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REVIEW ARTICLE

Exploring PCR Methodologies in Forensic DNA Profiling

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ABSTRACT

Polymerase Chain Reaction (PCR) techniques have revolutionized forensic DNA analysis, enabling the precise amplification of trace DNA samples. This abstract provides a concise overview of the pivotal role of PCR in forensic science. It delves into the principles of PCR, emphasizing its ability to amplify specific DNA sequences with remarkable sensitivity and specificity. Multiplex PCR, a variant technique, allows for simultaneous amplification of multiple genetic loci, enhancing the efficiency of forensic investigations. This abstract further highlights the broad applications of PCR in criminal investigations, paternity testing, and disaster victim identification. Recent advancements, such as MiniSTRs and Next-Generation Sequencing (NGS), are elucidated for their crucial contributions in addressing challenging forensic scenarios. Despite its instrumental role, PCR-based DNA analysis is not without challenges, with considerations including DNA contamination and low-template samples. The abstract concludes with a forward-looking perspective on the emerging field of forensic epigenetics and metagenomic analysis, offering a glimpse into the promising future of PCR techniques in forensic DNA analysis. PCR stands as an indispensable tool in modern forensic science, unraveling genetic identities from the most minute of genetic traces.

Keywords PCR, Forensic DNA analysis, Criminal investigation, Amplification, Multiplex PCR, Realtime PCR, Microfluidic PCR, Digital PCR

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INTRODUCTION

Forensic DNA analysis has become an indispensable tool in criminal investigations, providing crucial insights into genetic identities from trace biological samples [1]. At the heart of this revolutionary advancement lies the PCR, a transformative molecular biology technique that has redefined the boundaries of forensic genetics [2]. PCR's unparalleled ability to amplify specific DNA sequences with exquisite precision and sensitivity has revolutionized our capacity to extract meaningful genetic information from even the most minute traces[3].

The success of PCR in forensic applications is rooted in its robust principles, through a cyclic process of denaturation, annealing, and extension, PCR exponentially replicates target DNA regions, rendering previously imperceptible genetic material accessible for analysis [4]. The tailored design of primers, short DNA sequences that initiate the amplification process, is of paramount importance in ensuring the specificity of the procedure. In forensic contexts, the design of highly discriminative primers is essential to distinguish closely related DNA profiles [5].

Furthermore, the advent of multiplex PCR has significantly bolstered the efficiency and throughput of forensic DNA analysis. This technique allows for the simultaneous amplification of multiple DNA loci in a single reaction, enhancing our capacity to process limited or degraded samples [6]. By combining the power of PCR with the versatility of multiplexing, forensic scientists can extract a wealth of information from even the most challenging genetic materials [7].

This paper will delve into the myriad applications of PCR in forensic science, ranging from criminal investigations to paternity testing and disaster victim identification. In criminal cases, PCR-based DNA analysis serves as a linchpin for connecting crime scene evidence with known individuals, aiding in suspect identification and the exoneration of the innocent. Paternity testing, an area of critical social importance, relies extensively on PCR techniques to establish biological parentage with unmatched accuracy [8]. Moreover, in disaster scenarios characterized by fragmented or degraded remains, multiplex PCR assays play an instrumental role in victim identification, providing closure for grieving families [9].

As we embark on a comprehensive exploration of PCR techniques in forensic DNA analysis, it is imperative to recognize both the immense potential and the inherent challenges of this methodology [10]. This paper will delve into recent advancements in PCR, including the emergence of MiniSTRs and Next-Generation Sequencing (NGS), as well as address critical considerations such as DNA contamination and low-template samples. Looking forward, the study will also provide insights into the promising future of forensic epigenetics and metagenomic analysis, underscoring the continued evolution and refinement of PCR techniques in forensic genetics [11].

Objective:

- The objective of this review is to provide a comprehensive overview of the current state and future directions of PCR techniques in forensic DNA analysis. This paper will cover:
- The fundamental principles and mechanisms of PCR
- The diverse applications of PCR in criminal investigations, paternity testing, and disaster victim identification
- Recent advancements such as multiplex PCR, MiniSTRs, and Next-Generation Sequencing (NGS
- Ongoing challenges, including DNA contamination and the analysis of low-template samples

- Future directions in forensic genetics, including forensic epigenetics and metagenomic analysis

Principles of PCR in Forensic DNA Analysis

The principles of PCR form the foundation of its application in forensic DNA analysis. PCR is a molecular biology technique that amplifies specific segments of DNA, making it possible to generate millions of copies of a target DNA sequence from even minuscule samples. The principles of PCR in forensic DNA analysis involve three main steps: denaturation, annealing, and extension, which are repeated in cycles. Here is a detailed description of each step:

- **Denaturation:**

Denaturation is the initial step of PCR, where the double-stranded DNA template is heated to a high temperature (typically around 94-98°C) to break the hydrogen bonds between the complementary DNA strands.

This process results in the separation of the two DNA strands, yielding single-stranded DNA molecules.

Denaturation ensures that the DNA template is in a single-stranded form, making it accessible for the subsequent steps of the PCR reaction [12].

- **Annealing:**

Following denaturation, the temperature is lower to allow primers to anneal or bind to the complementary sequences flanking the target DNA region. Primers are short, single-stranded DNA molecules that are designed to be complementary to specific sequences on each end of the target DNA region. The annealing temperature typically ranges between 50-65°C, depending on the length and nucleotide composition of the primers. Proper primer annealing is crucial for the specificity and efficiency of PCR amplification, as it determines the location where DNA synthesis will initiate [13].

- **Extension:**

Once the primers are annealed, DNA polymerase synthesizes new DNA strands by extending from the 3′ end of each primer in the 5′ to 3′ direction. DNA polymerase is a heat-stable enzyme that catalyzes the addition of nucleotides to the growing DNA strand, using the single-stranded DNA template as a guide. The extension temperature is typically around 72°C, which is optimal for the activity of DNA polymerase. As DNA polymerase extends along the template strand, it synthesizes a new complementary DNA strand, resulting in the replication of the target DNA sequence. The extension step is crucial for amplifying the target DNA region and generating multiple copies of the desired DNA sequence [14].

- **Cycling:**

The denaturation, annealing, and extension steps are repeated in cycles, typically 20-40 times, depending on the desired level of amplification. Each PCR cycle doubles the number of DNA molecules, resulting in exponential amplification of the target DNA sequence [15]. After several cycles, the desired amount of amplified DNA is obtained, which can be analyzed using various techniques such as gel electrophoresis, sequencing, or real-time PCR [16].

Overall, the principles of PCR in forensic DNA analysis involve the repetitive cycling of denaturation, annealing, and extension steps to amplify specific DNA sequences from forensic samples. This powerful technique has revolutionized forensic science by enabling the analysis of trace amounts of DNA evidence, leading to advancements in criminal investigations, identification of suspects, and resolution of legal cases [17].

Factors Influencing PCR Cycling Conditions:

Optimizing PCR (Polymerase Chain Reaction) requires careful consideration of various factors that influence the success and efficiency of the amplification process. Here's a breakdown of some key elements:

TEMPLATE DNA:

• **Quality and Quantity:** The quality and quantity of the starting template DNA significantly impact PCR. Impurities like proteins or salts can inhibit the reaction, while insufficient DNA might lead to insufficient amplification [18].

• **Target Sequence Length:** Longer target sequences typically require longer extension times during PCR to ensure complete amplification [19].

PRIMERS:

• **Specificity and Annealing Temperature (Tm):** Primers need to be highly specific to the target DNA sequence for accurate amplification. The Tm, the temperature at which primers optimally bind to the template, is crucial. Conditions favoring primer-template annealing will improve amplification efficiency [20].

DNTPS (DEOXYNUCLEOSIDE TRIPHOSPHATES) :

• **Concentration:** dNTPs are the building blocks for new DNA strands during PCR. The concentration needs to be balanced to avoid limitations due to insufficient dNTPs or competition between primers and template for available dNTPs [21].

MAGNESIUM (MG2+ CONCENTRATION):

• **Cofactor for Polymerase:** Magnesium ions (Mg2+) act as a cofactor for the DNA polymerase enzyme, crucial for its activity. The optimal Mg2+ concentration depends on various factors, including primer design and buffer composition [22].

BUFFER SYSTEM:

• **pH and Ionic Strength:** The buffer system in the PCR reaction maintains the optimal pH and ionic strength for the DNA polymerase enzyme to function efficiently. Different buffer formulations are available for specific PCR applications [23].

DENATURATION TEMPERATURE:

• **DNA Melting:** Denaturation temperature refers to the temperature at which the double-stranded DNA template separates into single strands. A temperature high enough to ensure complete denaturation is essential, but excessively high temperatures can damage the template [24].

ANNEALING TEMPERATURE:

• **Primer Hybridization:** Annealing temperature is the temperature at which the primers specifically bind to their complementary sequences on the single-stranded DNA template. A carefully chosen annealing temperature optimizes primer-template hybridization, leading to efficient and specific amplification [25].

EXTENSION TEMPERATURE:

• **DNA Polymerase Activity:** Extension temperature is the optimal temperature for the DNA polymerase enzyme to extend the primers and synthesize new DNA strands. An appropriate temperature ensures efficient and accurate extension of the target sequence [26].

CYCLE NUMBER:

• **Amplification Efficiency:** The number of PCR cycles determines the final amount of amplified target DNA. A sufficient number of cycles is needed for adequate amplification, but too many cycles can lead to non-specific amplification artifacts [27].

Applications of PCR in Forensic Science:

PCR (Polymerase Chain Reaction) has become an indispensable tool in forensic science, revolutionizing the field by enabling analysis of minute DNA samples. Here's a breakdown of its key applications:

CRIMINAL INVESTIGATIONS:

• DNA Profiling: PCR amplifies trace DNA evidence left at crime scenes, such as blood, hair, or skin cells. This amplified DNA can then be analyzed to create a unique genetic profile, potentially linking suspects to the crime, or exonerating them [28].

PATERNITY TESTING:

• Biological Relationship Determination: PCR allows for the analysis of DNA from a child, mother, and alleged father. By comparing their genetic profiles, PCR can determine biological relationships with high accuracy [29].

DISASTER VICTIM IDENTIFICATION:

• Identification in Mass Casualties: In the aftermath of disasters, PCR can be crucial for identifying victims whose remains are fragmented or decomposed. By analyzing recovered DNA, it helps reunite families and support closure [30].

BODY FLUID IDENTIFICATION:

• Distinguishing Biological Materials: PCR can be used to distinguish between different body fluids found at crime scenes, such as blood, semen, or saliva. This information can be critical in directing the course of an investigation [31].

SEXUAL ASSAULT INVESTIGATIONS:

• DNA Evidence Analysis: In sexual assault cases, PCR plays a crucial role by analyzing DNA evidence collected from the victim and suspect. This helps identify perpetrators and bring them to justice [32].

Beyond these core applications, PCR also finds use in other forensic areas, such as investigating animal abuse and wildlife trafficking by analyzing DNA from animal remains.

d) **PCR Methods Applied on Forensic DNA Analysis**

i. Conventional PCR in Forensic DNA Analysis

Conventional PCR stands as a cornerstone in forensic DNA analysis, offering a robust method for amplifying specific DNA sequences from even the most minute samples. This technique, though simpler compared to its modern counterparts, holds significant relevance in forensic investigations due to its reliability, adaptability, and widespread use [33]. At the outset, conventional PCR begins with the careful selection of target regions within the DNA sample for amplification. These target regions typically include Short Tandem Repeats (STRs), Single Nucleotide Polymorphisms (SNPs), mitochondrial DNA, or other genetic markers commonly utilized for forensic identification purposes. Precision in target selection is crucial as it directly impacts the accuracy and specificity of the subsequent DNA analysis [34].

Following target selection, the next pivotal step involves the design of primers. Primers are short DNA sequences, usually comprising 18-25 nucleotides, strategically crafted to flank the target regions. Their complementary nature to the sequences on each end of the target region is meticulously curated to ensure precise binding and subsequent amplification during the PCR process. The efficacy of primers is paramount as they dictate the initiation points for DNA synthesis [35].

With the target regions identified and primers designed, the PCR reaction setup ensues. This involves the preparation of a PCR reaction mixture containing the DNA template harboring the target region, forward and reverse primers, DNA polymerase enzyme, nucleotides (dNTPs), buffer solution, and magnesium ions (Mg^{2+}). The DNA template can be extracted from various forensic samples like blood, saliva, semen, hair follicles, or tissue samples. Careful handling and preparation of PCR reagents are imperative to mitigate the risk of contamination and ensure the fidelity of results [36].

The heart of conventional PCR lies in its thermal cycling process. This entails a series of precisely controlled temperature changes to facilitate denaturation, annealing, and extension of DNA. The thermal cycling conditions are meticulously optimized based on the melting temperatures (Tm) of the primers and the target DNA region [37]. This iterative process of denaturation to separate the DNA strands, annealing to allow primer binding, and extension for DNA synthesis is repeated for a specific number of cycles, typically ranging from 25-35, depending on the desired level of amplification [38].

Upon completion of the PCR amplification cycles, the resulting DNA products undergo analysis. Techniques such as agarose gel electrophoresis, capillary electrophoresis, or real-time PCR are commonly employed for this purpose. Agarose gel electrophoresis, for instance, allows for the visualization and size-separation of the PCR products based on their molecular weights. The resulting DNA profiles are then scrutinized by qualified forensic scientists, who interpret the findings and generate reports detailing crucial information such as the number of alleles detected, their sizes, and any pertinent additional data [39].

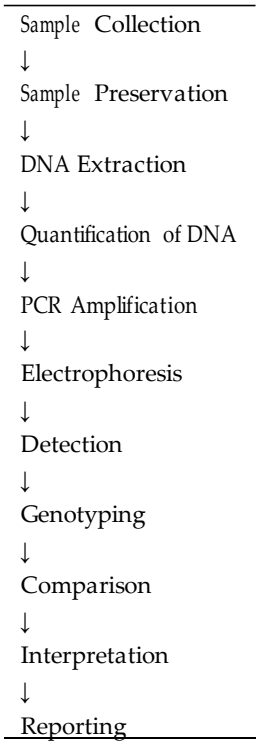

Figure 1: Workflow of conventional forensic DNA typing.

REAL-TIME PCR IN FORENSIC DNA ANALYSIS

Real-time PCR represents a groundbreaking advancement in forensic DNA analysis, offering unparalleled capabilities for simultaneous amplification and quantification of DNA in real-time. Unlike conventional PCR, which requires post-amplification analysis for result interpretation, real-time PCR provides immediate insight into the progress of DNA amplification during each cycle of the reaction. This real-time monitoring enables precise quantification of DNA samples, detection of low-copy number DNA, and assessment of PCR inhibition—all critical aspects in forensic DNA analysis [40].

The core principle of real-time PCR lies in the incorporation of fluorescent reporter molecules into the PCR reaction mixture. These reporter molecules emit fluorescence upon binding to the amplified DNA during each cycle of the reaction, allowing for continuous monitoring of DNA amplification. Commonly used reporter systems include fluorescent dyes such as SYBR Green, which binds to double-stranded DNA, or sequence-specific fluorescent probes like TaqMan probes and molecular beacons [41].

Real-time PCR assays typically involve the use of specialized instrumentation equipped with sensitive detectors capable of measuring fluorescence intensity in real-time [42]. The fluorescence signals generated during the PCR amplification process are captured and plotted against the number of amplification cycles, producing a profile known as an amplification plot or amplification curve [43]. This curve provides valuable information about the initial

DNA concentration, amplification efficiency, and cycle threshold (Ct) values—a parameter used for quantification and comparison of DNA samples [44].

One of the key advantages of real-time PCR in forensic DNA analysis is its ability to accurately quantify DNA samples over a wide dynamic range, spanning several orders of magnitude. This capability is particularly beneficial in cases involving low-copy number DNA or complex mixtures of DNA from multiple contributors, where precise quantification is essential for accurate interpretation of results. Real-time PCR also enables the detection of PCR inhibitors, which can adversely affect the efficiency and reliability of DNA amplification, thereby enhancing the robustness of forensic DNA analysis [45].

Moreover, real-time PCR offers multiplexing capabilities, allowing for the simultaneous amplification and detection of multiple DNA targets within a single reaction [46]. This feature enhances the efficiency, throughput, and cost-effectiveness of forensic DNA analysis by reducing the time and resources required for processing samples. Multiplex real-time PCR assays are commonly used in forensic casework for (STR) profiling, (SNP) analysis, and mitochondrial DNA sequencing, among other applications [47].

Table 1. Real-time PCR assay used in Forensic genetics for specific quantification of specific DNA targets [48,49]

Assay	DNA Target	Application	Advantages
Quantifiler™ Duo	Human and male DNA	Quantification of human and male DNA in forensic samples	High sensitivity, differentiation between human and male DNA
Quantifiler™ Trio	Human, male, and degradation index DNA	Quantification of human, male DNA, and DNA degradation assessment	Comprehensive analysis including degradation assessment
Quantifiler™ Human DNA	Human DNA	Quantification of total human DNA	High specificity for human DNA, minimizes cross-species contamination
Plexor® HY	Human and male DNA	Quantification of human and male DNA	Incorporates internal positive controls to ensure accuracy
Quantifiler™ HP	Human DNA	Quantification of human DNA	Higher precision and sensitivity for degraded or low quantity samples
Investigator Quantiplex	Human DNA	Quantification of human DNA	High accuracy, suitable for various forensic sample types
PowerQuant® System	Human, male, and degradation index DNA	Quantification of human and male DNA, and assessment of DNA quality	Provides data on DNA quality, aiding in decision-making for downstream analysis

MULTIPLEX PCR IN FORENSIC DNA ANALYSIS

Multiplex PCR stands out as a powerful and versatile technique in forensic DNA analysis, offering the ability to simultaneously amplify multiple DNA targets within a single reaction [50]. This capability has revolutionized forensic investigations by maximizing the

information obtained from limited or degraded forensic samples, enhancing the efficiency, throughput, and cost-effectiveness of DNA analysis [51].

At its core, multiplex PCR utilizes a single reaction mixture containing multiple pairs of primers, each targeting a specific DNA sequence of interest. These primer sets are carefully designed to ensure compatibility and specificity, allowing for the simultaneous amplification of multiple DNA targets without interference or cross-reactivity. The selection of appropriate primer sets is critical and often involves optimizing primer concentrations, annealing temperatures, and amplicon sizes to achieve optimal performance [52].

One of the primary advantages of multiplex PCR in forensic DNA analysis is its ability to amplify several types of genetic markers concurrently. For example, multiplex PCR assays can target (STRs), (SNPs), mitochondrial DNA, or other genetic markers commonly used for forensic identification. This versatility enables forensic scientists to obtain comprehensive DNA profiles from a single sample, thereby maximizing the probative value of forensic evidence [53].

Moreover, multiplex PCR offers significant time and cost savings compared to performing individual PCR reactions for each target region [54]. B1y consolidating multiple amplification reactions into a single multiplex assay, forensic laboratories can streamline their workflow, reduce reagent consumption, and increase sample throughput [55]. This efficiency is particularly advantageous in high-volume forensic casework, where rapid turnaround times and resource optimization are paramount [56].

Additionally, multiplex PCR enables the analysis of complex DNA mixtures and degraded samples, which are common challenges encountered in forensic DNA analysis [57]. By amplifying multiple DNA targets simultaneously, multiplex PCR increases the likelihood of detecting informative genetic markers, even in samples with low DNA quantities or poor DNA quality [58]. This capability enhances the sensitivity and reliability of DNA profiling, leading to more accurate forensic interpretations and outcomes [59].

In practical terms, multiplex PCR assays are typically performed using specialized PCR reagents and instrumentation designed for multiplexing applications [60]. The reaction conditions, including primer concentrations, annealing temperatures, and cycling parameters, are optimized to ensure efficient and reproducible amplification of all target regions [61]. Following amplification, the resulting PCR products are analyzed using techniques such as agarose gel electrophoresis, capillary electrophoresis, or real-time PCR to visualize and characterize the amplified DNA fragments [62].

MICROFLUIDIC PCR IN FORENSIC DNA ANALYSIS

Microfluidic PCR represents a cutting-edge approach to forensic DNA analysis, offering miniaturized and integrated systems that revolutionize the amplification of DNA samples.

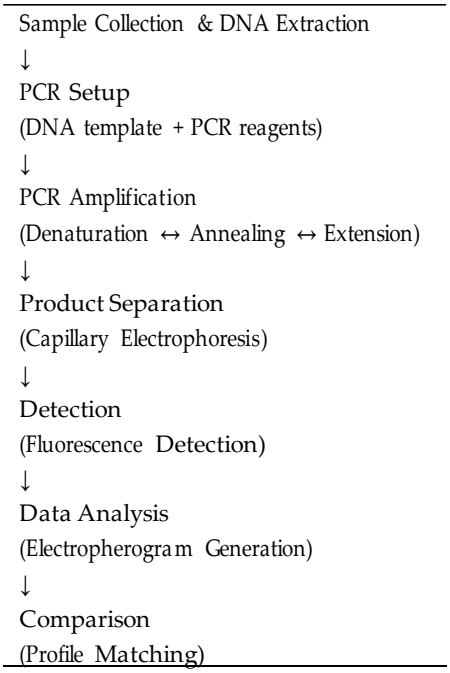

Figure 2: schematic view of multiplex PCR analysis of STRs.

This innovative technique harnesses the principles of conventional PCR within microscale devices, leveraging microfluidic channels to manipulate small volumes of reagents and samples with unprecedented precision and efficiency [63].

At the heart of microfluidic PCR lies the microfluidic chip, a tiny platform engineered to perform PCR reactions on a miniature scale. These chips typically consist of microchannels, and chambers fabricated using advanced microfabrication techniques, such as photolithography or soft lithography [64]. The microchannels serve as conduits for the flow of reagents and samples, while the chambers provide confined spaces for PCR amplification to occur [65].

One of the key advantages of microfluidic PCR in forensic DNA analysis is its ability to significantly reduce reaction volumes and reaction times [66]. By operating at the microscale, microfluidic PCR requires only nanoliters to picoliters of reagents and samples, compared to microliters in conventional PCR [67]. This miniaturization not only conserves precious DNA samples but also accelerates reaction kinetics, resulting in faster amplification times and shorter turnaround times for forensic analyses [68].

Moreover, microfluidic PCR offers precise temperature control and thermal uniformity, which are critical for efficient and reproducible PCR amplification [69]. Microfluidic devices are often equipped with integrated heating and cooling elements, such as thermoelectric heaters and temperature sensors, to maintain optimal thermal conditions throughout the PCR cycling process [70]. This precise temperature control minimizes thermal gradients and ensures uniform amplification across all regions of the microfluidic chip, enhancing the reliability and consistency of forensic DNA analysis [71].

Another notable feature of microfluidic PCR is its ability to multiplex PCR reactions within a single microfluidic chip [72]. By integrating multiple microchannels and reaction chambers on a single chip, microfluidic devices enable parallel amplification of multiple DNA targets simultaneously [73]. This multiplexing capability enhances the efficiency and throughput of forensic DNA analysis, allowing forensic scientists to analyze complex DNA samples and obtain comprehensive DNA profiles in a streamlined manner [74].

Furthermore, microfluidic PCR holds promise for on-site forensic investigations and decentralized forensic laboratories. The compact size, portability, and automation potential of microfluidic devices make them well-suited for field deployment, enabling rapid DNA profiling and real-time decision-making at crime scenes or remote locations [75]. This capability has significant implications for expediting forensic investigations, improving case resolutions, and enhancing criminal justice outcomes [76].

Table 2. applications of microfluidic PCR in Forensic DNA Analysis devices [77,78].

Application	Description	Advantages	Disadvantages
Rapid DNA Profiling	Quick generation of DNA profiles at the crime scene or in the lab.	- Fast turnaround time - On-site analysis - Minimal sample handling	- Limited throughput - Higher initial cost of devices - Requires specialized training
Low-Quantity Sample Analysis	Efficient amplification and analysis of low DNA quantities from trace evidence.	- High sensitivity - Reduces sample consumption - Effective for degraded or minute samples	- Potential for contamination - Requires optimization for low-template samples
Integration with Lab-on-a-Chip	Combining PCR with other analytical processes on a single microfluidic device for comprehensive analysis.	- Streamlined workflow - Reduced risk of contamination - Compact and portable	- Complex device fabrication - Integration challenges - High initial development costs
Automated Processing	Automation of DNA extraction, amplification, and analysis on a single platform.	- Reduces human error - Increases consistency and reliability - Saves labor and time	- Maintenance and troubleshooting can be complex - High initial investment
High-Throughput Analysis	Simultaneous analysis of multiple samples for large-scale forensic investigations.	- Increases sample processing capacity - Efficient use of reagents - Scalability	- Potential for cross-contamination - Requires sophisticated equipment and software
On-site Forensic Analysis	Portable devices for immediate analysis at crime scenes, disaster sites, or mass casualty events.	- Immediate results - Reduces backlog in forensic labs - Portable and easy to transport	- Limited by battery life and environmental conditions - Lower robustness compared to lab equipment
Sensitive Detection of Mixed Samples	Discrimination and analysis of mixed DNA samples from multiple contributors.	- High precision - Effective in separating mixed profiles - Sensitive to low-abundance DNA	- Complex data interpretation - Requires advanced software algorithms

Digital PCR in Forensic DNA Analysis

Digital PCR represents a groundbreaking advancement in forensic DNA analysis, offering unparalleled precision and sensitivity in the quantification and detection of DNA samples [79]. Unlike traditional PCR methods that rely on amplification of DNA in bulk, digital PCR partitions the PCR reaction into thousands of individual micro-reactions, allowing for absolute quantification of DNA targets without the need for standard curves or reference materials [80].

At the core of digital PCR lies the partitioning of the PCR reaction mixture into numerous discrete compartments, such as droplets or wells, each containing a single DNA molecule or a few DNA molecules at most. This partitioning is typically achieved using microfluidic devices or emulsion-based techniques, ensuring that each micro-reaction represents a unique and independent amplification event [81].

One of the key advantages of digital PCR in forensic DNA analysis is its ability to precisely quantify DNA targets across a wide dynamic range. By distributing DNA molecules into individual compartments, digital PCR enables the absolute counting of target DNA molecules, regardless of their concentration or amplification efficiency. This capability is particularly beneficial for accurate quantification of low-copy number DNA samples, where traditional PCR methods may be susceptible to inaccuracies or biases [82].

Moreover, digital PCR offers exceptional sensitivity and accuracy in detecting rare alleles and minor DNA variants [83]. The high level of partitioning ensures that even rare DNA molecules are captured and amplified in separate compartments, allowing for robust detection and discrimination of subtle genetic differences [84]. This sensitivity is invaluable in forensic DNA analysis, where identifying minor contributors to DNA mixtures or detecting trace amounts of DNA evidence can be crucial for investigative purposes [85].

Another advantage of digital PCR is its resistance to PCR inhibitors, which can interfere with amplification efficiency and compromise the accuracy of DNA quantification [86]. Because each PCR reaction occurs independently in a separate compartment, any inhibition affecting one micro-reaction is unlikely to affect others, minimizing the impact on overall quantification accuracy [87]. This resilience to inhibitors enhances the reliability and robustness of forensic DNA analysis, especially when working with challenging or degraded samples [88].

Furthermore, digital PCR offers multiplexing capabilities, allowing for the simultaneous detection and quantification of multiple DNA targets within a single digital PCR assay [89]. By incorporating multiple primer sets and fluorescent probes into the reaction, digital PCR enables comprehensive profiling of forensic DNA samples, enhancing the efficiency and throughput of DNA analysis [90].

Table 3. Advantages and disadvantages of different PCR techniques[91,92].

Aspect	Advantages	Disadvantages
Speed	- Allows for immediate identification and response to threats and incidents.	- May lead to hasty decisions without thorough analysis.
	- Reduces the time window for attackers to cause damage.	- Can overwhelm analysts with a high volume of data and alerts.
Accuracy	- Immediate data availability can improve the accuracy of identifying and verifying incidents.	- Real-time data may lack context, leading to potential misinterpretations.
	- Continuous monitoring helps in correlating events more accurately.	- False positives can be more frequent without proper tuning of monitoring systems.
Resource Utilization	Optimizes the use of resources by enabling Quick allocation based on real-time needs.	- Requires significant computational and storage resources to handle continuous data streams.
	- Allows dynamic adjustment of resources in response to detected threats.	- Can lead to resource exhaustion if not managed properly.
Complexity	- Enhances the ability to manage and mitigate complex and evolving threats.	- Implementation and maintenance of real-time systems are complex and costly.
	- Integrates with automated systems for efficient threat response.	- High complexity can lead to potential system vulnerabilities.
Legal and Compliance	- Helps in meeting compliance requirements by providing immediate evidence and audit trails.	- Real-time data handling needs stringent compliance with privacy and data protection regulations.
	- Facilitates transparent reporting and accountability.	- Legal challenges may arise from the rapid collection and analysis of sensitive data.

Advantages and disadvantages of different PCR techniques:

Recent Developments in PCR for DNA Profiling:

- **Multiplexing:** Amplifying more DNA regions at once for faster analysis.
- **Miniaturization:** Smaller reaction chambers for faster cycling and lower costs.
- **Real-time PCR:** Monitoring DNA amplification as it happens for quantification.
- **Digital PCR:** Detecting single DNA molecules for ultra-sensitive analysis.
- **Next-Generation Sequencing:** Potentially providing detailed genetic information beyond traditional methods.

These advancements offer benefits like:

- Increased sensitivity for analyzing limited DNA evidence.
- Faster analysis times for quicker investigations.
- More comprehensive DNA profiles with potentially richer genetic information.

Challenges remain in standardizing these techniques for widespread use and efficiently analyzing the vast amount of data generated by some methods. As these advancements are

refined, they hold great promise for the future of accurate and informative DNA profiling in forensics [93–97]

Challenges and Limitations

PCR techniques have revolutionized forensic DNA analysis, offering unparalleled sensitivity, specificity, and efficiency in amplifying, and analyzing DNA samples [98]. However, despite their numerous advantages, PCR techniques also face several challenges and limitations in forensic DNA analysis [99].

One significant challenge is the potential for contamination during sample handling, preparation, and PCR amplification. Even trace amounts of extraneous DNA can compromise the integrity of forensic DNA analysis results, leading to erroneous conclusions or false identifications [100]. Strict adherence to rigorous contamination control measures, such as using dedicated laboratory spaces, disposable equipment, and stringent sample handling protocols, is essential to mitigate this risk [101].

Another challenge is the presence of PCR inhibitors in forensic samples, which can interfere with the amplification process and inhibit DNA polymerase activity. Common PCR inhibitors include substances such as heme, humic acids, and polyphenols, which may be present in biological samples such as blood, soil, or environmental debris. Identifying and mitigating the effects of PCR inhibitors requires specialized purification methods, enzymatic treatments, or dilution strategies to ensure accurate DNA amplification and analysis [102].

Additionally, PCR techniques are susceptible to allele dropout, a phenomenon where certain alleles fail to amplify during PCR amplification, leading to incomplete or partial DNA profiles [103]. Allele dropout can occur due to various factors, including primer mismatches, DNA degradation, or stochastic effects during PCR amplification [104]. Mitigating allele dropout requires careful primer design, optimization of PCR conditions, and validation of PCR assays to ensure robust and reproducible amplification of all alleles of interest [105].

Furthermore, PCR artifacts such as stutter bands, non-specific amplification, and allelic dropout can complicate the interpretation of DNA profiles and lead to erroneous conclusions in forensic DNA analysis [106]. Stutter bands are false peaks that result from slippage of DNA polymerase during PCR amplification, leading to additional peaks in the DNA profile that may resemble true alleles [107]. Distinguishing between true alleles and PCR artifacts requires careful examination and validation of DNA profiles by experienced forensic scientists [108]

Another limitation of PCR techniques in forensic DNA analysis is their reliance on reference databases and population databases for allele frequency calculations and statistical interpretation of DNA profiles [109,110]. In populations with limited genetic diversity or inadequate representation in reference databases, calculating accurate match probabilities and

estimating the likelihood of DNA profile matches can be challenging [109]. Moreover, the potential for population substructure and genetic admixture further complicates the interpretation of DNA evidence in forensic casework [111].

Future Directions

The future of PCR techniques in forensic DNA analysis holds promise for continued innovation and advancement, with several emerging trends and technologies poised to shape the landscape of forensic science. One such direction involves the integration of PCR with next-generation sequencing (NGS) technologies, enabling comprehensive DNA analysis at the genomic level. NGS platforms offer unprecedented capabilities for high-throughput sequencing of DNA samples, allowing forensic scientists to obtain detailed genetic information beyond traditional DNA profiling markers. By combining PCR with NGS, forensic laboratories can enhance their ability to identify individuals, detect genetic variations, and uncover valuable forensic evidence from complex DNA samples [112].

Another future direction involves the development of portable and point-of-care PCR devices for on-site forensic investigations. Miniaturized PCR platforms equipped with integrated sample processing, amplification, and detection capabilities hold immense potential for rapid DNA profiling and real-time decision-making at crime scenes or remote locations. These portable PCR devices enable forensic scientists to perform DNA analysis directly in the field, thereby expediting forensic investigations, enhancing case resolutions, and facilitating timely justice outcomes [113].

Furthermore, advancements in microfluidic PCR technologies are expected to drive miniaturization, automation, and integration of PCR workflows for forensic DNA analysis. Microfluidic devices offer precise control over reaction conditions, reduced sample volumes, and accelerated reaction kinetics, making them ideal platforms for high-throughput DNA amplification and analysis. Future developments in microfluidic PCR are likely to focus on enhancing sensitivity, multiplexing capabilities, and compatibility with forensic sample types, further expanding the utility of microfluidic platforms in forensic science [114].

Additionally, the integration of PCR techniques with advanced bioinformatics and computational methods holds promise for enhancing data analysis, interpretation, and forensic intelligence [115]. Machine learning algorithms, statistical models, and data visualization techniques can aid forensic scientists in extracting meaningful insights from DNA profiles, identifying patterns, and predicting potential genetic relationships among individuals. By leveraging computational approaches, forensic laboratories can optimize DNA analysis workflows, prioritize casework, and generate actionable intelligence to support criminal investigations and legal proceedings [116].

Moreover, emerging trends such as digital PCR, single-cell PCR, and nanopore sequencing are expected to contribute to the advancement of PCR techniques in forensic DNA analysis [117]. Digital PCR offers absolute quantification of DNA targets, enhancing the accuracy and sensitivity of DNA profiling, while single-cell PCR enables analysis of individual

cells, providing insights into cellular heterogeneity and trace DNA evidence. Nanopore sequencing platforms offer rapid, real-time DNA sequencing capabilities, complementing PCR techniques for comprehensive DNA analysis in forensic casework [118].

CONCLUSION:

PCR techniques have revolutionized forensic DNA analysis, enabling precise identification, evidence analysis, and aiding criminal investigations. Over time, PCR has advanced from conventional methods to sophisticated technologies, each with unique benefits. \\

Conventional PCR, known for its simplicity and versatility, established the foundation by amplifying specific DNA sequences from small samples. Real-time PCR added the ability to monitor and quantify DNA in real-time, enhancing speed and accuracy. Multiplex PCR allowed the simultaneous amplification of multiple DNA targets, maximizing information from limited samples.

Microfluidic PCR brought high-throughput, automated, and portable DNA analysis, ideal for on-site investigations and rapid profiling. Emerging trends like digital PCR and single-cell PCR are further improving sensitivity, accuracy, and efficiency.

Despite these advancements, challenges such as sample contamination, PCR inhibitors, allele dropout, and complex profile interpretation remain. Addressing these requires ongoing research, technological innovations, and interdisciplinary collaboration to maintain reliability and validity.

Future PCR techniques will likely integrate advanced technologies, focusing on miniaturization, portability, automation, and enhanced data analysis. These innovations will optimize workflows, prioritize casework, and provide actionable intelligence for criminal investigations.

PCR has reshaped forensic science, providing tools to unravel DNA evidence, identify perpetrators, exonerate the innocent, and deliver justice. As technology advances, PCR will remain at the forefront of forensic DNA analysis, driving innovation and enhancing forensic capabilities.

Advancements and Future Directions in PCR Techniques for Forensic DNA Analysis:

The future of PCR in forensic DNA analysis looks promising with several key advancements. Integrating PCR with next-generation sequencing (NGS) will provide detailed genetic information, especially useful for complex cases. Enhancing sensitivity and specificity through innovations like digital PCR (dPCR) will improve detection of low-abundance and degraded DNA.

Microfluidic and lab-on-a-chip technologies will enable portable, automated systems for on-site DNA analysis, speeding up investigations. Increased automation and high-throughput capabilities will standardize procedures and handle large sample volumes effi-

ciently, crucial for reducing case backlogs and managing mass disasters.

Advanced bioinformatics and computational tools will improve the analysis of complex DNA profiles and mixed samples. Forensic epigenetics will explore DNA methylation patterns, offering insights into tissue origin, age, and environmental exposures. Point-of-care diagnostics will develop portable devices for rapid DNA identification, aiding immediate law enforcement decisions.

Establishing robust standards and protocols will ensure consistent, accurate analyses. Interdisciplinary collaborations will drive innovation, addressing complex challenges in forensic DNA analysis. These advancements will ensure PCR techniques remain at the forefront of forensic science, enhancing their capabilities and applications.

DECLARATIONS

1. Authors' contributions

Contributor Role	Degree of Contribution		
	Lead	Equal	Supporting
Conceptualization	MAH	OAM	
Data curation	MAH		
Formal analysis	OAM	SHM	NHB
Funding acquisition			
Investigation	MAH	OAM	
Methodology	OAM	NHB	RHA
Project administration	MAH	OMA	LME
Resources	OAM	SHM	SRJ
Software	MAH	NHB	RHA
Supervision	MAH	RHA	
Validation	OAM	SHM	NHB
Visualization			
Writing-original draft	MAH	OAM	SHM
Writing-review & editing	RHA	SRJ	LME

2. Conflict of interest

The authors declare no conflict of interest

3. Ethical approvals (institutional ethical approvals and informed consent)

This research does not conflict with our university's ethical standards, nor with any known ethical criteria.

4. Funding resources

The research is self-funded

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