages	This method is not intrinsically selective, but rather uses software algorithms to distinguish between mammalian DNA and RNA. The sensitivity is also limited, with a higher detection limit compared to fluorescence-based methods.	This process takes longer as it requires the preparation of reagents and samples before measurements are taken. In addition, no information is provided about the purity of the measured sample.

Quantitative analysis of nucleic acids using dPCR is applicable to various sample types, including liquid biopsies, environmental samples, and clinical specimens. Liquid biopsy specimens, such as blood, urine, cerebrospinal fluid, and saliva, contain circulating nucleic acids secreted by tumors or other clinical pathological conditions [33]. dPCR enables precise quantification of nucleic acid biomarkers in liquid biopsies, enabling noninvasive monitoring of disease progression, treatment response, and early detection of cancer or other disorders in a systematic and comprehensive manner. For example, dPCR can measure circulating tumor DNA (ctDNA) in blood samples to detect minimal residual disease, monitor tumor dynamics, and assess treatment efficacy in patients with cancer. By accurately measuring ctDNA levels, dPCR can help doctors make informed decisions regarding patient management and personalized treatment strategies. A schematic example of the dPCR analysis of various types of biopsies is presented in **Figure 8.**

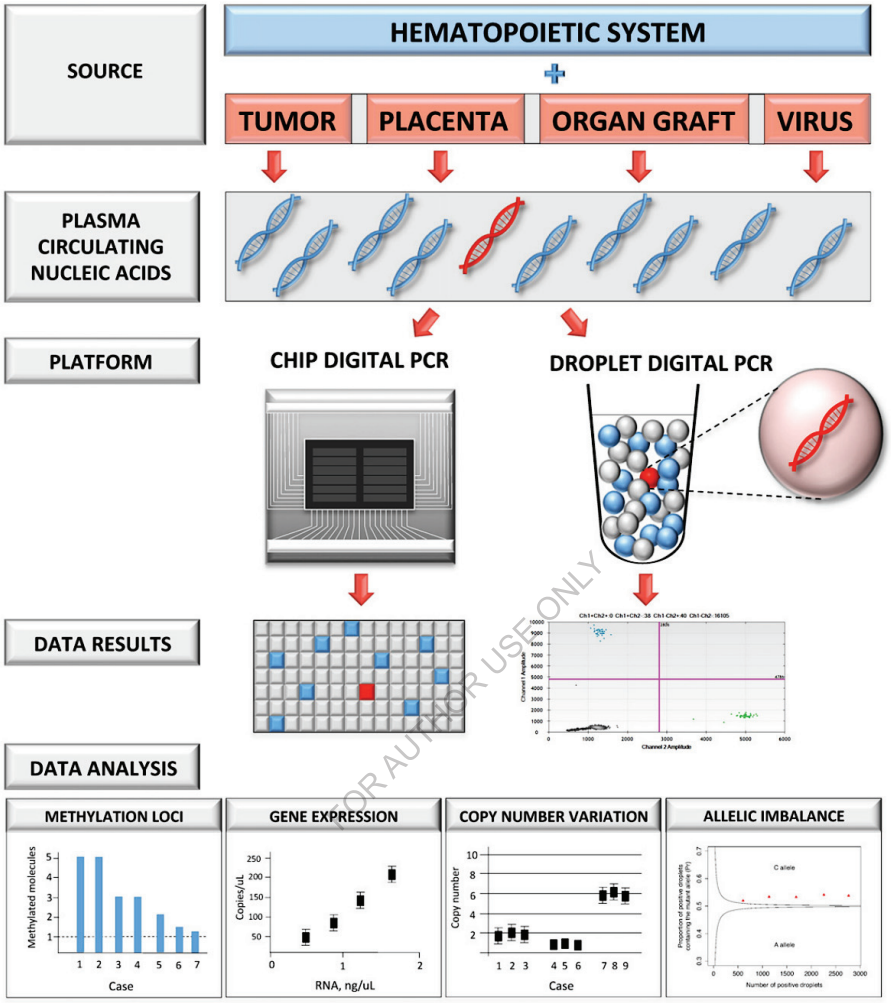


Figure 8. Illustration of the use of digital PCR (dPCR) in the identification of circulation in nucleic acid samples (Image courtesy of Hudecova [24])

Environmental samples such as soil, water, air, and microbial communities contain diverse nucleic acid targets that reflect environmental health, biodiversity, and microbial ecology [107,108]. dPCR enables the quantitative analysis of nucleic acids in environmental samples, facilitating the detection and monitoring of microbial populations, pathogens, and genetic markers associated with environmental

contaminants or pollutants. For example, dPCR can be used to quantify microbial populations, detect antibiotic resistance genes, and monitor viral or bacterial pathogens in environmental samples [109]. By providing precise quantification of target nucleic acids, dPCR supports environmental monitoring, risk assessment, and ecosystem conservation initiatives. The scheme for reading DNA or RNA targets in the biopsy samples is shown in **Figure 9.**

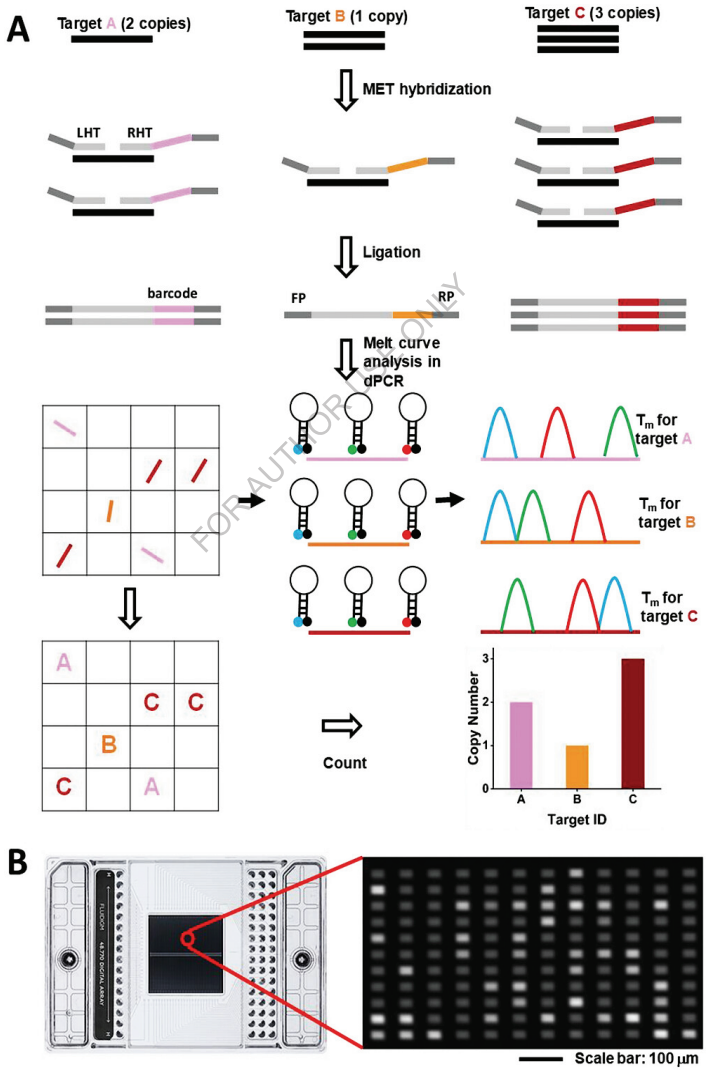


Figure 9. The scheme of reading DNA or RNA targets in biopsy samples.

Clinical specimens, including tissue biopsies, swabs, aspirates, and body fluids, are routinely analyzed for the diagnosis, prognosis, and management of various diseases. dPCR enables the quantitative analysis of nucleic acid biomarkers in clinical specimens, offering improved sensitivity, accuracy, and reproducibility over traditional PCR methods [110]. For example, dPCR can measure viral load in respiratory tract specimens to diagnose respiratory tract infections (e.g., influenza, COVID-19, tuberculosis), assess gene expression levels in tissue biopsies for cancer diagnosis, and detect genetic mutations in prenatal samples for noninvasive prenatal testing (NIPT) [38,62,80,111,112]. By providing precise quantitative data, dPCR can improve clinical decision-making, patient stratification, and therapeutic monitoring across diverse medical specialties. Examples of standard curve quantification in biopsies and clinical specimens are shown in **Figure 10.**

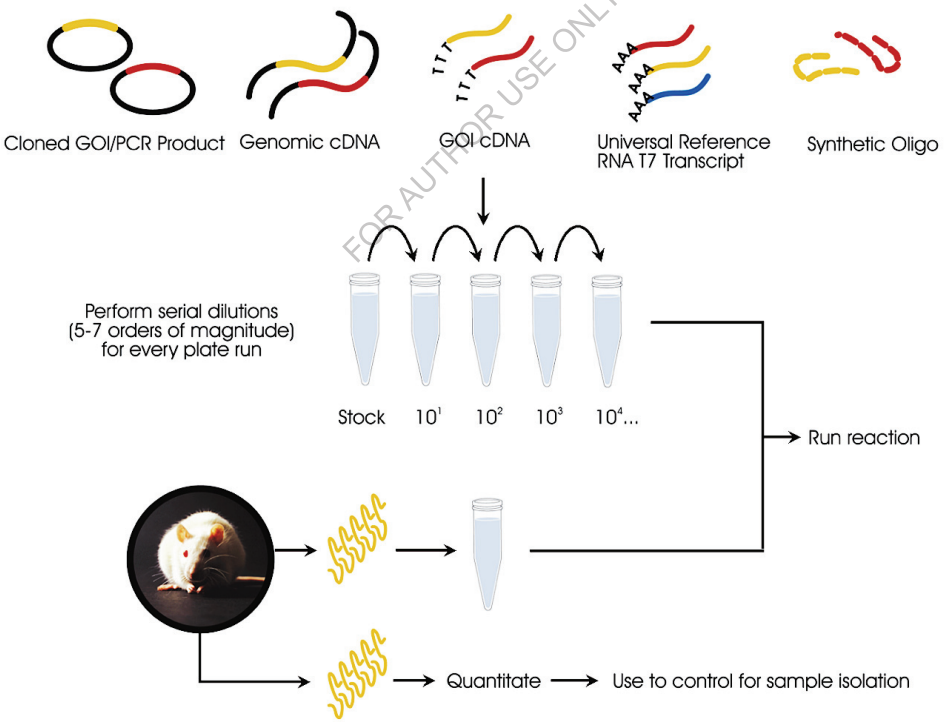


Figure 10. Scheme of standard curve quantification in biopsies and clinical specimens.

4.2. Application of digital PCR in infection control

The utilization of dPCR for infection control at the global level has become an integral part of infectious disease prevention, detection, and control efforts in the last decade. dPCR offers highly sensitive detection, accurate quantification, and analysis of genetic mutations relevant to disease-causing microorganisms, including viruses, bacteria, fungi, protozoa, and other ectoparasites. In its role as a medium for the detection, evaluation, and monitoring of infectious diseases, dPCR plays a significant role in various technical matters, especially infection control at the global level, including the following:

1. **Utilization of digital PCR for early detection**

Early detection of this disease is one of the key aspects of infection control. dPCR enables highly sensitive detection of pathogenic genetic material in biological or environmental samples associated with infectious events, including blood, saliva, and other body fluids. Thus, dPCR can be used to identify infections at an early stage, even before clinical symptoms appear [113]. This enables more effective prevention and control measures to prevent the spread of infectious diseases. An example of the use of dPCR for early disease detection is presented in **Figure 11**.

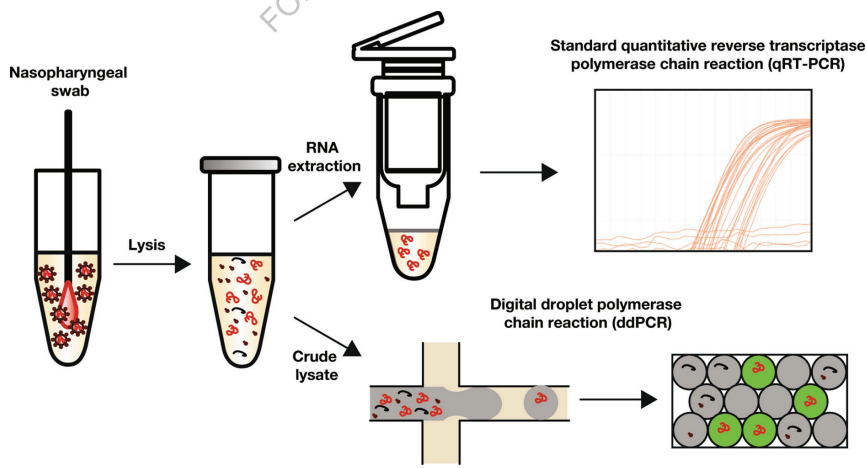


Figure 11. Early detection of SARS-CoV-2 using two testing models, top: qPCR, bottom: dPCR, with the result that dPCR detected better than qPCR.

2. Accurate quantification in disease epidemiology

The use of dPCR is important for measuring disease burden and understanding the epidemiology of a disease at the global level. With its ability to count the absolute number of gene targets in a sample, dPCR provides more accurate quantification than conventional PCR methods. This allows researchers and policymakers to estimate disease prevalence more precisely and to design control strategies appropriate to the situation at hand [114–117] .

At the global, national, and subnational levels, dPCR can be used as a basis for program development related to surveillance results to minimize the occurrence of outbreaks, endemics, or epidemics in a region. Precision quantification makes the expected target in accordance with the hypothesis so that it can be used as actual data when preparing recommendations for controlling infectious and communicable diseases [31,118].

3. Identification and monitoring of genetic variants and mutations

In the face of explosive outbreaks of pandemics due to infectious diseases, especially viruses, bacteria, fungi, protozoa, and other ectoparasites, the ability to identify genetic variants and mutations is very important. This is because the rapid ability of a microorganism to mutate has implications for increasing microbial immunity, which leads to difficulties in control efforts. A very rapid mutation can increase the burden of disease and mortality [81,83,105].

dPCR can be used to detect and monitor genetic changes in pathogens, such as influenza virus, coronavirus disease (COVID-19), dengue virus (DENV), chikungunya virus (CHIKV), and antibiotic-resistant bacteria [119–123]. This information is important for designing effective vaccines, developing accurate diagnostic tests, and selecting appropriate therapies according to the state of the evolving genetic mutation so that treatment can be targeted.

4. Evaluation and supervision of vaccination programs

Vaccination is one of the most effective strategies for controlling infectious and communicable diseases. On a global scale, dPCR can be used to evaluate the

effectiveness of vaccination programs by measuring the immune response of individuals or populations to vaccines. Through dPCR, health program administrators can detect the presence of the virus in samples, such as blood samples or nasal secretions from vaccinated individuals. This allows vaccination supervisors to monitor the level of viral infection in the vaccinated population and confirm the short- to long-term effectiveness and reactivity of the vaccine in the individual's body.

In addition to detecting the presence of the virus, dPCR can be used to quantify the amount of virus (quantification) in individual biopsy samples, allowing for a more accurate evaluation of the effectiveness of the vaccine in reducing the viral burden in the vaccinated population. Furthermore, long-term treatment generally has an adverse impact on individual health. dPCR can be used to detect mutations in viruses, as this may affect the effectiveness of vaccine delivery. By regularly monitoring viral mutations, policymakers can ensure that vaccines remain effective against emerging viral variants [82,124–126].

In contrast, dPCR can be used as a quality-control tool to ensure successful vaccine production. By testing for the presence of the target virus in vaccine batches, manufacturers can ensure that the vaccines produced meet the safety and effectiveness standards. These conditions were also evaluated by monitoring the immune response to vaccination by measuring the number and type of immune cells involved in responding to the vaccine.

5. Drug resistance assessment

The development of drug resistance is a serious challenge in infection control. dPCR can be used to monitor for the presence of genetic mutations associated with drug resistance in pathogens. This information is important for designing effective treatment strategies and for reducing the risk of further development of drug resistance [97,127,128]. With the utilization of dPCR technology, infection control at the global level can be improved using a more precise, responsive, and effective approach. From early detection to epidemiological monitoring, understanding of

genetic variants, and evaluating vaccination programs, dPCR is an invaluable tool for safeguarding public health.

6. Fast and accurate diagnostic testing

In an outbreak or pandemic, rapid and accurate diagnostic testing is essential to identify cases, trace contacts, and control the spread of the disease. dPCR can be implemented as an efficient and reliable diagnostic tool for detecting pathogenic genetic material within a short time [129–132]. Thus, dPCR supports a rapid response to global, national, and subnational health crises. The diagnostic stage of the disease is an important goal for accelerating disease control. dPCR has a large capacity to accelerate disease control through the detection of various sample types. The diagnostic stages generated from SARS-CoV-2 samples for the detection of the presence of the virus in nasopharyngeal swab samples are presented in **Figure 12**.

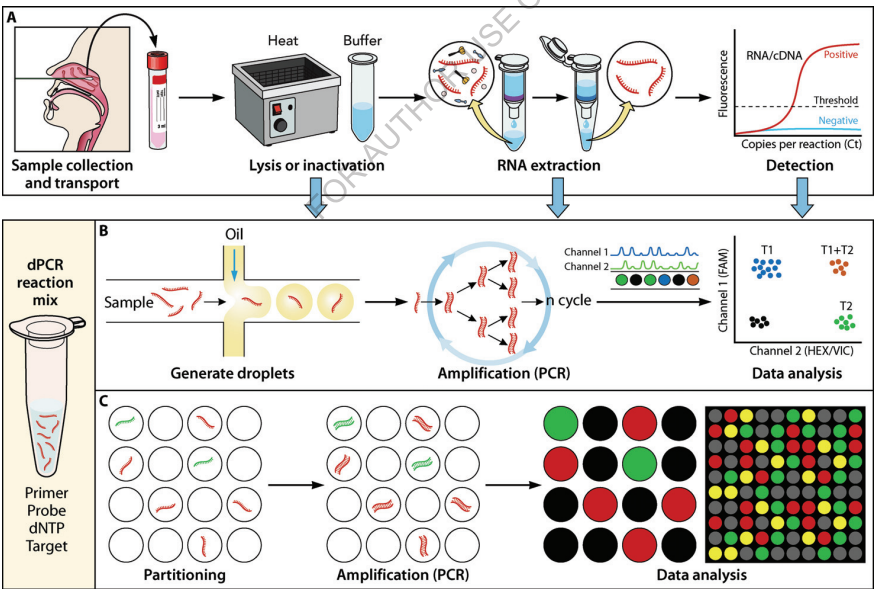


Figure 12. Processing of SARS-CoV-2 samples using dPCR. Description: (A) Nasopharyngeal swab samples were collected, inactivated, and processed for detection; (B) dPCR was used for analysis by the droplet method; and (C) the end point of sample processing and retrieval of findings.

4.3. Application of digital PCR in vector control

dPCR is a molecular method with important applications in vector control in endemic regions and at the global level. This technology enables highly sensitive detection and quantification of DNA or RNA targets with a high degree of accuracy. In vector control efforts, such as infectious diseases transmitted by mosquitoes, rodents, other insects, kuta, and ectoparasites, the use of dPCR can provide an in-depth understanding of the distribution and prevalence of disease-causing agents. **Figure 13** shows an example of the application of dPCR in the identification and control of disease-causing vectors to obtain various types of information.

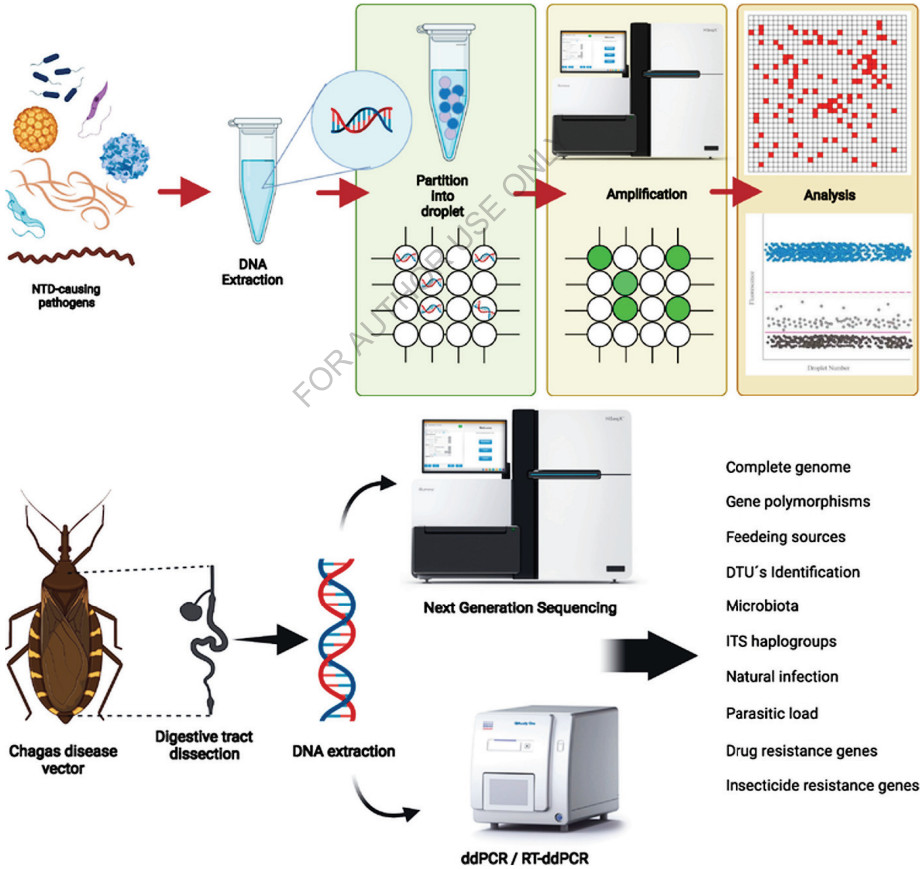


Figure 13. Example of the use of dPCR for the identification and control of parasite-based disease-causing vectors.

In particular, dPCR can be utilized as a medium for vector control, such as

1. **Detection and quantification of pathogenic agents**

dPCR enables the detection and quantification of pathogenic agents in environmental samples with a high degree of sensitivity. This makes it possible to accurately detect small amounts of pathogenic agents present in samples, such as water, blood, and other fluids. For example, in malaria vector control, dPCR can be used to precisely detect and quantify the number of malaria parasites in mosquito populations or human blood samples. In addition, whole blood component analysis for dengue virus detection was performed by reviewing the presence of viruses in the blood. This information is very useful for understanding the level of infection, the risk of disease transmission in endemic areas, and its spread within the same region [32].

2. **Monitoring of vector control programs**

dPCR can be used as a monitoring tool in vector-control programs. By regularly monitoring vector populations, the effectiveness of interventions such as fogging, insecticide use, or vaccination can be evaluated. Data obtained from dPCR can provide deep insights into changes in vector populations, including the potential development of resistance or geographical shifts, including genetic mutations in each region. These results are useful for developing the best vector control programs to improve public health outcomes [133,134].

3. **Drug resistance monitoring**

The use of drugs for vector control can lead to drug resistance. dPCR enables careful monitoring of the presence of genetic mutations associated with drug resistance, including mutation rate, resulting impact, and recommendations to minimize the occurrence of drug resistance in the long term. Thus, preventive measures should be taken to avoid the unwanted spread of drug resistance on a larger scale. For example, the use of insecticides can trigger the development of resistance in mosquitoes that transmit viruses that cause infections, such as dengue fever, chikungunya, or filariasis [135–138]. An in-depth understanding of the

dynamics of drug resistance is essential for designing effective control strategies and pursuing new types of natural drugs to control vector-borne diseases [97,128].

4. **Vector species identification**

In some cases, proper identification of vector species is crucial for planning appropriate control strategies. dPCR can be used to quickly and accurately identify vector species. This allows the tailoring of control strategies to consider the behavior and biological characteristics of the species in question. For example, the use of dPCR to identify Aedes mosquito species that transmit Zika or dengue viruses in real time can help in planning more specific and effective control measures that are well targeted [41,79,124,128,139].

5. **Controlling the spread of zoonotic diseases**

The use of dPCR can also be extended to the control of vectors involved in the spread of zoonotic diseases, which are transmitted between animals and humans. Examples of zoonotic diseases include leptospirosis transmitted through water contaminated by animal urine, monkeypox (MPX) transmitted from sexual contact with fellow people, coronavirus disease (COVID-19) transmitted from bats [98,140–142], and other zoonotic diseases transmitted through the sylvatic cycle [143]. In this case, dPCR can be used to detect the presence of disease-causing bacteria in vector animal populations or contaminated environments [34,144,145] .

Thus, dPCR has proven to be an invaluable tool for vector control in endemic areas and at the global level. Its ability to detect, quantify, and monitor pathogenic agents with a high degree of sensitivity and specificity makes it a very useful tool in vector-borne disease prevention and control. The utilization of dPCR is expected to be more effective in reducing disease burden and improving overall public health.

4.4. Application of dPCR in the investigation of extraordinary events

The utilization and application of dPCR in global outbreak investigations are important tools for understanding and addressing the challenges associated with disease spread, epidemiological surveillance, and research in healthcare. dPCR is an

innovative technology that enables the detection and quantification of genomic targets with a high degree of sensitivity and the ability to detect very low amounts of target molecules in samples. Investigating global outbreaks is critical given the complexity and speed of the spread of emerging diseases [108,146,147].

One of the key benefits of dPCR in global outbreak investigations is its ability to detect and monitor disease-causing microorganisms with greater sensitivity than conventional PCR methods. For example, in the face of a pandemic such as COVID-19, dPCR can be used to more accurately detect the SARS-CoV-2 virus even at low levels of virus in samples, allowing the identification of cases that may be missed by other detection methods [71,72,148]. This information is crucial for understanding the patterns of disease spread and for designing appropriate control strategies.

In addition, dPCR enables the monitoring of genomic mutations in disease-causing agents. In the context of a pandemic, this is crucial, as certain mutations can affect the transmission, virulence, and response to vaccines. With the ability of dPCR to detect and monitor mutations with high sensitivity, researchers can quickly identify new variants, which can aid in decision-making regarding disease control and the development of necessary vaccines. In addition to direct investigation of disease-causing agents, dPCR is also useful for epidemiological surveillance [149,150]. In this case, dPCR can be used to monitor the presence of pathogenic microorganisms in human, animal, and environmental populations with high sensitivity. This information is important for evaluating the risk of transmission, analyzing patterns of disease spread, and designing effective control strategies.

The implications of dPCR in global outbreak investigations are related to the development and validation of diagnostic tests [71,98]. The use of dPCR in diagnostic testing can improve the sensitivity and specificity of the tests, which in turn can result in more reliable and accurate detection of disease-causing agents. This has become crucial in global outbreak situations, where the speed and accuracy of diagnosis are important factors in preventing the spread of the disease. However, the use of dPCR in global outbreak investigations presents some challenges. One of them is the cost

and technical complexity associated with dPCR equipment and analysis. In addition, further development is needed for the standardization of dPCR methods and interpretation of results to ensure the consistency and validity of data among different laboratories.

The utilization and application of dPCR in global outbreak investigations have significant implications for understanding, detecting, and addressing diseases that affect human populations. With its high sensitivity and ability to detect very low amounts of target molecules, dPCR enables more effective surveillance, more accurate diagnosis, and better mutation monitoring of disease-causing agents. However, challenges, such as cost and technical complexity, remain to be overcome to maximize the potential of dPCR in fighting global outbreaks.

4.5. Application of dPCR in the detection of noncommunicable and degenerative diseases

The use of dPCR technology has changed the way we detect and identify noncommunicable and degenerative diseases globally. Nondiscommunicable diseases, such as various cancers, diabetes mellitus, heart disease, hypertension, and other degenerative diseases, such as Alzheimer's disease, Parkinson's disease, and dementia, are global health challenges that require early detection and accurate monitoring for better management [129,151]. dPCR is an innovative molecular diagnostic tool that offers sensitivity, accuracy, and the ability to detect markers of infectious and degenerative diseases, massive cell damage, mutations, and genetic variants, even in very low or complex samples.

dPCR divides a DNA or RNA sample into many individual stand-alone reactions, each containing at least one of the original samples. In each of these reactions, DNA or RNA was exponentially amplified, and the results were monitored separately. This is in contrast to conventional PCR, in which amplification occurs in a single tube. The digital approach allows the absolute measurement of molecular target concentrations, which means that dPCR can detect even relatively small amounts of the target. One of

the main applications of dPCR in the identification of noncommunicable and degenerative diseases is the detection and monitoring of cancer, tumors, or other disease progression.

dPCR enables the detection of specific mutations in genes associated with cancer, tumors, or other diseases at very low levels, allowing for more accurate early diagnosis and monitoring of disease progression. This is important because the treatment of noncommunicable and degenerative diseases is often more effective if started in the early stages of the disease rather than after infection, such as cancer. An example of dPCR utilization in noncommunicable and degenerative diseases, especially cancer detection, is presented in **Figure 14.**

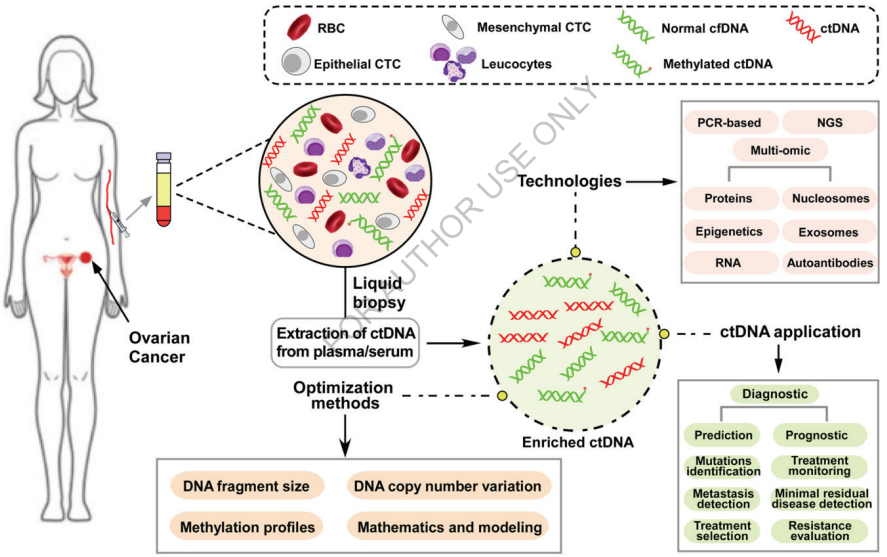


Figure 14. Detection of ovarian cancer in various types of liquid biopsies for prediction, prognosis, mutation detection, treatment programs, etc.

Digital PCR also plays an important role in diabetes management. By detecting and monitoring glucose levels and genes associated with insulin resistance, dPCR can assist in the timely and effective treatment of people with diabetes mellitus in an effort to control fasting, intermittent blood sugar, insulin or pancreatic damage, and other

complications [152]. In heart disease, hypertension, and other diseases, dPCR is used to detect genetic mutations associated with certain heart diseases and blood disorders, including abnormal hypertension, or to monitor the expression levels of genes involved in the development of heart disease and blood disorders, which helps in better diagnosis and management by clinical health personnel [29,78,82,153].

Furthermore, in neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease, dPCR can be used to detect biomarkers associated with the progression of these diseases. For example, dPCR can be used to detect and measure tau protein levels in cerebrospinal fluid, which is an early indicator and predictor of Alzheimer's disease progression [154]. In addition to disease detection, dPCR is important for monitoring therapeutic response and disease progression. By enabling more sensitive and quantitative analysis of specific targets in patient samples, dPCR can help evaluate treatment effectiveness and identify possible resistance or evolving disease changes [155].

Overall, dPCR has revolutionized the identification of noncommunicable and degenerative diseases at the global level [156]. With its high sensitivity and precision as well as the ability to work with very small or complex samples, dPCR enables early detection, more accurate diagnosis, therapeutic response monitoring, and a better understanding of the progression of these diseases. As such, it is a valuable tool for improving the management and treatment of diseases that affect global, national, and subnational health.

4.6. Application of dPCR in the development of cell and gene therapies

Recently, dPCR has become a growing molecular technique for the development of cell and gene therapies worldwide. dPCR enables the detection of genes or DNA targets with higher sensitivity than conventional PCR. This is important in the development of cell and gene therapies, where the number of targets is often very low in the analyzed sample, allowing for result bias and undetectable and less accurate assays. With increased sensitivity, dPCR can detect and quantify gene targets even in

very small amounts to evaluate the efficacy of therapies and monitor the progress of treatments provided by healthcare providers [56].

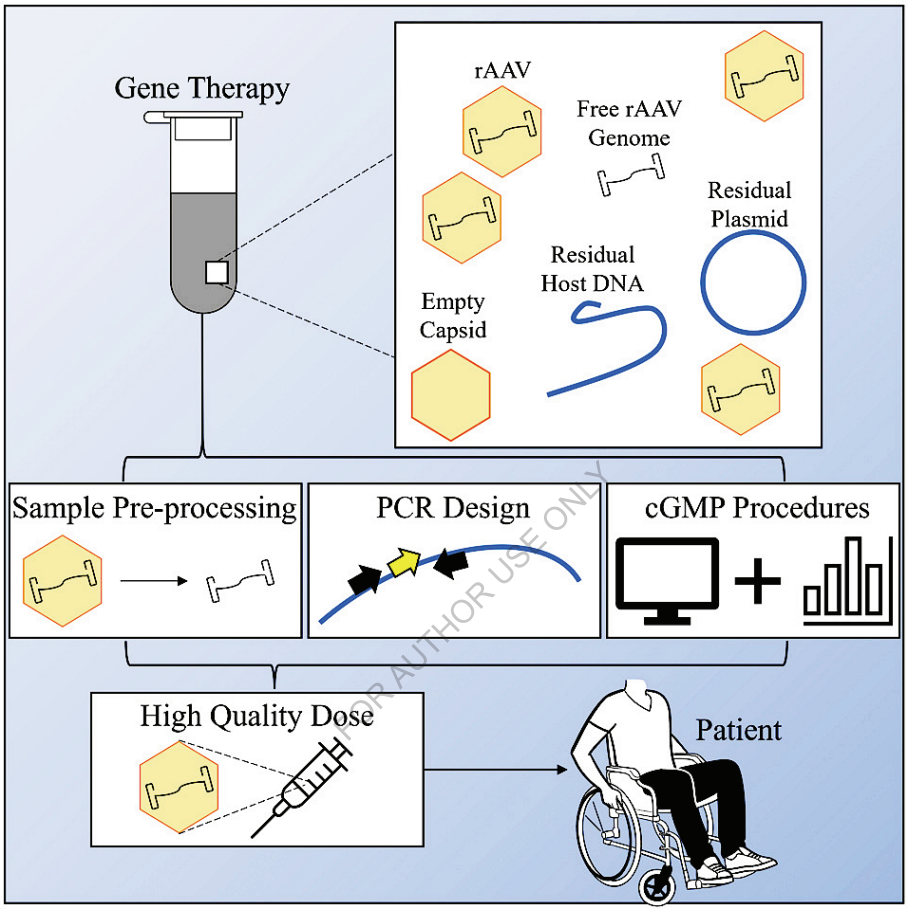


Figure 15. Generation of recombinant adeno-associated virus (rAAV) using dPCR technology

In addition, dPCR offers high accuracy and precision for gene target quantification. In cell and gene therapy, where the number of genetically modified cells or molecules must be accurately measured, this accuracy and precision are critical for generating recommendations for policy makers and clinicians. dPCR partitions the sample chamber into hundreds to thousands of small compartments, each containing multiple target genes or DNA molecules. Since each compartment works independently, dPCR

enables more accurate measurements and higher precision even for complex samples [35]. The framework for developing gene therapy using dPCR technology is presented in **Figures 15** and **16.**

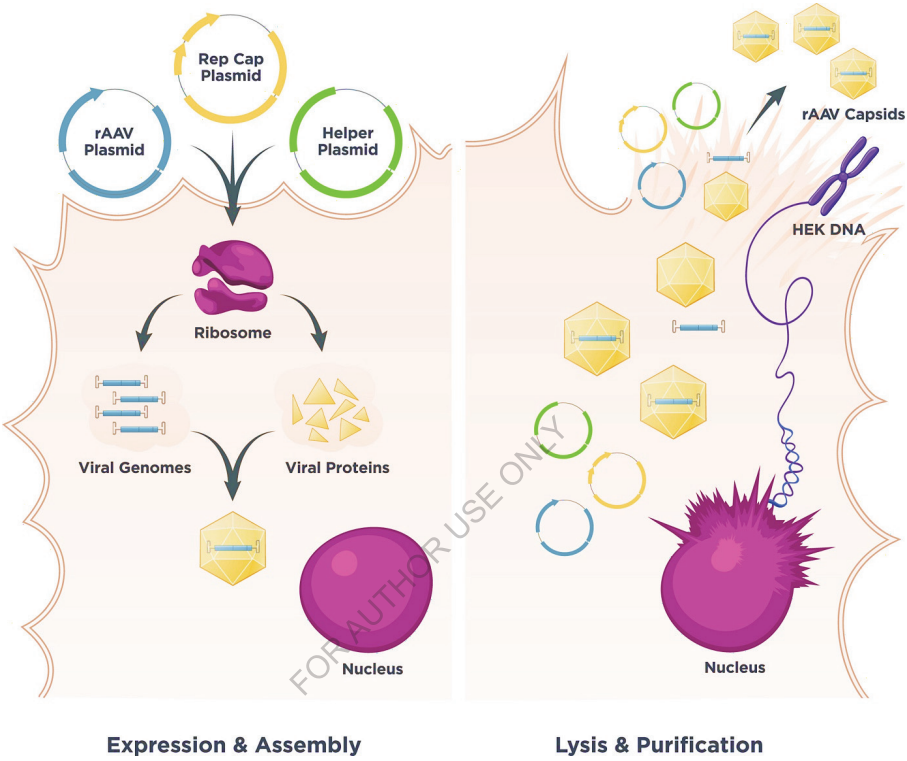
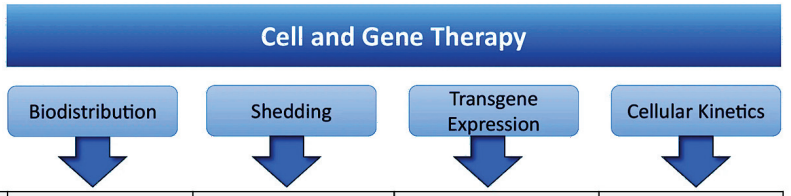


Figure 16. The production flow of recombinant adeno-associated virus (rAAV) is accompanied by the analysis process used in dPCR.

The applications of dPCR in cell and gene therapy development include genetic mutation monitoring, evaluation of viral vector transduction efficiency in gene delivery, and target gene expression monitoring. For example, in gene therapy, monitoring the expression levels of target genes after viral vector delivery is key to evaluating the efficacy of therapy [127,131]. dPCR enables accurate quantification of the number of RNA molecules amplified from target genes, providing a better understanding of gene expression levels before and after treatment in new data to measure the success rate

of treatment. In addition, dPCR has been used for the detection and quantification of circular DNA, such as extracellular circular DNA (eccDNA) or mitochondrial circular DNA [157,158]. Thus, it is relevant in the development of cell and gene therapies, as circular DNA may play a role in resistance to therapy and serve as a biomarker for disease prognosis [49,86]. The flow related to cell and gene therapy drug development efforts validated by the dPCR method is presented in **Figure 17.**



Purpose	Measures the gene/cell therapy product in target and non-target tissues and biofluids.	Measures the release of virus-based gene therapy products from the patient through excreta and secreta to understand exposure to the environment and transmission to other humans.	Measures the expression of the transgene that is delivered by the gene/cell therapy product in target and non-target tissues and biofluids.	Measures the pharmacokinetics of the cell therapy after administration.
Phase	Preclinical, Clinical	Preclinical, Clinical	Preclinical, Clinical	Preclinical, Clinical
Matrices*	Injection site, gonads, adrenal gland, brain, spinal cord, liver, kidney, lung, heart, spleen, blood, etc.	Urine, saliva, tears, feces, nasopharyngeal fluids, wounds, sores, etc.	Target tissues, gonads, adrenal gland, brain, spinal cord, liver, kidney, lung, heart, spleen, blood, etc.	Blood, PBMCs, bone marrow aspirates, etc.
Bioanalytical Assay Platforms	qPCR/dPCR, Flow Cytometry	qPCR/dPCR, cell-based infectivity	qPCR/dPCR (mRNA level), various platforms (protein level)	qPCR/dPCR, Flow Cytometry

Figure 17. Development direction of cell and gene therapy with dPCR

dPCR applications are becoming increasingly relevant not only in research but also in clinical testing and diagnostics in contemporary laboratories. dPCR has been used in noninvasive testing for cancer cell detection and monitoring, evaluation of minimal levels of clonal insertion in hematopoietic stem cell transplantation, and assessment of the level of viral vector integration in gene therapy. Thus, the utilization of dPCR in the development of cell and gene therapies has opened the door for significant advances in our understanding of cellular and molecular biology and has increased the possibility of developing more effective and safe therapies. Its presence in the global market continues to expand the scope of its applications and contribute to advancements in the field of cell and gene therapy.

4.7. Application of dPCR in wastewater and environmental surveillance

The utilization of dPCR in wastewater and environmental monitoring at the global level has changed the management of monitoring and managing available natural resources, including the management of diseases originating from the environment. The development of infectious diseases originating from animals and the environment has led to various studies on the development of disease detection methods. Research in the field of wastewater and environmental surveillance for infectious disease detection has become a growing subject, such as during the COVID-19 pandemic. Figure 18 shows the use of dPCR technology for the detection of pathogens and disease-causing microorganisms in wastewater and in the environment, especially during the COVID-19 pandemic [108,123,140].

This method involves monitoring the quality of wastewater and the environment to detect the presence of disease-causing pathogens such as viruses, bacteria, and parasites, which can be transmitted through contaminated water or the environment, and monitoring the transmission of disease-causing pathogens through water and light. One of the examination techniques used and developed includes RT–PCR, qPCR, dPCR, and next-generation sequencing (NGS), which allows for more complete monitoring, detection, and characterization of the genetic load of various pathogens in wastewater samples, particularly at the household level. The following applications of dPCR have been widely developed in people's lives:

1. **Detection and quantification of pathogenic microorganisms**

   One of the main applications of dPCR in wastewater surveillance is the detection and quantification of disease-causing pathogenic microorganisms such as *E. coli and V. cholerae*. Wastewater can contain various pathogens, such as bacteria, viruses, parasites, protozoa, and other ectoparasites, that can potentially cause diseases in humans and animals. Using the dPCR method, healthcare providers and environmental surveillance teams can identify and quantify the number of such pathogens in wastewater samples with a high degree of sensitivity [140]. This allows

quick and effective preventive measures to reduce the risk of wastewater-borne disease transmission [123,159–163].

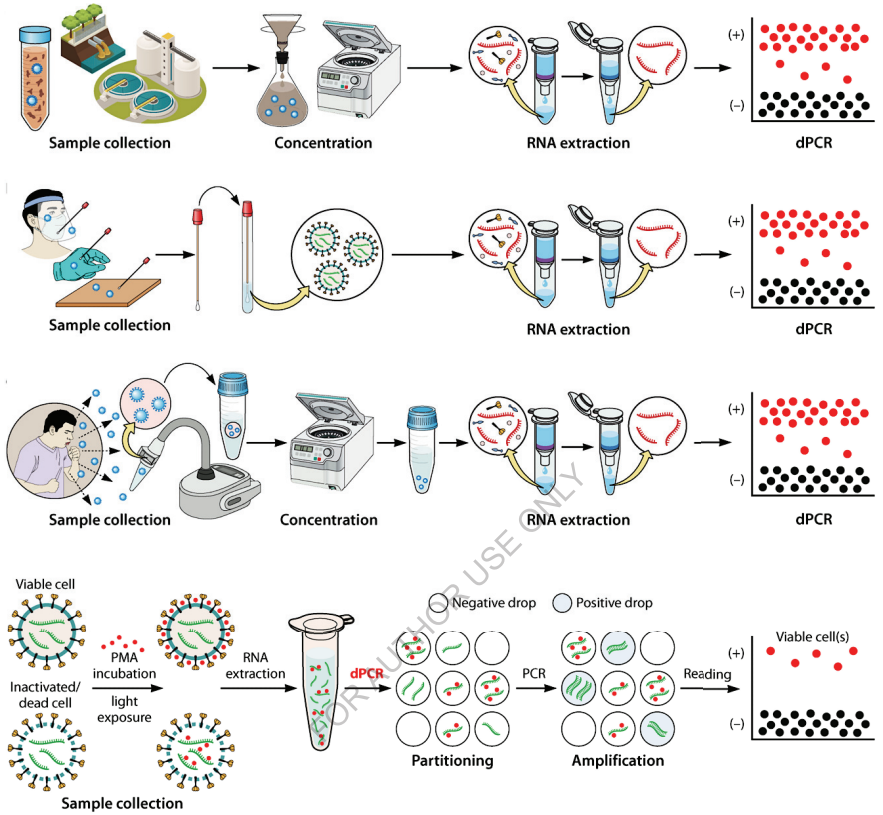


Figure 18. Application of dPCR to detect disease-causing pathogens in household wastewater surveillance results.

2. Monitoring of hazardous chemical content

In addition to pathogenic microorganisms, wastewater can contain various harmful chemicals, such as heavy metals, pesticides, and other synthetic organic compounds. dPCR can be used to detect and quantify the concentrations of these chemicals in wastewater. By regularly monitoring the content of hazardous chemicals in the surrounding environment, communities can ensure that the wastewater discharged into the environment does not exceed the limits set for the

environment and help reduce its negative impact on ecosystems and disease vector bionomics [164].

3. **Evaluation of effluent treatment effectiveness**

The use of dPCR can be extended to evaluate the effectiveness of sewage treatment systems, ranging from the household level to the country level. After wastewater is treated through various stages, such as physical, chemical, and biological treatments, it is important to ensure that all contaminants have been removed or reduced to safe levels. By comparing the concentrations of genetic targets before and after effluent treatment using the dPCR technique, we can evaluate the effectiveness of the treatment system in removing contaminants from wastewater. This was also clearly demonstrated by the reduction in cases, morbidity, and mortality due to pathogens.

4. **Support environmental protection policies**

The data obtained through dPCR can also be used by relevant policymakers to support the development of environmental protection policies. Information on wastewater quality obtained through dPCR technology can help governments and regulatory agencies make better decisions in terms of environmental regulation and management, protection of environmental sustainability, and planning and providing a safe environment for individual survival. By having a better understanding of environmental conditions, policies can be designed to minimize negative impacts on the environment and ensure the sustainability of natural resources, and people are more aware of changes that occur as a form of mitigation to the environment directly and indirectly.

5. **Monitoring environmental changes**

In addition, dPCR can be used to monitor environmental changes that occur over time, either due to climate change, changes in hydrometeorological elements, and/or environmental degradation. For example, the use of dPCR to monitor the presence of certain microbial species in wastewater can provide insights into how environmental changes, such as changes in temperature or pollution levels, may

affect these microbial communities. This information can be used to predict the impact of further environmental changes and to design mitigation strategies appropriate for field conditions [37,64].

Overall, the utilization of dPCR in global wastewater and environmental surveillance has significantly improved the understanding and management of these important natural resources. With its high sensitivity and accuracy, this technology has opened the door for more effective surveillance, evidence-based decision-making, and better environmental protection. In the face of current global environmental challenges, the integration of dPCR in wastewater surveillance and management strategies can be one of the most valuable tools for maintaining environmental sustainability for future generations.

4.8. Application of dPCR in immunotherapy development

The utilization and application of dPCR in the development of immunotherapy is becoming increasingly popular with the emergence of diseases that attack the immune system, such as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), Monkeypox (MPX), sexually transmitted infections (STIs), and HIV/AIDS, which require a complex understanding of immune responses and the design of more effective therapeutic strategies [165–167]. dPCR is a cutting-edge technology that enables the precise and sensitive detection and quantification of target molecules in samples. Immunotherapy development using dPCR offers various possibilities for measuring specific immune biomarkers used to suppress pathogens or enhance immunity, monitor patient response to therapy, and predict short to long-term therapy outcomes [167–169].

The application of dPCR is beneficial for immunotherapy development because it involves monitoring and measuring the expression levels of specific genes involved in the immune response. For example, in chimeric antigen receptor T-cell (CAR-T) therapy, dPCR can be used to measure the expression levels of target genes on modified T cells, enabling accurate monitoring of cell activity and the immune

response [170,171]. A better understanding of the expression of these genes can help optimize the design of therapies and improve their effectiveness. In addition, dPCR can also be used to monitor the levels of certain genetic mutations associated with the response to immunotherapy, such as driver mutations in cancer. With its ability to detect mutations with high sensitivity, dPCR can assist in identifying patients most likely to respond to a particular therapy as well as monitoring changes in the presence of mutations during treatment. This allows for greater personalization in the development of therapies tailored to the genetic profile of a patient.

Furthermore, dPCR is useful for monitoring the level of free circulating DNA (cfDNA) in a patient's blood [169,172,173]. cfDNA contains genetic information derived from cells undergoing death, including tumor cells. With dPCR, cfDNA can be measured with high sensitivity, enabling noninvasive monitoring of tumor dynamics during immunotherapy. A better understanding of changes in the amount and characteristics of cfDNA may help to evaluate therapeutic response and identify possible disease progression earlier. In addition to its monitoring function, dPCR has also been used in the development of vaccine-based therapies. In these cases, dPCR is used to monitor the immune response to the vaccine in target cells, including the expression levels of genes involved in the immune response, as well as to monitor the presence of target antigens in the sample. This will enable a better assessment of vaccine effectiveness and the development of more effective vaccine development strategies.

At the global level of immunotherapy development, dPCR offers significant benefits for understanding individual responses to therapy, thus enabling a more targeted and personalized approach in patient care [174,175]. Its ability to accurately measure specific immune biomarkers and genetic mutations, as well as to monitor tumor dynamics, provides valuable insights into the development of more effective therapies. In an effort to develop immunotherapeutic agents for various infectious and noncommunicable diseases, the use of dPCR technology helps provide information on immunotherapeutic agents that can be developed in terms of effectiveness, variability,

and complexity of development. Globally, there are various sources of immunotherapeutic agents for the treatment of diseases, such as monoclonal antibodies, which are proteins designed to bind to specific targets on tumor cells or immune cells. These antibodies can stimulate the immune system to destroy tumor cells and inhibit their growth. Examples include PD-1 (programmed cell death protein 1) or CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) receptor blockers, which increase the activity of cytotoxic T cells against tumor cells [169,174].

In addition, therapeutic vaccines stimulate an immune response against tumor cells by introducing specific tumor antigens into the immune system. This helps the body recognize and destroy tumor cells containing these antigens. dPCR was used to monitor the expression of genes associated with the immune response to vaccines and to measure the success rate of vaccination. Furthermore, the next immunotherapeutic agent, T-cell therapy, involves the collection, modification, and reinfusion of a patient's T cells that are genetically altered to express a specific antigen receptor (CAR) and then reinfusion into the patient to identify and destroy tumor cells. CAR-T cells have been found to be effective in treating several types of blood cancers, including leukemia and lymphoma [170,171,173].

Immunotherapeutic agents that are also widely used are cytokines, which are regulatory proteins produced by immune cells and are used in some immunotherapy therapies to modulate immune system activity and regulate immune system function. In this case, dPCR is used to monitor gene expression levels associated with the production and response to certain cytokines so that they are not toxic when used. In immunotherapy, cytokines can be used in several ways, including the following:

1. Interleukins (ILs) are immune stimulants, and various types of interleukins, such as IL-2 and IL-12, have been used therapeutically in the treatment of cancer. IL-2 has been used to treat certain metastatic cancers, especially melanoma and kidney cancer, and functions by stimulating the activity of T cells and other cells in the immune system to attack cancer cells. However, the use of IL-2 is limited because of its serious side effects.

2. Interferon (IFN) was developed as a treatment agent, and IFN is a cytokine that can be used as an immunotherapeutic agent. IFN has been used to treat melanoma and leukemia by stimulating the immune system to enhance antitumor responses.
3. Cytokine release syndrome (CRS) is a type of immunotherapy, such as CAR-T-cell therapy, and cytokine release may occur in response to modified T-cell activity. Thus, CRS may be a serious side effect of these therapies. In certain cases, regulating and controlling cytokine release is crucial for reducing the risk of adverse side effects, including fatality. However, it should be noted that the use of cytokines in immunotherapy can pose challenges, especially in relation to serious side effects. Therefore, research is ongoing to develop safer and more effective strategies for the use of cytokines in the treatment of cancer and other diseases. The use of dPCR should be maximized to identify the hazards and direct effects of the cytokines.

4.9. Application of dPCR in the development of phytotherapy

The development of phytotherapy or the use of medicinal plants for medicinal purposes has become an important focus of efforts toward a more natural and sustainable approach in healthcare. This is mediated by massive reports of drug resistance, side effects of treatment, ineffectiveness of synthetic drugs, and toxicity caused by long-term treatment [176,177]. One of the main challenges in phytotherapy development is ensuring the consistency and quality of the products produced; therefore, strengthening the technological capacity, validation, and specific testing are necessary to produce safe medicinal products [39,178,179]. dPCR is a technology that can facilitate accurate and sensitive analysis of genetic material, especially that found in medicinal plant parts, to improve phytotherapy development [39,180]. The applications of dPCR in phytotherapy development are as follows:

1. In the identification and validation of plant genera and species, effective phytotherapy often relies on the correct identification of plant species and an understanding of their chemical profiles. dPCR can be used to specifically identify genetic material associated with certain species and genera. This allows researchers

to verify the authenticity of plant materials used in phytotherapy formulations. By validating the exact plant composition, dPCR helps to avoid accidental contamination or substitution, ensuring product consistency and safety [181,182].

2. The quantification and monitoring of the active content and an accurate understanding of the active chemical content of medicinal plants are important elements in the development of effective phytotherapies. dPCR can be used for accurate quantification of genes related to the biosynthesis of specific active compounds. By monitoring the expression of genes involved in biosynthetic pathways, researchers can gain a better understanding of the factors that influence the concentration of active compounds. This enables the development of formulations that are consistent with the desired chemical content.
3. The detection and characterization of genetic variation can affect the quality and effectiveness of phytotherapy. dPCR can be used to detect and characterize genetic variation in medicinal plant populations. By understanding the genetic polymorphisms present, researchers can adjust the selection and cultivation processes to increase the production of desirable bioactive compounds or reduce the levels of undesirable compounds [183,184].
4. Quality control and product authenticity, two of the challenges in phytotherapy, involve maintaining product quality consistency from batch to batch. dPCR can be used as a quality control tool for monitoring product authenticity and consistency. By regularly testing product samples using this technique, manufacturers can ensure that their products are not contaminated with unwanted additives or are replaced by different plant species.
5. New developments and improvements in formulations, such as dPCR, can help in the development of new formulations or the improvement of existing formulations. By better understanding the genetic interactions involved in the biosynthesis of active compounds, researchers can design optimized formulations to improve the efficacy and consistency of phytotherapy. This opens the door to the development of more effective and innovative products for plant-based healthcare.

6. In terms of safety and efficacy, dPCR can be used for testing the safety and efficacy of phytotherapy products. By monitoring the expression of genes associated with toxicity or pharmacological activity, researchers can identify the potential risks and benefits of product use. This helps to ensure that phytotherapy products meet the necessary safety and efficacy standards before being sold to the market.

The application of dPCR in phytotherapy development provides new opportunities for improving product consistency, authenticity, and effectiveness. By harnessing the power of this technology for genetic identification, quantification of active compounds, and monitoring of genetic variation, researchers can produce standardized and high-quality products. This is beneficial not only to the pharmaceutical and traditional healthcare industries but also to clinical healthcare but also to consumers who rely on natural products for healthcare.

Recently, DNA barcoding has been developed to identify and analyze DNA sequences contained in plants as well as potential candidate agents for traditional and synthetic drugs. In the development of herbal medicines, DNA barcoding can be used to ensure the authenticity of the raw materials used and monitor the quality and authenticity of the final product of the medicinal raw materials. DNA barcoding enables the accurate identification of medicinal plants, even in processed forms such as simplisia, powders, extracts, or other preparations.

A technology that can be used in conjunction with DNA barcoding is dPCR [39,182,185]. dPCR is a DNA amplification technique that enables the detection and quantification of target DNA with high sensitivity. In herbal and/or traditional medicine development, dPCR can be used to detect and amplify DNA from medicinal raw materials as well as the final herbal medicine product. The application of dPCR-assisted DNA barcoding in herbal medicine has several advantages. First, it enables the identification of medicinal plant species with a high degree of accuracy, which in turn ensures the authenticity and quality of the final product. Second, dPCR has high sensitivity and can detect DNA, even in samples with low concentrations or in mixtures

with DNA from other species. This enables the detection of impurities or contamination of raw materials or final products of herbal medicines.

Shilin Chen [39] showed that DNA barcoding has been useful in the quality control of traditional medicine, especially herbal medicine. This study recommended several developments in the form of mini barcodes and super barcodes for sequencing and genomic organelles of traditional medicinal plants. The development and use of DNA barcodes for compound identification, genome characterization, and their potential are presented in **Figure 19**.

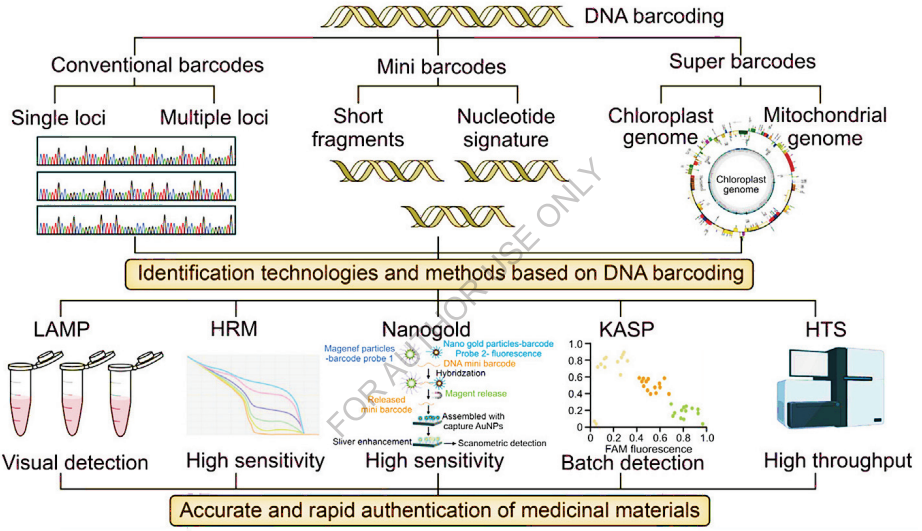


Figure 19. Development and use of DNA barcodes for identification of compound content, genome characterization and potential.

The process of developing herbal medicines using DNA barcoding and dPCR begins with the sampling of raw medicinal plant materials. DNA from these samples was extracted and amplified using conventional or dPCR techniques. Subsequently, the resulting DNA sequences were compared with a reference database to identify the medicinal plant species. A mismatch between the DNA sequence of the sample and the expected species could indicate contamination or substitution of the raw materials, leading to inaccuracies in the use of the species as medicine. A large variety of plant

species make this approach important for improving the quality of plant-sourced drugs [176,186].

In addition, this technology can be used to monitor the consistency of the quality of the final product over time and to detect changes in the genetic composition of the medicinal plant that may affect the efficacy or safety of the herbal medicine. By utilizing a combination of DNA barcoding and dPCR, herbal medicine development can become more targeted and qualified, thereby ensuring the authenticity, quality, and safety of the final product. This technology allows manufacturers to meet strict regulatory standards in the herbal medicine industry and increases consumer confidence in the products consumed.

4.10. Assessment of gene expression levels and epigenetic modifications

dPCR has emerged as the latest solution for more sensitive and accurate detection and quantification of gene targets and epigenetic modifications. In dPCR processing, DNA samples are split into separate reactions performed using microcameras. Each microcamera contained a sample with a low number of DNA molecules so that each DNA molecule could be reproduced separately. Thus, dPCR enables target detection with a high degree of sensitivity because of its digital nature, where DNA molecules are counted directly rather than estimated based on amplification [46,85]. The utilization of dPCR in the assessment of gene expression levels provides significant advantages for various molecular biology applications. For example, in cancer research, dPCR can be used to detect and quantify low gene expression levels, which may be difficult to detect using conventional PCR methods. This is important because low gene expression changes often have significant clinical implications in the detection, diagnosis, therapy selection, and monitoring of response to treatment. In addition, dPCR is particularly useful in epigenetic research for studying changes in gene activity that do not involve changes in DNA sequences.

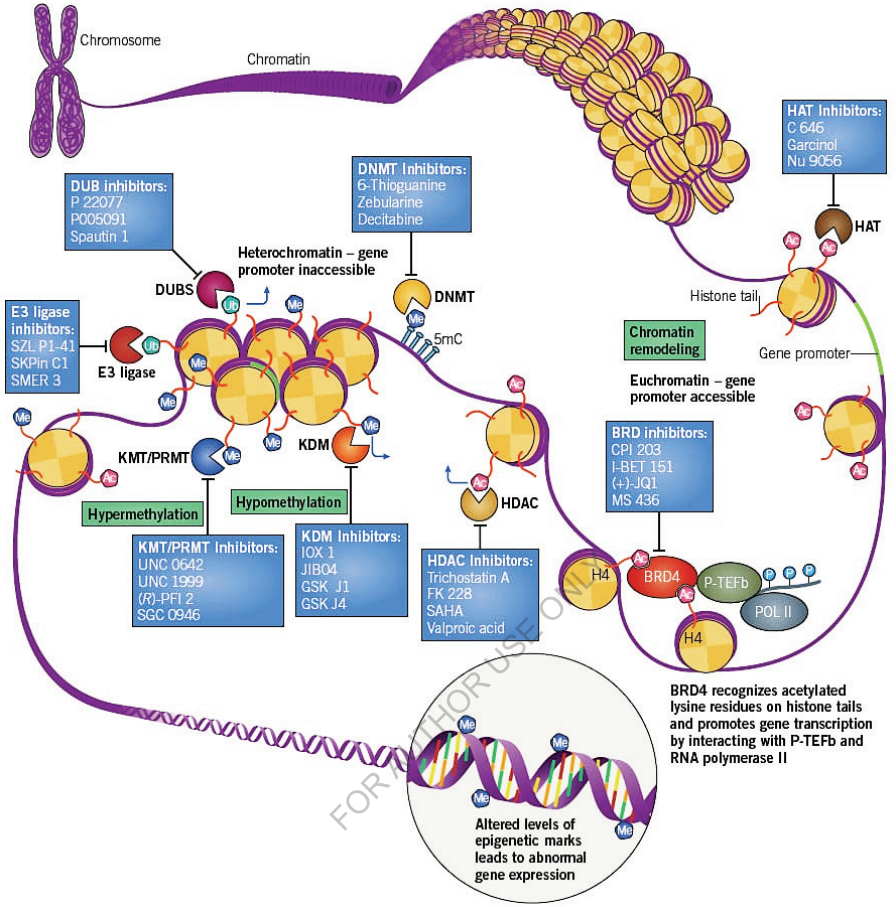


Figure 20. Epigenetic targets of cancer. **Remarks:** The basic structure of chromatin consists of nucleosomes, which are composed of two sets of histone proteins, namely, H2A, H2B, H3, and H4, which are tightly bound by DNA fragments. Changes in chromatin structure due to modifications after translation can regulate gene activity by forming heterochromatin or euchromatin, which generally inhibit or stimulate gene transcription. Posttranslational alterations include DNA methylation, methylation (Me), and acetylation (Ac) of histone tails. DNA methylation inhibits transcription by blocking the transcription complex from binding to gene promoters. Acetylation of histone tails generally loosens DNA from around the nucleosome, increases the accessibility of gene promoters to the transcription complex, and spurs the transcription process. However, methylation of histone tails can also suppress or stimulate gene expression, depending on the location, degree of methylation, and other histone modifications present in the surrounding environment. The pattern of posttranslational modification of nucleosomes determines the transcriptional profiles of adjacent genes. Disruptions

in the regulation of posttranslational modifications can result in unnatural gene expression, which in turn leads to oncogenesis and cancer development. **Abbreviations:** HAT, histone acetyltransferase; BRD, bromodomain; HDAC, histone deacetylase; KDM, histone demethylase; KMT/PRMT, lysine methyltransferase/protein arginine methyltransferase. DNMT: DNA methyltransferase; DUB: deubiquitinating enzymes.

The understanding of pathophysiological processes can be significantly improved through epigenetic analysis, which has potential in the diagnosis of predicted disease progression and the evaluation of susceptibility to disease. Epigenetic modifications such as DNA methylation can affect gene expression without changing the DNA sequence. dPCR technology can detect and quantify epigenetic modifications with a high degree of sensitivity [187–189]. For example, dPCR has been used in research on the relationship between DNA methylation and various diseases, including cancer, heart disease, diabetes, and neurological disorders. An example of a cancer epigenetic target mechanism is shown in **Figure 20**.

An important application of dPCR for epigenetic modification is DNA methylation mapping [188]. Traditional methods for measuring DNA methylation, such as bisulfite sequencing, require significant DNA amplification and are often insensitive to small changes in DNA methylation. In contrast, dPCR allows accurate detection and quantification of even small changes in DNA methylation, enabling a better understanding of the role of epigenetic modifications in genetic regulation and disease pathogenesis. In addition, dPCR can be used to monitor epigenetic modifications at the single-cell level. Using this technique, researchers can identify epigenetic variations between cells, which is important for understanding cellular heterogeneity during normal and pathological development. For example, dPCR has been used to understand how epigenetic modifications affect cell differentiation and disease development as well as to monitor cellular responses to epigenetic therapies.

dPCR can be used to measure the level of DNA methylation at specific CpG sites or regions of interest. Methylation-specific dPCR assays use methylation-sensitive restriction enzymes or bisulfite conversion to distinguish between methylated and

unmethylated DNA sequences [92,190,191]. This makes it possible to study DNA methylation patterns, epigenetic changes, and their implications for gene regulation, development, and disease. Moreover, in genetic modification, dPCR-based chromatin accessibility testing, such as digital restriction enzyme analysis of methylation accessibility (DREAM), allows the quantitative assessment of chromatin accessibility at specific genomic loci [12,189].

By measuring the accessibility of DNA to restriction enzymes or other nucleases, dPCR can provide insights into chromatin structure, transcriptional regulation, and epigenetic modifications associated with gene expression. Furthermore, dPCR can also be used to measure RNA modifications such as N6-methyladenosine (m6A), 5-methylcytosine (m5C), and pseudouridine (Ψ). By developing specific assays that target RNA modifications, dPCR enables the quantitative analysis of RNA epigenetic marks and their role in RNA processing, stability, and translational regulation. Therefore, the potential use of dPCR is highly promising for the development of molecular-based diagnostic, prognostic, and therapeutic methods [192].

4.11. Application of dPCR in the analysis of copy number variation

dPCR applications play a role in copy number variation (CNV) analysis because of its high sensitivity for detecting DNA copy number changes. dPCR enables CNV detection by amplifying the target DNA fragments into many separate partitions, such that each partition contains at least one target fragment. By monitoring the number of partitions containing the target fragment (positive), dPCR could accurately determine the DNA copy number in a small sample [29]. The implications of this method could provide better insight into the relationship between CNVs and disease conditions, improve the accuracy of genetic diagnosis, and monitor the response to disease therapy. In contemporary laboratories, the application of dPCR in copy number variation analysis has several practical implications, such as improved accuracy of test results, efficiency of analysis, sample and reagent savings, high scalability, and cutting-edge methods in current genetic research [128,193–195].

Chapter 5

Technological Advancements and Innovations

5.1 New Sensors and Detectors for dPCR

New sensors and detectors for dPCR have become a major focus of efforts to improve the sensitivity, speed, and accuracy of this technique. With advances in nanotechnology, optoelectronics, and sensor engineering, innovations have emerged that enable the detection of target molecules via dPCR with increased sensitivity and specificity. In this subchapter, we comprehensively describe some of the latest sensors and detectors that have enriched the capabilities of dPCR [2,54].

One important innovation is the development of more advanced signal detection sensors. The development of signal detection sensors is an important milestone in improving the performance and accuracy of digital polymerase chain reaction (dPCR). Signal detection sensors are responsible for detecting the fluorescence generated by PCR products during amplification [18,94]. Advances in signal detection sensors have brought about significant changes in the capabilities of dPCR, including improvements in sensitivity, resolution, and detection reliability. In this subchapter, several important aspects of the development of signal detection sensors are discussed.

First, it is important to understand the main role of signal detection sensors in dPCR [42,196,197]. As PCR progresses, DNA or RNA amplification products are usually tagged with fluorescent fluorophores. The signal detection sensor is responsible for measuring this fluorescence and converting it into a measurable signal that can be

used to determine the initial amount of the target molecules. Therefore, the performance of the signal detection sensor significantly affects the sensitivity and accuracy of dPCR. One important advancement in signal-detection sensors is the use of more advanced photodetection technologies. Modern photodetection sensors are highly responsive to the fluorescence produced even at very low levels. They also have a wide dynamic range, which enables their detection over a wide range of target concentrations. These sensors are also equipped with features such as noise reduction and sensitivity enhancement, which optimize signal detection performance under various experimental conditions [117,198,199].

In addition, signal detection sensors have been developed for design and integration with dPCR systems. Sensors specifically designed for various dPCR platforms, such as droplet-based, chip-based, or emulsion-based platforms, enable optimal and consistent detection. These customized designs consider factors such as sample volume, reaction geometry, and fluorescence efficiency, all of which affect signal detection performance. Digital signal processing technology is an integral part of the development of signal detection sensors. Advanced signal-processing algorithms and methods enable more accurate information extraction from complex fluorescence signals. These include noise reduction, precise detection threshold determination, and accurate statistical analysis. The integration of these technologies ensures that the data generated from the signal detection sensors can be interpreted precisely and quickly, thereby improving the reliability and validity of the dPCR results.

Furthermore, the development of signal detection sensors has involved the application of concepts and technologies from other fields, such as nanotechnology and optoelectronics. Nanotechnology-based sensors, for example, offer exceptional sensitivity to fluorescence changes at the nanolevel, enabling the detection of target molecules at very low concentrations [200,201]. In addition, the use of sophisticated optoelectronic components has increased the efficiency of signal detection and has optimized the use of energy in various fields.

Additionally, there is a need to develop innovative optical and photometric sensors. Optical sensors integrated with dPCR systems can provide more accurate and sensitive detection [202,203]. The dPCR system can capture fluorescence with high efficiency and produce stable signals, even under extreme variations in light conditions. These sensors are often equipped with automatic settings of detection parameters and noise reduction that improve the reliability and consistency of the results. Various efforts have been made to develop optical and photometric sensors that are more compatible with dPCR. The main factors that affect the performance of optical and photometric sensors are sensitivity, resolution, dynamics, and signal stability [204,205].

One of the major advances in optical and photometric sensors is the use of sensitive and responsive detection technologies. Modern sensors are equipped with advanced optical and photometric components, such as CCD or CMOS photodetectors, which have high conversion efficiency and a wide dynamic range. This technology enables the sensor to detect fluorescence, even at very low levels of the target molecule, significantly increasing the sensitivity and detection limit of dPCR. Optical and photometric sensors are often equipped with advanced features to improve the reliability and consistency of the results. For example, autofocus technology and the automatic setting of detection parameters ensure that the sensor is always operating under optimal conditions, resulting in consistent data from trial to trial [206]. Noise reduction systems and digital filters also help eliminate unwanted background signals, thereby improving the signal-to-noise ratio and measurement accuracy.

Innovative optical and photometric sensor designs also consider factors such as the reaction geometry and the type of dPCR platform used. Sensors optimized for various dPCR platforms, such as droplet-based, chip-based, or emulsion-based platforms, ensure optimal and consistent detection. These customized designs consider factors such as reaction geometry, light efficiency, and expected fluorescence levels, which all affect the optical and photometric detection performance.

The development of optical and photometric sensors also involves the application of concepts and technologies from other fields, such as microfluidics and nanotechnology. For example, sensors integrated with microfluidic systems enable real-time fluorescence measurements during PCR. The use of nanotechnology materials in optical and photometric sensors enables an increase in detection sensitivity and resolution, as well as a reduction in sensor size and manufacturing costs.

Furthermore, the development of advanced electronic detectors with increased sensitivity is needed [203]. Advanced electronic detectors are typically supported by more powerful hardware and more sensitive sensors. The use of advanced charge-coupled devices (CCDs) or complementary metal oxide semiconductor (CMOS) sensors, for example, enables detectors to capture fluorescence signals with high resolution and fast read speeds [207,208]. These sensors have a high conversion efficiency and low noise levels, resulting in accurate detection even at low fluorescence intensities or under complex light conditions. In addition, advanced electronic detectors are equipped with additional features that enhance the sensitivity and detection performance. The adjustable gain or exposure level setting feature allows the detector to adapt to a wide range of fluorescence intensity ranges and ensures that signals can be detected precisely, even at very low levels. The use of advanced noise reduction and digital signal processing technologies helps to increase the signal-to-noise ratio and improve the measurement accuracy [87,201].

The integration of advanced electronic detectors with sophisticated data analysis software is also an important part of dPCR development [209,210]. These detectors are often connected to computer systems or data analysis software, which allows for real-time signal processing and accurate extraction of information from the resulting data. Advanced signal processing algorithms and methods allow the detector to capture useful information from complex fluorescence signals, thereby improving the reliability and validity of the dPCR results.

Advanced electronic detectors can be equipped with additional features to improve the operating reliability and extend the lifetime of dPCR. Features such as

efficient cooling systems or protection against excessive light ensure that the detector can operate under optimal and stable conditions and can also help prevent damage to the sensor or electronic components due to thermal stress or photodegradation, which can compromise the detection performance in the long run. The development of advanced electronic detectors involves the application of the latest technologies to electronics and optoelectronics.

For example, the use of electronic components based on new semiconductor materials or microelectronic manufacturing technologies can lead to the production of smaller, lighter, and more efficient detectors [65,117,211]. This enables the integration of detectors with more compact and portable dPCR platforms, thereby expanding the application of this technique in a variety of laboratory and field contexts. Nanotechnology-based sensors offer sensitivity to molecular changes at the nanoscale as well as the detection of very small amounts of target molecules. The unique characteristics of nanotechnology materials, such as high conductivity, improve sensor performance in terms of sensitivity, resolution, and reliability.

Several studies have successfully utilized nanomaterial structures such as carbon nanotubes or quantum dots as fluorescence sensors for dPCR. Nanomaterial structures have a large surface area and high fluorescence sensitivity, enabling the detection of target molecules at very low concentrations. In addition, the use of nanomaterials in dPCR detection often provides a flexible and customizable platform, enabling the integration of various sample preparation methods and detection systems with high-precision molecular targets. With the continued development and integration of these technologies, dPCR has the potential to become an important tool in a wide range of applications in the fields of life sciences, preventive medicine, epidemiology, public health, and clinical care.

5.2 New Software and Data Analysis for dPCR

Current data analysis software for dPCR has become the key to understanding and extracting valuable information from the generated data. With the increasing

complexity of dPCR experiments and the availability of large amounts of data, sophisticated software is required to manage, analyze, and interpret data efficiently and accurately [212]. The introduction of the latest data analysis software has led to significant changes in dPCR capabilities, allowing researchers to explore and understand deeper information from experimental data. An important feature of the latest data analysis software is its ability to manage and integrate data from various dPCR platforms. Most modern software has broad compatibility with various dPCR platforms, including droplet-based, chip-based, and emulsion-based platforms, allowing users to analyze data from multiple sources with consistency and uniformity. This will help expand dPCR applications and allow researchers to perform comprehensive and unified analyses [213,214].

In addition, the latest data analysis software often has an intuitive and user-friendly user interface, which makes it easy to use and navigate through various analysis features. These features include interactive data visualizations, advanced data processing tools, and the ability to customize analysis parameters according to the user's specific needs [104,195,206]. With this easy-to-use interface, researchers can easily perform analyses and quickly generate relevant results. Some data analysis software also includes features that enable collaboration and efficient data sharing between different users. These include integrated online data storage, real-time collaboration tools, and the ability to create and manage collaborative projects. This will facilitate effective teamwork and enable continuous and collaborative dPCR research across the scientific community.

Furthermore, developments in algorithms and data analysis methods are integral to advances in dPCR [215,216]. New and improved data analysis algorithms and methods have been designed to overcome the challenges of data complexity and to obtain useful information accurately and efficiently. With the increasing complexity of dPCR data and the need for deeper interpretation, the latest data analysis algorithms and methods are key to understanding the biological implications of the experimental results.

One type of algorithm often used in dPCR data analysis is the clustering algorithm for single-molecule data analysis. Clustering algorithms are used to identify and separate signal clusters representing target molecules from background noise and experimental artifacts. Using advanced clustering techniques, these algorithms enable researchers to identify and count target molecules with a high degree of accuracy, even in complex samples.

In addition, the new and improved algorithms and data analysis methods incorporate advanced statistical techniques to evaluate the reliability and validity of the results [210,214,217]. Methods such as nonlinear regression analysis, multivariate analysis of variance, and statistical hypothesis testing were used to validate the results and determine the statistical significance of the differences between experimental groups. These techniques can ensure that the results obtained from dPCR data analysis have high statistical reliability and are biologically relevant.

The use of artificial intelligence (AI) in dPCR data analysis has become an added focus for improving the efficiency, accuracy, and reliability of analysis. AI enables the development of algorithms that can learn from existing data to identify complex patterns and trends that are difficult or impossible to identify manually. By applying AI techniques to dPCR data analysis, researchers can gain deeper insights and accelerate the data interpretation process [209,218,219].

One of the main applications of AI in dPCR data analysis is its use in predicting the quantification of standardized target molecules. Through the development of prediction models that use machine learning techniques, AI can predict the number of target molecules in a sample based on complex fluorescence signal patterns. The model can learn from existing data and identify patterns that correlate with the number of target molecules, enabling accurate predictions, even in complex or noisy samples. AI is also being used in the development of automated algorithms for dPCR data processing and analysis. Automation algorithms use machine learning techniques to identify and classify data, reduce human involvement in the analysis process, and

increase overall efficiency. This allows researchers to save valuable time and resources and focus their attention on the interpretation and use of results.

Thus, the development of data analysis software, algorithms, and the use of AI brings good prospects for the refinement of dPCR capabilities and applications. Through the use of advanced software, the development of reliable algorithms, and the application of AI techniques in data analysis, the scientific community can gain deeper insights and accelerate the research process in the fields of molecular biology and diagnostics, patient sample handling, and improved diagnostic capacity [220]. By continuously improving this technology, dPCR has the potential to become an even more powerful tool for various applications in the fields of life sciences, biology, medicine, and other fields for the advancement and welfare of mankind.

5.3 Integration of dPCR Technology with Other Laboratory Systems

The integration of dPCR technology with other laboratory systems has been the focus of development for improving efficiency, reliability, and productivity across a wide range of contemporary laboratory applications. This integration allows dPCR to become an integral part of the broader laboratory workflow, making it possible to automate processes, expand analytical capabilities, and improve interactions between different laboratory technologies [73,91,130].

Interoperability with current laboratory systems is key to ensuring that dPCR technology can seamlessly integrate with other existing systems and devices within the laboratory, involving broad compatibility with the various devices and platforms used in laboratory workflows, including sample extraction devices, automated pipette devices, and data analysis devices. One way to achieve interoperability is through the standardization of protocols and data formats. This standardization allows different devices and systems to communicate and interact seamlessly, ensuring accurate and consistent data exchange between various devices. Standards such as the Laboratory Information Management System (LIMS) communication standards and laboratory data file standards (such as XML or JSON file formats) support interoperability within

the laboratory. Additionally, the use of open application interfaces (APIs) enables easier integration between the dPCR system and other laboratory systems. With APIs, users can easily connect the dPCR system with LIMS systems, automated pipette devices, or other data analysis devices, enabling real-time data transfer and seamless process integration.

Furthermore, increased connection and integration with laboratory automation systems can maximize the efficiency and productivity of dPCR, which involves the use of hardware and software to enable the automation of laboratory processes, including sample preparation, PCR, and data analysis. One way to achieve this integration is to use laboratory robots connected to dPCR systems. These robots can be programmed to perform various laboratory tasks, such as sample pipetting, reagent mixing, and sample loading into the dPCR device. Using laboratory robots, laboratory service users can automate time-consuming processes and free up time and manpower for other activities. Integrated laboratory automation software plays a role in improving efficiency and productivity. The software allows users to automatically plan, organize, and monitor laboratory processes, from sample preparation to data analysis. Using automation software, users can save time, reduce human error, and improve the consistency of the results obtained.

This effort was made by developing strategies to maximize efficiency and productivity with integrated technologies involving the development of strategies that optimize the use of dPCR in laboratory workflows, including the development of integrated experimental protocols, the use of automation software, and the implementation of best practices in laboratory data management. One effective strategy is to develop integrated experimental protocols. These protocols are designed to ensure that the various stages in the laboratory workflow, from sample preparation to data analysis, are well coordinated and integrated. This allows researchers to run experiments with high efficiency and maximize the use of laboratory resources.

Additionally, the use of advanced automation software is an effective strategy for maximizing efficiency and productivity. This software can be used to automatically plan

and organize various stages of the laboratory workflow, including sample preparation, PCR, and data analysis. In addition, the implementation of best practices in laboratory data management, such as the use of an integrated LIMS system, advanced data management practices, and strict data security policies, helps maximize efficiency and productivity. Using these practices, laboratory managers can efficiently manage data, optimize the use of available information, ensure comprehensive data reliability and security, and become the laboratory application of choice in the future.

5.4 Combination of ChatGPT and other AIs with dPCR technology

Integrating ChatGPT and other AI technologies with digital PCR (dPCR) offers a transformative approach to molecular biology research and diagnostics. The natural language processing capabilities of ChatGPT enhance data analysis by allowing complex dPCR results to be interpreted in real time, providing insights and aiding experimental design through interactive conversations. In addition, AI algorithms can automate the process of creating experimental protocols, optimizing PCR conditions and primer sequences for maximum efficiency.

Predictive modeling supported by AI enables researchers to forecast experimental results, guide decision-making, and prioritize research efforts. In addition, AI-based quality control mechanisms ensure the accuracy and reliability of dPCR data, thereby enhancing experimental integrity. By integrating dPCR data with existing biological knowledge, AI facilitates comprehensive insights and cross-disciplinary collaboration. AI-powered interactive learning tools educate researchers and students on the principles and applications of dPCR, encouraging knowledge retention and skill development. In clinical environments, AI-powered dPCR analysis offers real-time decision support for personalized diagnostics and treatment, assisting healthcare professionals in interpreting results and guiding treatment decisions. The combination of ChatGPT, other AI technologies, and dPCR is revolutionizing molecular biology research, data analysis, and clinical diagnostics, accelerating scientific discovery and improving healthcare outcomes.

Chapter 6

Future Directions and Opportunities

6.1 Expansion of dPCR applications in personalized medicine

In the era of advanced medicine, technology continues to play an important role in improving personalized medicine or treatments tailored to the unique characteristics of each individual. Among the various available technologies, dPCR has emerged as a highly promising tool for supporting personalized medicine in the present and future. Owing to its advantages in terms of sensitivity, accuracy, and ability to detect target molecules in very small amounts of sample, dPCR offers great potential for the expansion of its application in personalized medicine.

Considering its benefits and potential, the expansion of dPCR applications is necessary based on evidence, and several reasons, such as dPCR, allow the detection of very small amounts of target molecules with high sensitivity so that early identification of diseases can be performed even in the early stages of disease occurrence or at very low levels of presence, which is very important in personalized medicine, especially for early detection and timely intervention. In addition, dPCR technology enables multiplex testing, which enables the detection of multiple molecular targets in a single reaction. This means that dPCR can be used for a more comprehensive analysis of an individual's biomarker profile, which is critical for determining appropriate personalized treatment strategies. This was supported by the high consistency and reproducibility of the analytical results generated from dPCR,

thus minimizing the variation between different assays. It is important to ensure that clinical decisions are based on consistent and reliable data directly sourced from the cause of the disease. dPCR technology is important not only for diagnosis and treatment but also for the development of translational research that attempts to link scientific discoveries with clinical applications in the field. This technology enables further research on biomarkers relevant to specific clinical conditions, thereby allowing the development of more targeted therapies.

dPCR technology has been applied in various health fields for disease diagnostics. dPCR is more sensitive and specific than traditional PCR methods for identifying genetic mutations. With high resolution, dPCR can detect gene mutations in small amounts of DNA, which is useful in personalized medicine, particularly in designing therapies that match the patient's genetic profile. For example, cancer-specific mutations in certain genes can affect responses to certain therapies. dPCR is useful for more in-depth testing to identify these mutations and design targeted, comprehensive treatment plans.

In addition, dPCR is useful for disease monitoring at the molecular level, which indicates that it can detect and quantify the amount of certain disease-related molecules in patient biopsies. For example, in the treatment of HIV, measuring the level and amount of the virus in the blood is crucial. dPCR provides high sensitivity in measuring the amount of virus, allowing for more accurate monitoring of the response to antiretroviral therapy (ARV). It is also intended to provide personalized care for individuals with infectious diseases, such as HIV, hepatitis, and tuberculosis.

In addition, dPCR technology can be used for the measurement of gene expression to determine gene expression patterns that may be related to the response to therapy; the more recognizable the gene patterns are in patients, the more accurate the treatment provided, and the greater the cure and safety rates are. dPCR is used to measure specific mRNA levels in samples, which provides a deeper understanding of an individual's genetic activity, both recessive and dominant. This knowledge helps in predicting a patient's response to a particular therapy so that the therapeutic approach

can be tailored to the patient's needs. These results are particularly useful for pharmaceutical and drug service providers to identify interactions between individual genetics and drug responses, known as pharmacogenomics. dPCR is used to identify genetic variants that may affect drug metabolism or the response to a particular drug. Thus, dPCR can help in designing an appropriate drug dosage and more effective therapy based on the patient's genetic profile.

In addition to its applications in personalized medicine, dPCR is useful for the rapid detection of pathogens. It can enable faster diagnosis and intervention in infectious situations that require quick action, such as nosocomial infections that often occur in healthcare settings (such as hospitals, clinics, and public health centers). dPCR can quickly and sensitively identify and quantify the number of pathogens in a sample for more efficient treatment. Recently, it has been reported that dPCR can be used in the development of gene-based therapies in which the delivery of specific genes is used to address genetic disorders or diseases that cannot be addressed by conventional methods.

dPCR can be used to monitor the efficiency of gene delivery to target cells and measure gene expression levels after therapy. This allows for a more accurate assessment of the success of therapy and guides subsequent therapy adjustments. With high precision and sensitivity, dPCR enables in-depth molecular analysis, which is important for designing appropriate therapies and monitoring treatment response. With further developments in this technology, it is expected that the application of dPCR will continue to expand, bringing greater benefits to personalized medicine and overall patient care.

6.2 Development of portable dPCR as a future mini laboratory

dPCR technology has a broad positive impact, especially for disease diagnostics in healthcare settings, such as clinical and environmental laboratories. The future direction of dPCR is promising owing to its multifaceted advantages. The future development of dPCR requires portable dPCR devices that enable on-site testing and

rapid diagnosis of various diseases, infections, and genetic disorders in clinical settings, remote areas, and resource-limited environments [104]. By accurately measuring target nucleic acids with high sensitivity, dPCR facilitates the early detection of pathogens, monitoring of treatment response, and screening of genetic markers associated with disease risk, even under limited conditions.

In addition, dPCR applications in point-of-care diagnostics include testing for infectious diseases (e.g., HIV, hepatitis, tuberculosis, sexually transmitted infections, and influenza), prenatal screening, oncology (e.g., cancer biomarker detection), and personalized medicine (e.g., pharmacogenomic testing and gene therapy). Mini dPCR platforms integrated with microfluidic cartridges or disposable chips provide a simple and easy-to-use interface for sample processing, amplification and result analysis, thus enabling rapid and cost-effective diagnostic testing outside the traditional laboratory setting [221]. Contemporary laboratories must be able to provide as much space and capacity as possible despite limited resources. The advent of dPCR has overcome this shortcoming. The slip chip concept is illustrated in **Figure 21**.

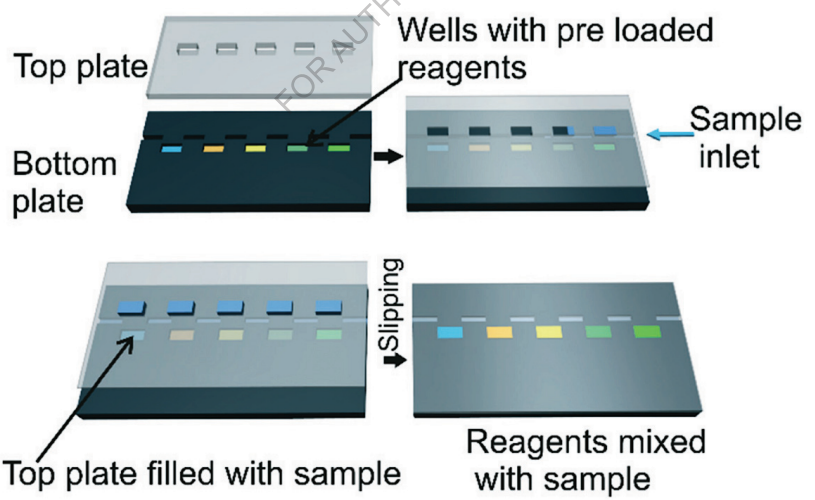


Figure 21. Concept of a slip chip in the development of an integrated mini dPCR. **Remarks:** This image is courtesy of Sreejith et al. [59].

On the other hand, the use of dPCR has expanded to include environmental health monitoring, environment-based disease epidemiology, and the application of one health concept for disease detection from animals to the environment and humans or others. Currently, dPCR is increasingly used for environmental monitoring, surveillance, and quality assessment of air, water, soil, and food samples. The development and production of portable dPCR instruments will facilitate real-time and integrated detection and quantification of environmental contaminants, microbial pathogens, and genetic markers indicative of pollution, contamination, and ecological health. A scheme of liquid biopsy testing using integrated portable dPCR is shown in **Figure 22**.

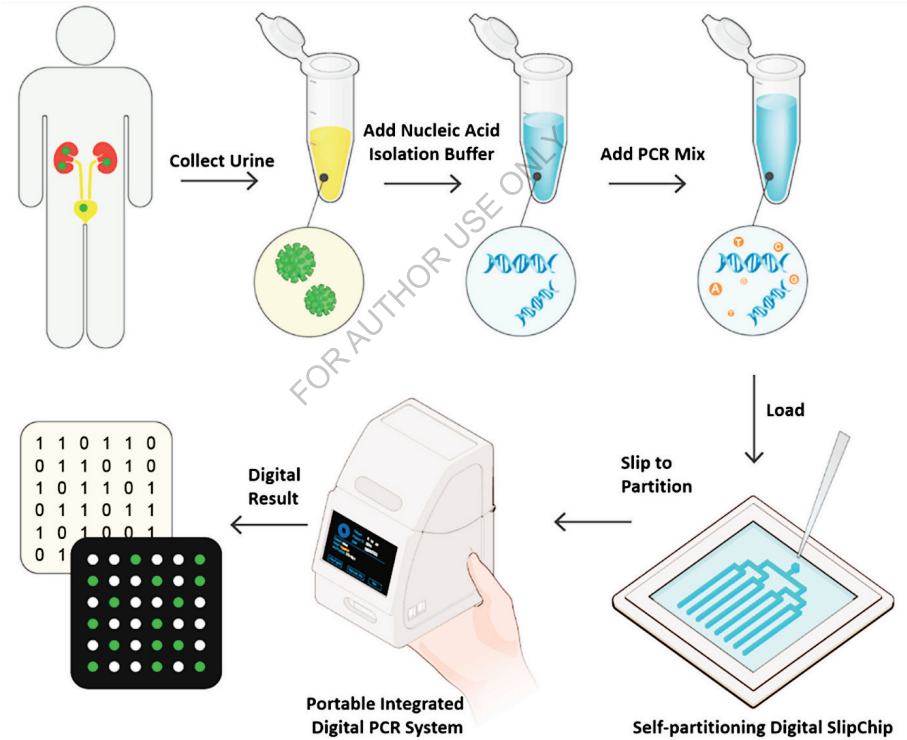


Figure 22. Schematic of liquid biopsy testing using integrated portable dPCR [104].

The applications of dPCR in environmental monitoring include water quality testing (e.g., detection of bacteria, viruses, and parasites in drinking water sources), air

pollution monitoring (e.g., quantification of microbial pollutants and airborne pathogens), soil analysis (e.g., detection of genetically modified organisms and pesticide residues), and food safety testing (e.g., identification of foodborne pathogens, allergens, and adulterants). By providing rapid, sensitive, and accurate results, dPCR can contribute to environmental risk assessment, regulatory compliance, and public health protection initiatives.

A portable dPCR platform designed for field-deployable applications enables on-site testing and real-time monitoring of environmental samples at remote or inaccessible locations [195]. This rugged instrument features battery-powered operation, durable construction, and a simplified workflow, making it suitable for fieldwork, disaster response, and environmental surveillance under challenging conditions. The field-deployable dPCR platform supports a wide range of environmental monitoring applications, including biodiversity assessment, ecosystem monitoring, pollution tracking, and disease surveillance and spread. By providing timely and actionable information, the platform empowers environmental scientists, conservationists, and public health professionals to make informed decisions, mitigate risks, and protect ecosystems and human health based on current results.

In addition, the integration of dPCR will focus on future remote sensing and networked monitoring. Networked dPCR monitoring systems enable remote sensing and real-time data transmission for continuous monitoring of environmental parameters and biological indicators across large geographic areas. This networked platform integrates multiple dPCR instruments, sensor nodes, and data communication technologies to create a distributed monitoring network that captures the spatial and temporal variations in specific environmental conditions. Remote sensing and network monitoring with dPCR support applications such as disease outbreak early warning systems, invasive species tracking, biodiversity hotspot monitoring, and climate change impact assessment. An example of research combining geospatial-based dPCR techniques is shown in **Figure 23.**

The results show that dPCR technology can be well integrated in terms of remote sensing and the spatial distribution of aquatic insects in a particular river in Dongjiang, China. These results provide new insights into the importance of environmental DNA monitoring for the identification of biodiversity sustainability and environmentally based disease sources [222].

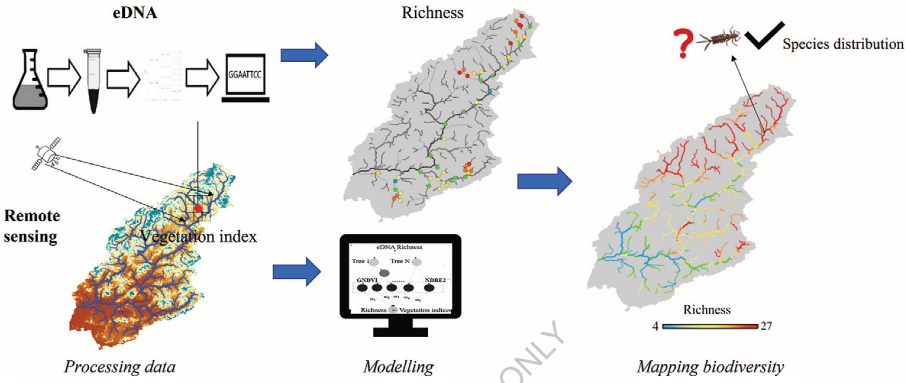


Figure 23. Application of dPCR technology for environmental DNA identification and GIS-based spatial distribution of aquatic insects. **Remarks**: This image is courtesy of Wu et al. [222]

By harnessing the power of data analytics, artificial intelligence, and geographic information systems (GIS), this integrated monitoring platform provides actionable insights for environmental management, conservation planning, sustainable development initiatives, and early management of disease sources [37].

6.3 The direction of collaboration with multiple disciplines in the development of dPCR

DPCR development must involve collaboration among multiple fields and disciplines that contribute unique expertise and perspectives to advance the capabilities and future applications of dPCR technology. By fostering interdisciplinary collaboration, researchers can harness the full potential of dPCR technology to address complex scientific challenges, improve human health, and protect the environment for

the benefit of society as a whole. The development directions of dPCR in multidisciplinary science are as follows:

1. Molecular Biology and Genetics

Collaboration with molecular biologists and geneticists is essential for the design and optimization of dPCR assays that target specific nucleic acid sequences. Molecular biologists have expertise in primer and probe design, reaction optimization, and assay validation, ensuring the reliability and accuracy of dPCR results. Geneticists provide insights into target selection, genetic variation, and allele-specific analysis, enabling the detection and quantification of genetic mutations, copy number variations, and rare alleles with high sensitivity and specificity.

2. Microbiology and Infectious Disease Research

Collaboration between microbiologists and infectious disease researchers enhances the application of dPCR for microbial detection, pathogen quantification, and infectious disease diagnosis. Microbiologists have expertise in sample preparation, microbial culture, and pathogen characterization, enabling the development of dPCR assays to detect bacterial, viral, and fungal pathogens in clinical, environmental, and food samples. Infectious disease researchers have contributed to the understanding of disease epidemiology, transmission dynamics, and host– interactions, guiding the selection of molecular targets and assay strategies for detecting infectious agents with clinical relevance and public health significance.

3. Oncology and Cancer Research

Collaboration with oncologists, cancer researchers, and molecular pathologists has expanded the utility of dPCR for cancer detection, monitoring, and personalized therapy. Oncologists have provided clinical insights into tumor biology, disease progression, and treatment responses, guiding the development of dPCR assays for detecting cancer-specific mutations, monitoring minimal residual disease, and predicting treatment outcomes. Cancer researchers have expertise in tumor

genetics, biomarker discovery, and molecular profiling, enabling the identification of actionable mutations, drug resistance mechanisms, and therapeutic targets for precision oncology interventions.

4. **Environmental Science and Ecology**

Collaboration between environmental scientists and ecologists has advanced the application of dPCR in environmental monitoring, biodiversity assessment, and ecosystem health. Environmental scientists have provided expertise in sample collection, environmental sampling techniques, and pollution monitoring, facilitating the development of dPCR assays to detect microbial contaminants, environmental pathogens, and pollutants in air, water, soil, and food samples. Ecologists have contributed to the understanding of ecosystem dynamics, species interactions, and ecological indicators, guiding the selection of molecular targets and assay approaches for assessing environmental health, tracking invasive species, and monitoring biodiversity changes over time.

5. **Bioinformatics and Computational Biology**

Collaboration between bioinformaticians and computational biologists enhances the analysis, interpretation, and visualization of dPCR data. Bioinformaticians have developed algorithms, software tools, and analytical pipelines for processing dPCR data, normalizing fluorescence signals, and quantifying target molecules with accuracy and precision. Computational biologists apply statistical methods, machine learning algorithms, and data mining techniques to identify patterns, correlations, and biomarker signatures from large-scale dPCR datasets, providing insights into disease mechanisms, treatment responses, and molecular pathways.

6. **Biomedical Engineering and Instrumentation**

Collaboration between biomedical engineers and instrumentation specialists drives innovations in dPCR technology, platform development, and instrument design. Biomedical engineers have designed microfluidic devices, droplet generators, and reaction chambers for dPCR platforms to optimize partitioning

efficiency, reaction kinetics, and thermal cycling performance. Instrumentation specialists have developed fluorescence detection systems, imaging technologies, and data acquisition systems for dPCR instruments to enhance sensitivity, dynamic range, and data resolution. Collaborative efforts among scientists, engineers, and instrument manufacturers have led to the creation of next-generation dPCR platforms with improved performance, usability, and scalability for diverse research and clinical applications.

7. Clinical Medicine and Translational Research

Collaboration with clinicians, clinical researchers, and translational scientists has accelerated the translation of dPCR technology from bench to bedside. Clinicians provide access to clinical samples, patient cohorts, and disease expertise, facilitating the validation of dPCR assays for diagnostic, prognostic, and therapeutic applications in clinical settings. Clinical researchers have conducted validation studies, outcome assessments, and clinical trials to evaluate the performance and clinical utility of dPCR assays in real-world patient populations, guiding regulatory approval, and healthcare adoption. Translational scientists bridge the gap between basic research and clinical practice by facilitating the translation of dPCR discoveries into clinical assays, biomarkers, and precision medicine interventions to improve patient care and outcomes.

6.4 Continued refinement of dPCR methodologies and platforms

The refinement of dPCR methodologies and platforms is a continuous process driven by advances in technology, instrumentation, and experimental techniques. By utilizing new technologies and collaborative efforts, researchers, academics, and relevant stakeholders can advance dPCR technology and pave the way for new discoveries in various scientific fields. Various improvements are required in current dPCR technology.

1. **Assay optimization and protocol standardization** and continuous refinement of dPCR assays involve optimizing primer and probe designs, reaction conditions,

and amplification protocols to enhance assay sensitivity, specificity, and robustness. Standardization efforts aim to establish consensus protocols, quality control criteria, and performance benchmarks for dPCR assays across platforms and applications.

2. **With improved instrumentation and detection systems,** next-generation dPCR platforms incorporate advanced instrumentation, detection systems, and data acquisition technologies to improve assay performance, throughput, and user experience. Innovations in droplet generation, fluorescence detection, and microfluidic systems have enhanced partitioning efficiency, signal-to-noise ratios, and result accuracy in dPCR experiments.
3. **Multiplexing and** parallelization and advances in multiplexing and parallelization enable simultaneous detection and quantification of multiple targets within a single dPCR. Multiplex dPCR assays leverage multiplexed primer and probe sets, fluorophores, and detection channels to expand the analytical capacity and throughput of dPCR platforms, enabling comprehensive analysis of complex biological samples and molecular pathways.
4. **Integration with next-generation sequencing (NGS)** and integration of dPCR with next-generation sequencing (NGS) technologies facilitate comprehensive genomic analysis, variant validation, and allele quantification in complex biological samples. Combined dPCR and NGS workflows enable targeted sequencing, mutation detection, and copy number analysis with enhanced sensitivity, specificity, and cost-effectiveness, thereby enabling researchers to leverage the strengths of both technologies for advanced molecular diagnostics and research applications.
5. **Automation and high-throughput capabilities**, automation of dPCR workflows, high-throughput platform streamlined sample processing, reaction setup, and result analysis increase experimental efficiency and scalability. Automated dPCR systems incorporate robotics, liquid-handling systems, and software-driven

workflows to minimize hands-on time, reduce experimental variability, and increase assay throughput for large-scale studies and clinical applications.

6. **Miniaturization and portable platforms,** miniaturization of dPCR platforms and development of portable instruments enable decentralized testing, point-of-care diagnostics, and field deployable applications. Compact dPCR devices integrated with battery-powered operation, rugged construction, and simplified workflows empower researchers, clinicians, and field scientists to perform molecular analysis in remote or resource-limited environments, expanding access to dPCR technology and accelerating translational research efforts.
7. **Data analysis and bioinformatics tools and** advancements in data analysis algorithms, bioinformatics tools, and computational resources support the interpretation, visualization, and integration of dPCR data with genomic, clinical, and environmental datasets. Bioinformatics pipelines for dPCR data analysis offer solutions for data normalization, error correction, statistical analysis, and visualization, facilitating meaningful insights and hypothesis generation from large-scale molecular datasets.

6.5. Challenges and Opportunities in the Future of dPCR Technology

The challenges and opportunities in the future of dPCR technology include various aspects that influence the development and application of this technology in contemporary laboratories. With an in-depth understanding of the main challenges faced and innovation opportunities available, policymakers, practitioners, academics, and clinicians can determine the future direction of dPCR technology development. The main challenges in the development of dPCR technology include the following.

1. Cost and Accessibility: One of the major challenges in the development of dPCR technology is the high cost and lack of accessibility for laboratories with limited budgets. Although dPCR technology offers advantages in terms of sensitivity and accuracy, the cost of its hardware and consumables remains a barrier for many laboratories.

2. **Complexity of data analysis**, with the increasing complexity of data generated by dPCR technology, data analysis has become a significant challenge. Understanding and extracting useful information from large and complex datasets requires the development of sophisticated data analysis algorithms and software.
3. **Standardization and reproducibility**, standardization of protocols, and analysis methods are required to ensure reproducibility of the results between laboratories and experiments. Achieving consistent and internationally recognized standards remains a major focus in the development of dPCR technology.
4. **Due to its compatibility with clinical applications**, dPCR technology has great potential for disease diagnosis, but challenges in validating and adopting this technology in clinical practice remain. Further research is needed to demonstrate the reliability and usefulness of dPCR technology in various clinical contexts.

Innovation opportunities for further improvement of dPCR include the following.

1. **The development of small portable devices** has led to the development of smaller, more compact, and portable dPCR devices. This will open the door for the use of dPCR in the field or in areas with limited access to laboratory facilities.
2. **Increased speed and efficiency**, innovations in amplification technology, and molecular detection can significantly improve the speed and efficiency of dPCR analysis. The development of more efficient and stable reagents, enzymes, and fluorophores can improve the overall dPCR performance.
3. **Integration with current technologies** and opportunities to integrate dPCR technology with current technologies such as artificial intelligence (AI), microfluidics, or more sensitive detection sensors will open up new opportunities in dPCR analysis and applications.
4. **The development of clinical applications**, the focus on developing innovative clinical applications, and the validation of dPCR technology for use in disease diagnosis will open up new opportunities for the application of this technology in personalized medicine and disease management.

Chapter 7

Summary, Implications and Updated Insights

7.1. Recap of the significance of dPCR in current laboratory practice

dPCR has emerged as a powerful tool in laboratory practice, offering precise, sensitive, and reliable quantification of nucleic acids across diverse research fields and applications. Its significance lies in its ability to provide absolute quantification without relying on standards, enhanced precision and sensitivity, improved detection of rare mutations, and reduced susceptibility to PCR inhibitors. dPCR enables the quantitative analysis of nucleic acids in various sample types, including liquid biopsies, clinical specimens, and environmental samples. It facilitates the detection and monitoring of infectious diseases, genetic disorders, cancer, gene expression levels, epigenetic modifications, viral loads, and pathogen quantification. Furthermore, dPCR enables the analysis of copy number variations and rare alleles and facilitates integration with next-generation sequencing for comprehensive genomic analysis. Its automation and high-throughput capabilities, along with emerging trends in instrument design and assay development, have contributed to its widespread adoption and utility in laboratory research.

7.2. Summary of Key Findings and Update Insights

This book highlights the versatility and significance of dPCR technology in modern laboratory practice. This finding underscores the advancements in dPCR

methodologies, platforms, and applications, as well as its integration with advanced analytics, artificial intelligence, and multidisciplinary fields, such as microbiology, infectious disease, oncology, environmental science, and remote sensing. Key findings include the enhanced precision, sensitivity, and absolute quantification capabilities of dPCR and its role in personalized medicine, environmental monitoring, and point-of-care diagnostics. Insights from these books emphasize the importance of standardization, quality control, and collaboration in dPCR research, as well as the potential for future advancements in assay optimization, data analysis, and clinical translation.

7.3. Implications for Future Research Directions and Clinical Applications

This book underscores the need for continued research and innovation in dPCR technology to address current challenges, expand its application capabilities, and realize its full potential in clinical practice. Future research directions may include a) further optimization of dPCR assays, platforms, and workflows to improve sensitivity, specificity, and scalability; b) development of standardized protocols, quality control measures, and reference materials to ensure reproducibility and reliability of dPCR results; c) exploration of novel applications and interdisciplinary collaborations in areas such as infectious disease surveillance, environmental monitoring, and precision medicine; d) integration of dPCR with emerging technologies such as single-cell analysis, spatial transcriptomics, and liquid biopsy techniques for comprehensive molecular profiling; and e) translation of dPCR assays and technologies in clinical practice through validation studies, regulatory approvals, and adoption by healthcare providers. Overall, this book highlights the transformative potential of dPCR in laboratory research and clinical applications, paving the way for future advancements in molecular diagnostics, personalized medicine, and environmental monitoring. By addressing technical challenges, fostering collaboration, and embracing emerging opportunities, dPCR is poised to make significant contributions to biomedical research, healthcare delivery, and public health initiatives in the future.

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