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From Trace Evidence to Genetic Identification: Addressing Bottlenecks in Forensic DNA Analysis

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Abstract

Forensic DNA analysis is an enormous field that rapidly evolves and increases, which highlights a various number of disciplines such as biology, genetics, instrumentation. homicide, biophysics, statistical, recombinant DNA technology and more, that aims to resolve legal matters which revolves around rape, murder, robbery, paternity dispute or identification of human remains. A subfield of forensics is Criminalistics which includes the application of principle of branch of sciences like physical, chemical and biological sciences in civil and criminal law. Currently this subfield turned to DNA typing, DNA profiling, DNA fingerprinting and DNA analysis due to which these methods direct towards the identification of originator of a particular DNA at the crime site and offers scientifically vigorous methods for the identification of the originator of the biological material/s at the crime scene. Methods like these mostly rely on genetic polymorphisms commonly STRs (Short Tandem Repeats) which shows the highest degree of accuracy between different set of genetic information. Specificity, sensitivity and new changeless are introduced by these methods and machines which gives fewer human errors. Human errors, contamination, temperature below room temperature (24˚-25˚C), low quality DNA, data analysis and misinterpretation are some challenges faced by forensic DNA analyst. This paper highlights the most crucial and important problems which DNA analyst have to deal which can compromise the validity of the result of any forensic sample and also how analysts can overcome these bottlenecks in DNA analysis. Every step and stage in DNA analysis from case receiving to sequences has problems one can face due to which they can affect the result.

Keywords: Forensic DNA Analysis; Criminalistics; STRs (Short Tandem Repeats)

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Introduction

DNA (Deoxyribose Nucleic Acid) is the most important biomolecules for the continuation of life in different living organisms. DNA stores and transfer genetic information from one generation to another generation in living organisms. DNA is not only one source for transfer of genetic information, RNA {Ribonucleic Acid} is also the source for the transfer of the genetic information. DNA is universal and predominant genetic material as it is more stable than RNA. In human beings, DNA stores genetic information and carry it to their offspring and hence it decide the skin, hair color, height, appearance etc [1, 2].

Importance of DNA

Since DNA includes all the information an organism needs to function and reproduce, it is frequently referred as the blue print of life [3]. There is a lot of DNA in every individual`s genome that could be the subject of DNA [3]. DNA is peculiar to each individual, excluding identical twins, which enables its use for identification in legal and forensic contexts. This individuality is especially useful in criminal investigations where DNA profiling can link a suspect/accused to biological evidence at a crime scene [4]. DNA can persist in biological samples for decades or even centuries. This has enabled scientists to analyze ancient human remains and solve decades-old cold cases [5]. Modern molecular biology relies on DNA as the core substrate for various analytical techniques. PCR, STR analysis and NGS. PCR isa technique used to amplify small quantities of DNA into millions of copies. This is essential when forensic or biological samples are minimal or

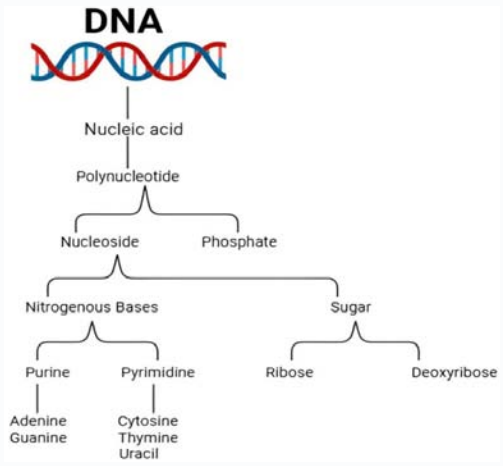

Figure 1: Nucleotides.

degraded. Short Tandem Repeats (STRs) are repeating sequences of 2-6 base pairs in DNA. The number of repeats at specific loci varies significantly between individuals [6]. Next-Generation Sequencing (NGS) allows for high-throughput sequencing of whole genomes or targeted specific gene panels. It offers deeper resolution and can detect mutations, variants, and even microbial DNA in a single run.

Composition of DNA

DNA is made from nucleic acid, also called polynucleotide which means multiple nucleotides and therefore made up of nucleotides. Nucleotides consist of phosphate ion (PO_4^{3-}), nitrogenous bases (purine and pyrimidine) and sugars (deoxyribose and ribose). Nitrogenous bases joined with sugar are called nucleosides and nucleosides joined with phosphates are called nucleotides [1, 2, 7-10] (Figure 1).

Nitrogenous bases: There are derivatives of nitrogenous bases from two parent compounds which are purine is a 9 membered ring and pyrimidine is a 6 membered ring. These bases are heterocyclic compounds containing carbon, hydrogen, oxygen and nitrogen atoms, where atoms of carbon and nitrogen are conventionally numbered for the easy numbering, naming and identification of these derivatives compound [7-9].

Purine have two types of bases: Adenine (A:- 6-aminopurine –NH_2 on C6 position of the ring) and Guanine (G:- 6-oxy-2aminopurine – amino group at C2 and carbonyl group at C6 position), both are present in both DNA and RNA whereas, pyrimidine have three types of bases: Cytosine (C:- 2-oxy4-aminopyrimidine – a hydrogen atom at the C5 position and an amino group at C4 position), Thymine (T:- 5-methyl-2,4-dioxypyrimindine – a methyl group at C5 position and a carbonyl group at C4 and C2 position) and Uracil (U:- 2,4-dioxypyrimidine – similar to thymine but uracil lacks a methyl group at C5 position), where Cytosine and Thymine are present in DNA and Cytosine and Uracil are present in RNA instead of Thymine [1,2, 7-10] (Figure 2).

Sugars: Sugar, sometime known as pentose, which are commonly denoted by (`) called prime (e.g. Two prime (*2`*)) to be easily differentiated from nitrogenous bases. Nitrogenous bases are attached to sugars by a covalent bond called N-*β*-glycosyl bond in which *1`* (one prime) carbon of sugar is joined to N-1 in pyrimidine and N-9 in purine. This bond is formed by the removal of water molecule, from pentose a hydroxyl group and a molecule of hydrogen from the base

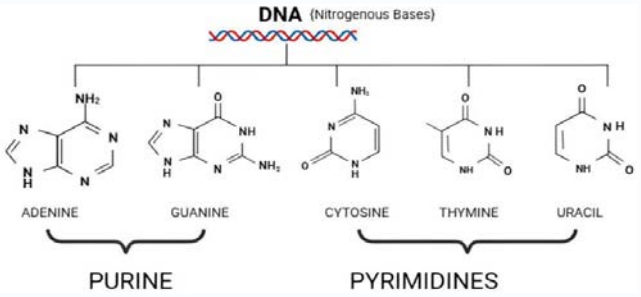

Figure 2: Purine and Pyrimidine.

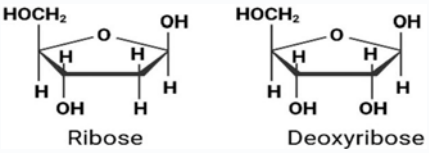

Figure 3: Sugar: Ribose and Deoxyribose.

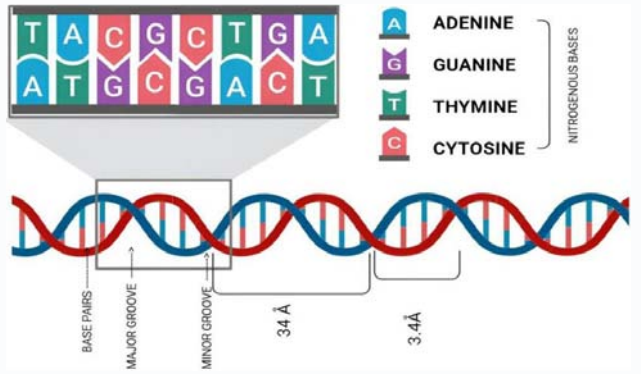

Figure 4: Double helical structure of DNA.

[1, 10] (Figure 3).

Phosphate: DNA forms double stranded helical structure most of the time and these double strands need a backbone to have a stable structure. This backbone is made up of sugar and phosphate, linked with nucleotides by phosphodiester bonds which gives directionality to whole helical structure [1, 8, 10]. Phosphate functions as a carrier of energy in the form of ATP (Adenosine Triphosphate) in which the high-energy bonds of phosphate allow the transfer of energy in the metabolic reactions [1].

Phosphate has negative charge on it that helps in the sustain the DNA solubility in environment like aqueous solutions and interacting DNA in company of histone protein. It also balances intracellular *pH* [11].

Double helical structure of DNA

In 1953, Watson and Crick gave the double helical structure of DNA by the help of data of *X*-ray diffraction of DNA fibers which was achieved by Rosalind Frankiln and Maurice Wilkins. They got Nobel price in1962 for Medicine for discovering the molecular structure of DNA [1, 2, 10] (Figure 4).

Organization of DNA into chromosomes

Each nucleated human cell contains two full copies of the genome. Humans have 23 pairs of chromosomes, which store 3,200,000,000

base pairs (bps) of information. Each parent contributes one copy of each of the two sets of chromosomes, for a total of 46 chromosomes [12, 13].

Classification of human genome

The human genome is classified into the following kinds based on its structure and function as shown in the figure 5:

1. Coding and regulatory regions: Genes are the sections of DNA that both encode and control the synthesis of proteins. One fifth of the 20,000-25,000 genes that make up the human genome are involved in protein encoding.

2. Noncoding: Although 23.5% of the genome is categorized as genetic sequence, this portion does not enclose proteins; instead, it is mostly involved in the control of genes, including polyadenylation signals, enhancers, promoters, and repressors.

3. About 75% of the genome is extragenic, with 45% consisting of interspersed repetitions and 50% consisting of repetitive DNA. Short interspersed elements, long interspersed elements, long terminal repeats, and DNA transposons are the four most prevalent forms of interspersed repetitive elements. There are three forms of tandem repeats: satellite DNA, minisatellite DNA, and microsatellite DNA.

Forensic science and it`s brief history

One ubiquitous technique for confirming the accuracy of forensic investigations is forensic identification. As integral components of forensic identification, criminalities and medico-legal identification both have probative value. The specialist's ability to match traces left at the crime scene with traces found on other materials, such reference evidence, is what gives an identification approach its worth. This process allows one to compare biological samples from the victim and traces of blood, saliva, or any other material left at the crime scene with samples taken from the suspect's clothing. Scientific methodologies or inherent scientific methods borrowed from other sciences, typically biomedical sciences, serve as the foundation for medico-legal identification.

The role of professionals in identification has been and still is highlighted by scientific advancements over the past 30 to 40 years. Their role demonstrates its importance in civil, family, and criminal law matters as well as in situations involving mass casualties (e.g., accidents, natural disasters, terrorist attacks, and wars). In the field of forensic genetics, Sir Alec Jeffreys employed the Polymerase Chain Reaction (PCR), which Mullis discovered in 1983, by analyzing a collection of DNA fragments that demonstrated distinct traits that were intrinsic and nonrecurring for every individual, with the exception of identical twins. These reaction products were dubbed "genetic fingerprints" by Alec Jeffreys [14]. PCR procedure is correct as per the reference.

Brief History of Forensic Genetics

- Karl Landsteiner identified the primary blood types in 1900 and noted that people may be categorized.
- Into various groups according to their blood type. This marked the beginning of the field of forensic hemogenetics [15].
- In 1915, Leone Lattes explains how ABO genotyping is used to settle paternity cases [15].
- 1931 saw the development of theabsorption–inhibition of ABO genotyping procedure. This was followed by thecharacterization of soluble blood serum protein markers and other blood group markers [15].
- In the 1960s and 1970s, researchers were able to analyze DNA sequences thanks to advancements in molecular biology, enzyme restriction, Southern blotting, and Sanger sequencing [16, 17].
- 1978: Southern blotting was used to detect DNA polymorphisms [18].
- The first polymorphic locus was discovered in 1980 [19].
- 1983: The invention of the PCR technique, which can amplify particular DNA areas, was a significant advancement in the history of forensic genetics. It was developed by chemist Kary Mullis, who went on to win the Nobel Prize in 1993 [20].
- In the field of forensic genetics, Alec Jeffrey introduced DNA fingerprinting in 1984. He demonstrated.
- That certain DNA regions contain repeating sequences that differ from person to person. This discovery.
- led to the use of DNA analysis to solve the first forensic case [21].

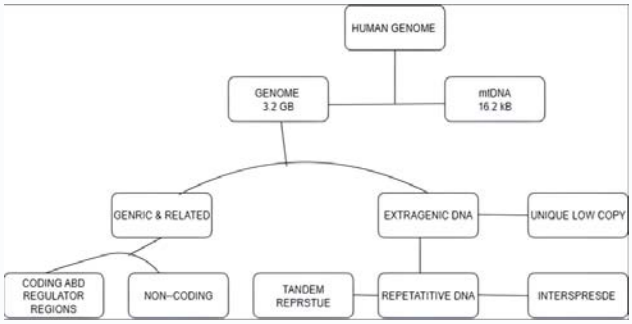

Figure 5: Classification of human genome.

DNA in forensic

DNA loci that are to be used for forensic genetics should have the following ideal properties:

- Should be highly polymorphic.
- Should be easy and cheap to characterize.
- Should be simple to interpret and easy to compare between laboratories.
- Should have a low mutation rate.

Any of the 3.2 billion bps that make up the genome can be analyzed thanks to recent developments in molecular biology technology [15]. The three most crucial processes for biological material are gathering, characterizing, and storing. Biological Evidence Sources With the exception of red blood cells, the majority of the trillions of cells that make up the human body are nucleated cells. Two copies of the individual's genome are present in each nucleated cell, which can be utilized to create a DNA profile. Samples typically exhibit some degree of deterioration, but more cellular material is required to create a DNA profile when the degree of degradation is significant [22].

Forensic genetic profiling requires biological samples containing

nucleated cells, such as: [23]

- Liquid blood or dry deposits.
- Liquid saliva, semen, or dry deposits.
- Hard tissues like bone and teeth.
- Hair with follicles.

Collection and Handling of Material at the Crime Scenes: One of the most common sources of DNA is thought to be whole blood. It is first kept for five to seven days at 4°C in an anticoagulant (EDTA ethylenediamine tetra acetic acid). Following this time frame, DNA samples are stored for a few weeks at -20°C or for extended lengths of time at -80°C. Sterile brush or bud is used to extract epithelial cells from crime scenes. Following harvest, they are stored at room temperature in a dry area after being wrapped in a paper or plastic envelope [24]. It is crucial that the right precautions are taken, such as preserving the crime scene's integrity and donning face masks and complete protective gear while the area is being investigated [25-27] because improper handling of the evidence may have catastrophic repercussions. In the worst situations, cross-contamination causes a high degree of sample degradation, which might cloud or prevent the evidence's final outcome.

DNA nalysis

DNA analysis, a crucial technique in genetic research, forensic science, and paternity testing, has substantial difficulties with sample quality, data analysis, and interpretation [9]. DNA analysis is the study and examination of an individual's deoxyribonucleic acid to extract biological, forensic, or medical information. This analysis has transformed modern science by enabling researchers to identify individuals, diagnose diseases, and understand genetic relationships. DNA's universality, stability, and specificity make it perfect for a variety of uses, from customizing cancer treatments to solving crimes.

Methods of DNA Analysis

1. Polymerase Chain Reaction (PCR): PCR amplifies specific DNA sequences, enabling detailed study even from minimal or degraded samples. It is foundational to diagnostics and forensic identification.

2. Short Tandem Repeat (STR) Profiling: STRs are highly variable among individuals, making them ideal for identity verification. This method is routinely used in criminal forensics and paternity testing [28].

3. Next-Generation Sequencing (NGS): NGS enables simultaneous sequencing of millions of DNA fragments, offering unprecedented depth in analyzing genetic variation, cancer mutations, and rare diseases.

4. Combinatorial and Graph-Theoretic Techniques: Advanced mathematical models like Levenshtein distance and graph theory have been applied to optimize sequence alignment and oligonucleotide selection for synthetic biology and diagnostics [29].

Steps involved in DNA analysis

DNA analysis involves steps four basic steps, which are the following:

- DNA Extraction: DNA extraction is the initial and critical step in molecular biology for isolating Deoxyribonucleic Acid (DNA) from biological samples such as blood, tissues, plants, or microorganisms. This purified DNA serves as the foundation for downstream applications like PCR, DNA sequencing, genotyping, and forensic analysis. This includes cell lysis, removal of proteins and contamination, DNA precipitation and DNA purification. The chemical and reagents are different for different types of material used as the source of DNA which varies from blood, hair, saliva or even a piece of cloth [30].

Table 1: Various sources of biological evidence.

Type of sample	Amount of DNA
Liquid blood	30,000 ng/ml
Stain of blood	200 ng/cm^2
Liquid saliva	5,000 ng/ml
Hair (with root) shed	1-12 ng/root
Hair (with root) plucked	1-750 ng/root
Liquid semen	250,000 ng/ml
Postcoital vaginal swab	0-3,000 ng/swab
Oral swab	100-1,500 ng/swab
Urine	1-20 ng/ml
Bone	3-10 ng/mg
Tissue	50-500 ng/mg
Teeth	20-200 ng/mg

- DNA Quantification: It is ideal to measure the quantity and quality of DNA extract precisely after it has been extracted. PCR produces the highest quality in the shortest amount of time when the right amount of DNA is added. A profile that is difficult or impossible to read will be produced by adding varying amounts of DNA [31, 32]. The type of model determines how much DNA can be retrieved from a sample [33]. Table 1 displays the amount of DNA in various biological samples.

- DNA Amplification: Although there are eight methods based on DNA and RNA, PCR and reverse transcription-PCR have been the most widely used. The most popular technique for amplifying DNA is PCR. Certain DNA template sections are amplified by PCR; 30 rounds of amplification can even increase the size of a single molecule by a billion times [34-45]

- DNA Sequencing: DNA sequencing is the process of determining the exact order of nucleotide bases (A, T, C, G) in a DNA molecule. It serves as a fundamental tool in genomics, enabling researchers to decode genes, identify mutations, and understand biological functions at the molecular level. The ability to "read" DNA has revolutionized medicine, forensics, agriculture, and evolutionary biology [35].

Accuracy, efficiency and error mitigation

Accuracy in DNA analysis refers to the generation of correct DNA profile, which is affected by several factors such as platform used, sequence read length or errors during amplification or sequencing [36]. For example, BWA and Bowtie2 are commonly used for short-read alignment and have been benchmarked for their accuracy using Illumina-like datasets. BWA aln showed the highest accuracy, followed by BWA mem and Bowtie2 [36]. Efficiency of DNA analysis includes human speed, the types of sources in DNA or the types of kit we are using. Efficiency of PCR influences on amplification of DNA. For an instance, blood in EDTA vial takes around 1 or 1.5 hour to extract DNA whereas DNA extraction from cloth or tissue or swab etc., takes more than 3 hours.

Literature Background

Scientists working in forensics are always searching for new ways to enhance DNA typing. Data backlog problems can be resolved with the aid of more accurate and user-friendly data analysis software, more throughput, improved sensitivity, and automation. These enhancements will also make data interpretation easier and provide current protocols more resilience. This essay will examine a number of recent developments in DNA profiling technology and go over their benefits over the forensic DNA profiling techniques used today [40].

DNA analysis and its history

Scientists discovered that DNA molecules are extremely polymorphic in the middle of the 1980s. Specifically, DNA molecules have sections where a brief sequence is repeatedly replicated in tandem. Each person has a different number of these repetitions. In the end, a combination of several DNA segments with tandemly repeating sections enables strong individual discrimination. In 1985, Alec Jeffreys, an English scientist, introduced the forensic community and other human identification services to the analysis of Variable Number of Tandem Repeats (VNTR), which is based on Restriction Fragment Length Polymorphism (RFLP) [46, 47]. Following thorough validation studies, the FBI applied this DNA fingerprinting approach to forensic casework in 1988. Many identical tandemly repeated sequences are found in a typical VNTR region, which isa DNA fragment that is a few thousand bases long. Usually, the repetition unit size is between 8 and 80 bp. Slab gel electrophoresis is used to separate the VNTR fragments, which are then moved to a nylon membrane using the Southern blot technique and marked with radioactive or luminous probes for further detection. A different approach that makes use of shorter repeating sequences has replaced VNTR forensic investigation within the past ten years. The total length of Short Tandem Repeats (STRs), which are typically between 100 and 450 bp, is substantially shorter than that of VNTRs. Their repeat units are 2-7 bp long.

The STR approach, which is based on PCR technology, uses less template DNA - usually 1 ng. Because the segments of interest are shorter and have a higher likelihood of remaining intact than VNTR, it enables study of partially degraded DNA. The most frequent application of tetranucleotide repeat sequences is in forensics. Silver staining was used to first identify amplified STR fragments on a slab gel. Fluorescence detection, which is more practical and sensitive, took the place of this method. Since PCR may be performed in a single tube with distinct primers and fluorescent tags, multiple STR segments can be amplified at the same time. These pieces can be detected consecutively and simultaneously in a single lane thanks to multiplexing. Following slab-gel electrophoresis, STR fragments are examined offline using fluorescence scanners or with sequencers based on slab gel or capillary electrophoresis. The primary method for forensic DNA identification is still STR analysis. We will go into further detail about modern STR methods and forensic needs in the section that follows [40].

RFLP and VNTR

Restriction Fragment Length Polymorphism (RFLP) and Variable Number of Tandem Repeats (VNTR) were among the earliest DNA profiling techniques adopted in forensic science. These methods analyze differences in DNA sequences between individuals, enabling unique identification from biological evidence such as blood, hair, or saliva found at crime scenes. RFLP involves cutting DNA into fragments using restriction enzymes. The resulting fragment lengths vary based on individual genetic differences. These are separated by gel electrophoresis and visualized with labeled probes. VNTRs are regions of DNA where short sequences are repeated multiple times. The number of repeats varies among individuals, making these markers highly polymorphic and ideal for identity testing [41]. VNTRs are analyzed using RFLP or hybridization probes to produce banding patterns unique to individuals. This forms the basis of DNA fingerprinting in forensic investigations. VNTR patterns are inherited; hence, they are used to determine familial relationships. DNA extracted from forensic samples (nails, blood, hair, etc.) Is matched against known profiles to identify or exclude suspects. VNTRs and RFLP methods have been used historically for identifying fragmented remains when newer methods like STR were not available [42, 43].

Even through VNTR and RFLP methods offers a high accuracy result, still have a few limitations such as required large amount of high-quality DNA samples, these methods are very time consuming, need intense human labor and RFLP and VNTR are incompatible with degraded or trace samples. This limitation can easily be replaced by PCR-based techniques such as STR (Short Tandem Repeats) which is way faster, doesn`t require high quality DNA samples. RFLP and VNTR analysis laid the foundation of forensic DNA profiling, enabling individual identification with high specificity. Although largely replaced by faster, more sensitive technologies like STR, these techniques played a pivotal role in early forensic breakthroughs and remain historically significant in the evolution of DNA-based identification [44].

STRs, SNPs, Mitochondrial DNA

STRs: The process of separating male sperm cells from swabs that include a combination of male and female cells after a sexual assault is known as differential extraction. The significance of recording overall recovery was illustrated by a study on the efficacy of various extraction strategies [48]. Additionally, various techniques for differently lysing the cells have been investigated. Martinez et al. showed how to efficiently extract epithelial cells from a swab using an immunomagnetic cell capture step before isolating sperm cells using pressure cycling and alkaline lysis [49]. In order to retrieve sperm cells, cell capture techniques were also investigated. Using magnetic streptavidin-coated beads with bound ligands modified with biotin, Katilius *et al.,* created an affinity-based sperm purification technique.

These beads were used to bind the sperm cells while removing the epithelial DNA from the mixes of sperm and epithelial cells [50]. Differential extraction has also been done with microfluidics. Inci *et al.,* created a chip that separates sperm cells from epithelial cells by binding them with an oligosaccharide. Depending on concentration, between 70 and 90 percent of sperm cells were collected [51].

A set of short tandem repeats found in the DNA template are amplified using the Polymerase Chain Reaction (PCR) in Short Tandem Repeat (STR) typing. The DNA amplicons are then separated using Capillary Electrophoresis (CE). Additional loci that facilitate sex determination and enhance overlap with current international databases have been incorporated into a number of commercial assays. These consist of the Applied Biosystems globalfiler PCR amplification kit, the QIAGEN Investigator 24plex QS, and the Promega powerplex Fusion 6C System [52-54].

The DNA of the female victim may outweigh that of the male

attacker in cases like fingernail scrapings or sexual assault. Because the female victim's DNA will not interfere as much in these circumstances, using information from the Y chromosome can be crucial [31]. As a result, attempts to discover and expand Y chromosomal STR loci have been ongoing. A Chinese research team recently created and verified a typing technique that uses capillary electrophoresis with six dye chemistry to multiplex amplify 37 Y-strs. The intention was to strengthen the ability to discriminate against men [55].

SNPs: When the retrieved DNA is severely damaged, single nucleotide polymorphisms are especially helpful. Because snps are useful in phenotyping and ancestry research, the use of these markers is increasing [31]. Guidelines for conducting SNP typing snapshot assays were recently reviewed. Several useful mixture deconvolution tips were presented in the paper [56]. The use of SNPs to forecast phenotypic features has also been extensively studied. A kit created by Qiagen includes techniques for predicting the color of the skin, hair, and eyes. Since the advent of better sequencing and genotyping methods, the use of SNPs in sex chromosomes has expanded.

Since Y-SNPs can be utilized to distinguish between people and determine ancestry, their significance has been well-established [57]. In forensic casework, ancestry and lineage markers are especially crucial when working with unidentified offenders. Paternity testing and haplogroup designation were performed using the Ampliseq Identity Panel [58, 59]. To boost database size and discrimination power, Y-SNP variations have also been identified in particular populations. In a study of the Flemish population's Y-SNPs, the variant alleles were found in 270 male samples [60]. In order to achieve high resolution subtyping of the haplogroup r1b-DF27, which exhibits high frequencies in Iberian and Iberian-influenced groups, fifteen additional SNPs were created [61]. Likewise, studies on X-SNPs have been conducted. To investigate a range of XSNPs in various populations, two distinct study groups used the MALDI-TOF MS techniques on their samples. These two studies shared the goal of increasing population data in order to boost the technique's ability to discriminate [31].

Mitochondrial DNA; Different from nuclear DNA, Mitochondrial DNA (MtDNA) isa tiny, circular DNA molecule that is present in the mitochondria. MtDNA is maternally inherited, which means it is only passed down from mothers to their children, in contrast to nuclear DNA, which is inherited from both parents. Mtdna is a useful tool in forensic investigations because of this characteristic, as well as its high copy number and resilience to degradation, particularly when working with limited, old, or deteriorated biological materials [45].

The use of MtDNA can be advantageous for forensic samples like hair and bone. Since each cell contains hundreds of copies of MtDNA, the process is significantly more sensitive than autosomal DNA. Since male MtDNA is not transferred during fertilization, it can also be utilized for lineage research. However, because there is no sexual recombination, it is less probative than autosomal STRs and autosomal Single Nucleotide Polymorphisms (SNPs). Sequencing techniques are commonly used to examine MtDNA, although other methods, including snapshots, can also be used to probe MtDNA snps. For instance, to genotype a panel of 52 phylogentically informative MtSNPs, a snapshot process was created. The technique produced an effective haplogroup classification process and may be helpful in forensic analysis [62]. Strobl *et al.,*'s paper seems intriguing examined the MtDNA found in teeth, hairs, and bones that had previously been examined using massively parallel Sanger sequencing. The findings shown that samples held over years can yield whole genomic profiles [63].

ISO Standard-17025

With members from 162 nations, the ISO is an autonomous, non-governmental, global organization. It was established in 1947 with the goals of facilitating global cooperation and bringing industry standardization together. The creation of international standards is currently the responsibility of 784 technical committees and subcommittees [64]. Both the International Electrotechnical Commission (IEC) and the International Organization for Standardization (ISO) have published these guidelines [65]. General requirements for testing and calibration laboratories' proficiency are outlined in ISO/IEC 17025. The standard used in forensic labs is ISO/IEC 17025 [66].

The ISO/IEC 17025:1999 was divided as follows: [66]

1. Objective
2. Normative References
3. Terms and definitions
4. Management requirements
5. Technical requirements

In 2017, an updated version of ISO/IEC 17025 was released to bring it into compliance with other modern standards, such as ISO 9001 [66]. The updated version added competency, impartiality, and consistent laboratory operation standards in order to achieve this goal. 18 The structure of the new document differs from that of the previous edition, and it does not separate technical requirements from management requirements [66]. The following divisions apply to the 2017 version:

1. Scope
2. References to norms
3. Definitions and terminology
4. General specifications
5. Requirements for structure
6. Requirements for resources
7. Requirements for processes
8. Requirements for management systems

As previously stated, the 2017 publication of the revised ISO/IEC 17025 was necessary to bring the document up to date and bring it into compliance with the most recent iterations of other standards, such as ISO 9001. Therefore, it is possible to identify certain key distinctions between the upgraded version (2017) and the old version (2005) in this context. A body that does at least one of the three activities listed as "laboratory activities" testing, calibration, and sampling followed by testing or calibration is considered to be "laboratory," according to the definition provided in the 2017 edition (3.6). It's clear that sampling is portrayed as a lab exercise [67, 68].

ISO/IEC 17025 is the international standard that outlines the general requirements for the competence of testing and calibration laboratories. In forensic science, this standard is crucial to ensure that laboratories produce results that are technically valid, legally defensible, and globally recognized. Accreditation under ISO/IEC

Table 2: The domains which are mentioned in ISO 17025.

Category	Requirements
Technical Competence	Staff must be qualified, trained, and periodically evaluated for competency in techniques like DNA extraction, PCR, STR analysis, or mtDNA sequencing [69].
Method Validation	All DNA methods (e.g., STR, MtDNA) must be scientifically validated and fit-for-purpose before use.
Equipment Calibration	Instruments like thermocyclers, spectrophotometers, and sequencers must be calibrated and maintained per international standards.
Quality Assurance	Internal audits, proficiency testing, and peer review of results are required to maintain consistency and accuracy [70].
Chain of Custody	Sample handling must be meticulously documented to ensure evidence integrity throughout analysis.
Traceability	Reagents, reference standards, and measurements must be traceable to recognized national or international standards.
Risk Management	Labs must identify and mitigate risks to test validity (e.g., contamination, human error).

17025 is often mandatory for forensic labs submitting DNA evidence in court (Table 2).

Accreditation under ISO/IEC 17025 ensures that forensic laboratories operate with consistent procedures, skilled personnel, and validated technologies. For DNA and MtDNA analyses especially those submitted in legal proceedings such accreditation is essential to guarantee accuracy, reliability, and public trust [71].

Testing and calibration labs must be aware of the instruments to guarantee quality control and dependable results and implement them in their everyday operations since the measurement findings they provide are mostly used to support socially significant decision making. As demonstrated, the QMS suggested for ISO/IEC 17025 aids in the development of a quality assurance-based routine. Asa result, testing and calibration facilities need to implement quality control and metrological traceability, as well as be accountable for their actions and their effects [66].

Methodology Overview

The results of DNA analysis dependents on the all the methods which are involved in this process and these processes become the reason for misinterpretation of the results. Handling, collections and preservation are the main three tasks which in each step can lead to wrong and misinterpreted results.

Collection of evidence

On the crime scene, there are a few biological evidence that are commonly found which are blood, semen, saliva, teeth, bone, urine, soft tissue or hairs (with roots and shaft or without root) [72]. For each department, there are separate types of evidence (Table 3):

Biological evidences which are preserved on the basis of time of storage: short term storage – up to 72 hours and long-term storage-more than 72 hours [72].

Short term storage: up to 72 hours [72].

- Liquid blood is needed to be refrigerated where the temperature should between 2˚C-8˚C and between 15.5˚C-24˚C is acceptable until 24 hours.
- Urine is best at or below -10˚C and between 2˚C-8˚C is acceptable until 24 hours.
- Dry biological materials such as stained clothes are best between 15.5˚C-24˚C and acceptable on room temperature.
- Blood and stained items which cannot be dried are best at or below -10˚C, acceptable between 2˚C-8˚C
- and between 15.5˚C-24 ˚C is acceptable until 24 hours.
- Bones are acceptable at or below -10˚C, between 2˚C-8˚C and between 15.5˚C-24˚C until 24 hours.
- Hair between 15.5 ˚C-24 ˚C and acceptable on room temperature.
- Wet swabs are best between 2˚C-8˚C and Dry swabs are best between 15.5˚C-24˚C.
- Vaginal smears are best between 15.5˚C-24˚C.
- Feces are best at or below -10˚C.
- Buccal swabs are best between 15.5˚C-24˚C.

Long term storage: more than 72 hours [72].

- Liquid blood best between 2˚C-8˚C.
- Urine is best at or below -10˚C.
- Dry biological materials such as stained clothes are best between 15.5˚C-24˚C.
- Bones are best between 15.5˚C-24˚C.
- Hairs between 15.5˚C-24˚C are best and are acceptable on room temperature.
- Dry swabs are best between 15.5˚C-24˚C.
- Vaginal smears are best between 15.5˚C-24˚C.
- Feces are best at or below -10˚C.
- Buccal swabs are best between 15.5˚C-24˚C.
- Liquid DNA extracts are best at or below -10˚C and between 2˚C-8˚C are acceptable.
- Dried DNA extracts between 15.5˚C-24˚C are acceptable.

Biological evidence, whether direct or indirect, settles on the surface or target of transfer. Generally, liquid-state evidence is absorbed and solid-state evidence sticks. The method of collection is determined by the state and condition of the biological evidence [72] (Table 4).

Crime scene management is an important step for forensic experts and investigators. It can play a vital role in uncovering the crime, collecting evidence and getting the culprit punished during investigation and trial [72].

Case receiving

The case receiving process in a Forensic Science Laboratory (FSL) marks the formal entry of evidence into the forensic workflow. Upon arrival, the case is registered using details from the

Table 3: Handling physical evidences.

S. No.	Department	Physical evidence	Types of crime	Packaging of evidence
1.	Serology	Blood, saliva, other body fluids, skin tissue and more	Rape, murder, torture	Stains of biological fluids should be made and packed separately. Use sterile paper envelopes/cardboard boxes to pack the evidence.
2.	Biomedical	Skull, skeletal remains, skin tissue and more	Suicide and murder	Clean the skull/scalp remains and pack separately in dry condition. Pack the skin and tissue in natural condition in separate screw-cap sterile glass/plastic tubes and label them. Avoid using polythene bags.
3.	DNA	Blood, blood stains and swabs, slides and swabs (vaginal, urethral, cervical, vulval and semen), bones, tissues, fetus, hairs (with or without roots), pubic hairs, armpit hairs, teeth, saliva, skeletal remains, stained clothes, etc.	Rape, murder, paternity, swapping of newborn babies, identification of unknown bodies	Liquid blood is collected in EDTA vials and donor's name and date of collection is added; it is kept in an ice container. Stained clothes should be dried under shade and packed separately. Embryo/tissue should be preserved in 0.9% normal saline and sent to the laboratory. Other evidence can be packed in natural state. Avoid using polythene bags. Use sterile paper envelopes/cardboard boxes for keeping evidence.
4.	Biology	Hairs, fibers, semen, semen stains and slides and swabs taken during medical examination, diatoms, tobacco products, other plant material, pollen, leaves, wood, seeds, fruits, flowers, insects, flies, etc.	Rape, murder, suicide, fraud, drowning	The exhibits should be dried under shade and packed in natural state in screw-cap sterile glass/plastic tubes and labeled. Avoid using polythene bags.
5.	Forensic Physiology	Suspect, witness or complainant	Burglary, robbery, theft, arson, rape, murder, white-collar crime	Court consent, medical certificate, FIR, crime scene sketch or photos, etc.

Table 4: Collections and preservation methods of different sources of DNA.

S. No.	Evidence Stage		Location/Source	Collection and Preservation Methods
1	Blood	Liquid	Person	2-3 ml blood in EDTA vial in thermocol box with ice gel pack within 72 hours of collection. Always use disposable syringe for blood collection. Or collect it on FTA card.
		Liquid	Object/site	Collect into EDTA vial using a syringe. Transfer to a cotton cloth and dry in the shade. Or collect it on FTA card.
		Wet/Moist	Clothes	Dry in the shade at room temperature and pack in a paper envelope.
		Wet/Moist	Ice	Pick up as much blood-soaked ice as possible and put it in a plastic bag.
		Wet/Moist	Water	Collect the sample with a syringe, place it in a plastic container and freeze it or pack it in a thermos with ice.
		Clot	Scene of incident	After inserting the push into sterile tube, add an equal amount of normal saline into the tube. Transfer the clot to a cotton cloth and dry it in shade.
6	Dried Blood	Scab/spot	Small Object/Weapon	Collect and pack the entire exhibit.
		Scab/spot	Carpets/Curtains/Wood etc.	Cut out the pigmented portion and pack it in a paper envelope. Collect the sample that is not pigmented.
		Scab/spot	Stable Surface/Wall	Scrape the sample and collect it in a paper envelope. Dry it in the shade and pack it separately.
		Splatter	Stable Surface	Pick it up on the tape and collect it in a container.
10	Saliva	Liquid	Person	Collect it directly in container and refrigerate or keep in ice. Or collect it on FTA card.
		Liquid	Scene of incident	Transfer it to a test tube with the help of a syringe and refrigerate or keep in ice.
		Smear	Scene of incident	Collect the stained part. Collect the unstained part separately. Pack in paper envelope.
		Smear	Clothes/Item	Collect as it is.
14	Tissue	Fresh	Scene of incident	Store in a sterile container and refrigerate or keep in ice. Collect about 100 gm of brain tissue in a glass or plastic container containing 0.9% DNS (available in medical stores) and keep in ice or with cyanide (salt) preservative. Formalin should not be used.
15	Bone/Teeth	Fresh/Moist/Dry	Scene of incident	Dry it in air and pack it by wrapping it in paper or cotton cloth.
16	Hair	Tissue including hair	Scene of incident	Collect the hair along with the tissue and keep it in the refrigerator or in ice.
		Blood-Stained Hair	Scene of incident	Separate the hairs from the blood, dry it in shade and keep it in a paper/packet envelope.
		Whole Hair	Scene of incident	Pick it up with the help of clean tweezers and place it in a paper envelope/packet.
		Piece Hair	Scene of incident	Pick it up with the help of tape and pack it in a paper packet.
		Known Sample	Person	Comb at least 20 hairs including the roots.

17	Semen	Liquid	The Victim	Collect the sample by making a swab using sterile cotton/bandage/cotton cloth and after drying in shade pack it in a paper bag.
		Liquid	Object/Scene of incident	Collect the liquid solution in a tube and keep it in ice. Transfer it to a cotton cloth, dry it in shade and pack it in a paper envelope.
		Moist/Wet	Clothes	Dry it in shade and pack it in a paper envelope.
		Dry spot	Clothes	Pack separately.
			Carpets/Curtains/Wood etc.	Cut the blot and pack it. Pack the unstained specimen separately.
			Stable Surface	Pack the scraps in an envelope. Collect the uncolored ones too
23	Vaginal/Oral/Anal swabs	Smear	Victim	After completely drying the swab, pack it in a paper envelope or new clean glass bottle. Make a hole in the lid of the bottle in which you keep it and request the medical officer to make a swab.

accompanying requisition letter or FIR, and the condition of the evidence seal is carefully inspected and documented. Any broken seals or discrepancies are noted immediately. Once verified, the case is assigned a unique ID and logged into the chain of custody record, ensuring that every movement is traceable. The evidence is then securely stored and digitally tracked, often through a Laboratory Information Management System (LIMS), before being assigned to the relevant department or analyst. This process ensures legal accountability, prevents contamination, and maintains the integrity of the evidence from receipt to final reporting.

Case opening

Case opening is a time taking process which include the checking if all the sample mentioned are available to the laboratory, next align them in sequence which is mentioned in the case file and make a digital record of each of the sample and evidence by clicking photos of them and having them printed and punched into the case file. These evidences are needed to be written in detail such as the color of envelope the evidence was in and number of samples in an envelope, color of clothes if there is any etc. And at last blood are kept in refrigerator so it would be preserved better.

DNA extraction

The process of separating DNA from proteins and other cellular material, ideally in enough amount and purity for use in later processes, is known as DNA extraction [73]. Friedrich Miescher carried out the first DNA extraction in 1869 while researching the biological function of leucocytes [74]. Since then, researchers have developed a number of extraction techniques that are simpler, more affordable, dependable, quicker, and yield more. The three steps of a typical DNA extraction procedure today are (i) lysis of the cell membrane to liberate DNA into the lysis solution, (ii) protein denaturation and/or enzymatic digestion to release DNA from nucleoproteins, and (iii) DNA separation from inhibitors and other cellular components. DNA can be extracted using a variety of techniques, but there isn't a single optimum technique that works for the wide range of substrates that biological evidence can appear on [73]. The biological sample in question frequently determines the best approach. For instance, DNA extraction from forensic crime scene samples must be successful in eliminating a wide range of environmental pollutants and inhibitors, such as tannins, humic acid, and denim colors, that impede subsequent PCR operations. However, for reference blood or buccal samples, the DNA extraction procedure is simpler and can even be omitted completely to save time [75, 76]. The need for trustworthy and effective DNA isolation techniques that can produce sufficient amounts of high-quality DNA with few contaminants has increased with the development of gene editing and customized medicine. Table 5 lists several different extraction techniques.

Quantification (RT-PCR/QC RT-PCR) and PCR amplification

Thermal cycling is used in qPCR, and sample cooling usually limits its pace, which decreases as sample volumes rise. For a number of years, Cetus Corporation's (later sold to Chiron Corporation; CA, USA) patent protection restricted the commercial use of PCR. By using novel nucleic acid amplification techniques, researchers attempted to get around cooling issues and circumvent patent protection. Since there is no cooling procedure needed, isothermal amplification solves the cooling issues. Isothermal amplification was used to develop a number of methods, including the now-popular recombinase polymerase amplification [89] and Loop-mediated isothermal Amplification (LAMP) [88]. Compared to rapid PCR, isothermal nucleic acid amplification has the advantage of a simpler device design and reduced power consumption, and it doesn't require temperature cycling [90].

Using the S' nuclease assay and real-time detection, a novel method for quantitative reverse transcriptase polymerase chain reaction (QC RT-PCR) has been created. One quick and effective method for amplifying DNA *in vitro* is the Polymerase Chain Reaction (PCR) [91, 92]. Small amounts of DNA have been quantified using quantitative PCR (QC PCR), and mRNA has been measured using QC RTPCR. The internal control used in QC PCR and QC RT-PCR is coamplified with the target sequence [93-98].

Gel electrophoresis must be used to separate each sample for RT-PCR amplifications. The PCR products must then be isotopically labeled or stained with ethidium bromide to quantify the bands. Fluorescence hybridization probes and a sequence detector are used in the RT-PCR process. Because target mRNA and internal control RNA are introduced to the same tube, this method reduces the possibility of inconsistent results due to possible variations in reverse transcription and amplification efficiency. Both the internal control RNA and the target mRNA's amplification efficiency will be impacted by any amplification inhibitors that are present in the sample.

Additionally, both target mRNA and internal control RNA are transcribed and amplified using a same set of primers; nevertheless, distinct hybridization probes are employed to further guarantee the assay's correctness. Two separate reactions were made, one with the internal control RNA probe and the other with the target mRNA probe. This method was applied to improve the assay's dynamic range. The assay is sensitive and specific since a signal can only be

Table 5: Various methodological approaches.

S. No.	Methods of Extraction	General Steps	Advantages	Disadvantages
1	Organic [77]	1. Cell lysis with enzyme and detergent. 2. Extraction with phenol-chloroform (DNA in aqueous layer). 3. Alcohol and salt precipitation of DNA pellet. 4. Rehydration of DNA.	1. Considered gold standard in DNA extraction. 2. Accommodate wide range of sample and substrate types. 3. No upper limit to DNA yield. 4. Generally effective removal of inhibitors.	1. Use of toxic volatile reagents. 2. Labour-intensive with multiple tube-to-tube transfers. 3. Increased risk of contamination. 4. Lengthy procedure.
	Differential extraction with DTT [77, 78]	1. Selective digestion of non-sperm cells in the absence of DTT. 2. Separate intact sperm from digested cellular material. 3. Organic extraction of sperm, with DTT.	1. Enrichment of spermic DNA. 2. Adaptable to automation.	1. Same limitations as the organic solvent extraction method. 2. Enrichment, rather than segregation of sperm from other cell types.
	Chelex resins [79, 80]	1. Cells lysed through boiling in 5% Chelex mixture. 2. Polyvalent ions are sequestered by Chelex resins. 3. Lysate can be used for PCR after…	1. Rapid and simple procedure. 2. Single-tube extraction, thus reducing risk of contaminations. 3. Not labour-intensive. 4. Adaptable to automation.	1. Does not remove contaminants e.g. resins and hematin effectively. 2. Extracted DNA may not be stable in Chelex for long-term storage.
	FTA paper [76, 81, 82]	Centrifugation.	1. Rapid and simple procedure. 2. Inactivates bacteria and virus. 3. Provides long-term compact dry storage of sample at room temperature. 4. Adaptable to automation.	—
2	Paramagnetic DNA capturing matrix [83-88]	1. Cell lysis with enzyme and detergent. 2. Affinity binding of DNA to matrix. 3. Washing of DNA-bound matrix. 4. Release of DNA in a low salt buffer with pH > 7.5.	1. Rapid procedure. 2. Generally able to remove most contaminants. 3. Adaptable to automation.	1. DNA yield limited by amount of matrix used. 2. Silica-binding inhibitors reduce DNA yield.

produced by the specific hybridization of primers and probe.

By employing an exon/intron junction spanning forward primer, where the *5′* end of the primer is complementary to the *3′* end sequences of one exon and the *3′* end of the primer is complementary to the *5′* sequences of the subsequent exon, amplification of the DNA contained in the total RNA preparation is prevented [99].

qPCR involves the following steps:

- Sample preparation by extraction of DNA.
- Reaction setup has many components such as DNA template (cDNA), forward and reverse primers, DNA polymerase (mostly Taq), dNTPs, buffer with MgCl2, fluorescent dye (e.g., SYBR Green) or probe (e.g., TaqMan).
- Thermal Cycling (also called amplification) this step involves three sub-steps: Denaturation which occurs at ~95˚C separate double-stranded DNA, Annealing which occurs at ~50˚C-60˚C DNA binds to primer and Extension which occurs at ~72˚C synthesize new strand by the help of DNA polymerase.
- Data Acquisition in which fluorescence signal increase with each cycle and is plotted as a real-time amplification curve.
- Quantification & Analysis includes relative, absolutes quantification and melt curve analysis (if SYBR Green is used).

RT-PCR combines two processes:

1. Reverse Transcription: Converts RNA into complementary DNA (cDNA)

2. PCR Amplification: Amplifies the cDNA to detectable levels

RNA Extraction: Isolate high-quality total RNA from the sample (e.g., cells, tissues, swabs) ensure the RNA is free from DNase and RNase contamination.

- Reverse Transcription (RT): Convert RNA into cDNA using Reverse Transcriptase enzyme, Primers (options: oligo-dT, random hexamers, or gene-specific primers), dNTPs (deoxynucleotides), Buffer with $MgCl_2$. This reaction usually occurs at 37-55˚C for 15-60 minutes.
- cDNA Amplification by PCR: The cDNA serves as the template for PCR, which proceeds through standard thermal cycling and repeated for 30-40 cycles (Table 6).

A basic method for amplifying particular areas of Deoxyribonucleic Acid (DNA) through an enzyme reaction is the Polymerase Chain Reaction (PCR). Deoxynucleotide triphosphates (dNTPs), thermostable DNA polymerase, template DNA, primers, and a buffer containing potassium and magnesium are the five essential ingredients needed for PCR amplification [100-102]. The sequence-specific primers in the reaction and the strict cycling conditions used are primarily responsible for the great specificity of PCR.

The three fundamental steps of denaturation are the same in PCR programs: heating to separate the DNA molecule's double strands; annealing to enable primers to attach to their complementary target sequence; and extension, in which the sample is heated to a temperature marginally higher than annealing, which is ideal for DNA polymerase to create the new double stranded molecule [101-105]. Until enough target DNA is generated to be detectable, these three procedures are repeated.

An entirely new family of devices, including flow-through miniaturized and rapid PCR, was developed as a result of the use of microfluidic systems. The very early cyclers used a space- domain technology. This issue was swiftly taken on by silicon micromachining researchers, who created portable, miniature end point PCR and later qPCR instruments [106].

Table 6: Amplification of DNA in PCR.

Step	Temperature	Duration	Purpose
Initial Denaturation	94-95°C	2-5 min	Unwinds DNA strands
Denaturation	94-95°C	15-30 sec	Separates DNA
Annealing	50-65°C	20-40 sec	Primers bind to target
Extension	72°C	30 sec-1 min	DNA polymerase synthesizes new strand

In accredited forensic laboratories,the DNA present in a sample must be quantified before it progresses to STR PCR for DNA profiling. The dynamics of quantitative PCR (qPCR) are substantially different to those of an STR PCR. Commercially available qPCR kits, such as QuantiFiler Trio (Thermo Fisher Scientific, Waltham, MA, USA) or Investigator Quantiplex Pro (QIAGEN, Hilden, Germany), use TaqManR probes [107, 108]. Two fluorescent dyes are used to mark these probes: a quencher dye at the *3′* end and a reporter dye at the *5′* end [109, 110]. Because the probe is tiny, the two dyes are near together when it is intact (before polymerization). The quencher dye's proximity to the reporter dye results in an energy transfer between the two, which suppresses the reporter dye's fluorescence. The inverse sixth power of the intermolecular spacing of the two dyes determines the efficiency of fluorescence Resonance Energy Transfer (FRET), which is the basis for this energy transfer [110, 111].

During the annealing phase of a qPCR program, the probes attach to the target sequence and the reporter dye fluorescence is inhibited. The reporter dye at the *5′*-end is separated from the quencher dye at the *3′*-end during the extension step, however, since the bound TaqMan probes are displaced by the *5′*-exonuclease activity of DNA polymerase [109-111]. The reporter molecule can now fluoresce because of the separation, which eliminates the energy transfer that inhibited fluorescence when the two fluorophores were close to one another [109-111]. Since more reporter dye molecules are cleaved and more fluorescence is measured when there is more DNA present in the reaction, the amount of fluorescence detected and the amount of DNA present in the reaction are directly correlated. Usually, an amplification curve is created using these fluorescence data as the qPCR procedure goes on and the DNA increases exponentially. These data are then used to determine the DNA concentration at the conclusion of the qPCR session. A sample can proceed to STR PCR and produce a DNA profile if it is determined that it has enough DNA to yield valuable genetic information [112].

Rapid PCR is the third PCR procedure that is frequently employed in forensic science. Rapid PCR use the same fluorescent primer chemistry as STR PCR, but it can produce DNA profiles considerably more quickly thanks to specific tools, PCR software, and reaction chemistries. Rapid PCR procedures use condensed PCR scripts to generate DNA profiles rapidly, but often omit the DNA quantification step. This approach makes it possible to swiftly construct relevant DNA profiles, but samples must have a significant amount of high-quality template DNA, like that found in reference swabs, in order to accomplish this [113, 114].

Capillary Electrophoresis/Sequencing

DNA sequencing has transformed forensic science, enabling the identification of individuals with high precision using genetic material from crime scenes. This paper explores the evolution of sequencing technologies from Capillary Electrophoresis (CE) to current Next-Generation Sequencing (NGS) platforms and their forensic applications. We also examine advancements in sensitivity, speed, and sample handling that are driving the future of forensic genomics [115]. DNA sequencing in forensic science plays a critical role in criminal investigations, missing persons cases, and disaster victim identification. Early sequencing techniques based on Sanger's method and capillary electrophoresis laid the groundwork for today's Next-Generation Sequencing (NGS) approaches, which allow massively parallel analysis of genetic markers [115].

The earliest automation of DNA sequencing involved fluorescently labeled Sanger sequencing, followed by separation *via* capillary electrophoresis. CE offered rapid, high-resolution separations, read lengths up to 1000 bases under optimal conditions and compatibility with Short Tandem Repeat (STR) profiling, the forensic gold standard. The development of high-speed, four-color CE instruments improved sequencing throughput, while optimization of polymers and temperature control improved read-length accuracy [116].

However, CE required multiple PCR amplifications, individual STR locus targeting and labor-intensive sample prep, limiting scalability. NGS technologies particularly Illumina, Ion Torrent, and Oxford Nanopore, are now being adopted for forensic applications due to their ability to sequence multiple markers in parallel (e.g., STRs, SNPs, MtDNA), degraded or low-template DNA and ancestry- and phenotype-informative loci. Recent platforms like Illumina MiSeq FGx are specifically designed for forensic use which providing Integrated workflows, Automated library prep and Kits validated per ISO 18385 and ISO/IEC 17025 standards [117].

Advantages of modern sequencing includes NGS detects minor contributors in DNA mixtures, enhancing discriminatory power [118], massive parallel sequencing reduces costs and time per case, whole MtDNA sequencing is now routine, especially in cases involving aged remains [119]. NGS data interpretation requires bioinformatics expertise and forensically curated databases. Forensic labs must be accredited to ensure method validation, contamination control and reproducibility of DNA results [120] (Table 7).

From capillary electrophoresis to next-gen platforms, DNA sequencing has reshaped forensic science. While CE remains integral for STR profiling, NGS is the future, offering unprecedented sensitivity, scope, and scalability. Continuous innovation in sample preparation, data analysis, and regulatory frameworks will define the next era of forensic genomics.

Bottlenecks in Forensic DNA Analysis

DNA analysis is a cutting-edge, innovative tool in the field of forensic science that is crucial to crime investigation. There are several steps involved in DNA analysis, which we covered previously in the study. Even though this technology is so strong, there are still obstacles and limitations. The purpose of this study is to clarify the complexities and challenges of DNA analysis, with an emphasis on sample quality, data analysis, and interpretation all of which are essential for precise and trustworthy results [121, 122].

Table 7: Application of genotyping.

Application	Technology	Description
STR Typing	CE, NGS	CE is standard; NGS allows multi-locus analysis in one run
MtDNA Sequencing	Sanger, NGS	Useful in degraded samples; NGS enables full MtGenome analysis
SNP Panels	NGS	For ancestry inference, phenotyping, and lineage
Y-STR and X-STR	CE, NGS	Helpful in sexual assault cases and kinship testing

The foundation of DNA analysis is sample quality. The accuracy of the outcome depends on the integrity of the DNA sample. Numerous factors provide difficulties, including contamination, environmental deterioration, and the difficulty of managing limited or mixed DNA samples. These problems might produce unclear or inaccurate results, which can make forensic investigations very difficult. Another area with many difficulties is the analysis of DNA data. Complex genetic data interpretation calls for advanced software and analytical abilities. However, human mistake and the limits of current technologies can cause data to be misinterpreted, which could have major ethical and legal repercussions.

Additionally, interpreting the results of a DNA study isa sensitive procedure that is frequently impacted by privacy concerns, ethical considerations, and the possibility of misuse. Interpretations must be accurate and trustworthy because they might have significant ramifications for both personal and legal decisions [123, 124].

Each step of DNA analysis has enormous chances of contamination in DNA which lead to misinterpreted result in DNA sequencing. Addressing these challenges is crucial for the continued success and reliability of DNA analysis [9].

Sample contamination and switch

Sample contamination is one of the biggest problems with DNA analysis. From collection to analysis, contamination can happen at any point and come from a variety of sources, including handling, the environment, and cross-contamination from other samples. This can lead to misleading results, where DNA profiles may be incorrectly attributed or excluded [9]. The quantity of DNA available is often limited, especially in forensic cases where samples might be sourced from minute biological traces like hair follicles or skin cells. Low-quantity samples are challenging to analyze and are more prone to contamination and errors in DNA amplification. Furthermore, the success of DNA analysis depends critically on the quality of the DNA, which is influenced by variables such as the sample's age and exposure to environmental conditions [9]. It can be difficult to distinguish individual profiles in forensic instances where samples contain DNA from several people. This is most prevalent when there have been several people touching an object or when there has been sexual assault. Complex analysis and interpretation are necessary for mixed samples, which raises the possibility of misunderstandings [9].

Complex genetic data produced by DNA analysis can be difficult to understand. The intricacy of the analysis is increased by the existence of many alleles, differences in genetic markers, and the complexities of human genetics. When dealing with mixed DNA samples, this intricacy is increased even more, making it difficult to identify individual contributors [9]. Subjectivity is frequently present in DNA analysis, especially when interpreting difficult or subpar material. The same data collection may lead to disparate findings from different analysts, which could result in discrepancies. This subjectivity may be affected by things like training, experience, or the particular techniques used by various labs [9].

Low-Template and degraded DNA

Environmental elements including heat, moisture, and sunlight can cause DNA samples to degrade. Partial profiles are frequently produced by degraded DNA, which makes analysis difficult and may jeopardize the accuracy of the findings. When samples are badly damaged, it is very difficult to extract a comprehensive DNA profile, which reduces the sample's usefulness for forensic examination [9].

The analysis of Low-Template (LT-DNA) and degraded DNA poses significant technical, interpretative, and procedural hurdles. These challenges arise mainly due to the physical fragmentation, chemical modification, and scarcity of DNA templates, often resulting from age, environmental exposure, or suboptimal storage. Genetic analysis of low-template and degraded DNA, commonly arising from aged, environmentally exposed biological material, presents significant challenges. Samples may contain less than 0.1 ng of amplifiable DNA, resulting in stochastic PCR effects (e.g., allele dropout, dropin, peak imbalance, stutters) that hinder reliable STR genotyping [125].

Exposure to heat, UV radiation, humidity, microbial activity, and prolonged environmental exposure degrades DNA via strand breaks, depurination, and chemical modifications. Over time, fragment lengths drop below ~200 bp, minimizing success of standard STR amplification [126].

Over time or under environmental stress (e.g., heat, UV light, microbialdegradation), DNA undergoes strand breakage, depurination, and oxidative damage, resulting in small fragments - often under 200 base pairs (bp). Most traditional genotyping systems (like STRs) require intact DNA of 100-450 bp. When DNA is fragmented larger STR loci fail to amplify, leading to incomplete profiles, amplification is biased toward shorter loci, reducing profile balance and resolution [125] and forensic identification power drops as fewer loci can be reliably detected.

Low-template conditions - typically defined as <100 pg of DNA (i.e., <17 genome copies):

- Allelic Dropout is one allele at a heterozygous locus fails to amplify, making it appear homozygous.
- Allelic Drop-in is spurious alleles appear due to contamination or mis-priming.
- Peak Imbalance is heterozygous alleles show highly unequal peak heights in capillary electrophoresis.
- Elevated Stutter Peaks is minor peaks mimicking true alleles complicate interpretation.

These stochastic errors reduce confidence in matching profiles and complicate mixture interpretation in forensic contexts [127, 128].

Degraded samples are often collected from non-sterile

environments (crime scenes, burial sites, disaster zones), making them highly susceptible to contaminant DNA, which may compete during amplification, PCR inhibitors like humic acid, calcium, or heme from degraded tissues that suppress DNA polymerase activity and bacterial and fungal DNA, which can dominate sequencing reads, especially in metagenomic contexts. This complicates accurate human DNA profiling and reduces overall amplification efficiency. Nuclear DNA is often present in very low copy numbers or entirely degraded. In such cases standard STR or whole-genome sequencing becomes infeasible and analysts must shift to alternative targets (e.g., mitochondrial DNA, SNPs), which are less informative for individual identification [128].

Due to the unpredictable nature of low-template samples, replicates of the same sample can produce inconsistent profiles, especially with stochasticdropout. There is higher dependence on analyst discretion, increasing subjectivity. Interpretation of mixtures (e.g., multiple contributors) becomes difficult, especially if contributors are at low proportions. This raises legal and scientific concerns regarding evidentiary reliability, particularly in forensic contexts [129]. Before genotyping, laboratories typically assess DNA quantity and integrity using qPCR or fluorometric methods. In degraded samples quantification becomes inaccurate or undetectable due to DNA fragmentation and integrity metrics (e.g., degradation index) often fail to predict amplification success, especially if inhibitors are present. This affects downstream decisions on assay selection and interpretation thresholds.

Most standard forensic and biomedical assays are optimized for high-quality, high-quantity DNA. With degraded/low-template DNA STR multiplexes exhibit locus dropouts, SNP assays may require redesign of primers for ultrashort amplicons and MtDNA sequencing, while more robust, offers lower discriminatory power and is maternally inherited, limiting individual specificity. Traditional STR assays produce 100-450 bp amplicons, which often fail when DNA fragmentation is severe. Low-template conditions exacerbate stochastic variation and partial or null profiles. Allelic dropout and imbalance become frequent, eroding match probabilities and forensic discrimination power [125-129].

PCR inhibition

Although there are eight methods based on DNA and RNA, PCR and reverse transcription-PCR have been the most widely used. The most popular technique for amplifying DNA is PCR. PCR increases specific portions of the DNA template; 30 cycles of amplification can increase the size of a single molecule by 1 billion times [130]. PCR cycles typically range from 28 to 32, however they can reach 34 cycles if the amount of DNA is very low [131]. The three steps of the cycling phase - denaturation, annealing, and extraction - are when DNA amplification takes place.

Here are some additional techniques: [132]

- The amplification technique based on nucleic acid sequencing.
- Amplification of strand displacement.
- Amplification by recombinase polymerase.
- Amplification depending on strand invasion.
- Amplification of many displacements.
- Chain reaction of hybridization

PCR (Polymerase Chain Reaction) is the backbone of most DNA analysis workflows, from forensic genotyping to disease diagnostics. However, the presence of PCR inhibitors in biological or environmental samples is a significant barrier to successful amplification. These inhibitors interfere with enzyme activity, DNA denaturation, or primer annealing, leading to false negatives, reduced sensitivity, or complete PCR failure. Especially for restricted or damaged materials, the advent of this sophisticated DNA recovery and amplification methods has revolutionized the field. DNA analysis has been transformed by methods such as Polymerase Chain Reaction (PCR), which allow for the amplification of minute amounts of DNA. Specialized PCR techniques, including extended STR analysis, have been developed for degraded samples to effectively assess even severely fragmented DNA [9].

From DNA extraction to analysis, mistakes can happen at any point during the data processing process. Results may be erroneous or deceptive due to contamination, sample mis-labeling, or technical problems with lab apparatus. Furthermore, mistakes or artifacts may occasionally be introduced during the PCR amplification process, making the analysis more difficult [9].

PCR inhibitors originate from a variety of biological and environmental sources, making them difficult to predict or avoid endogenous inhibitors (from within the sample such as hemoglobin in blood: chelates Mg^{2+} and interacts with DNA polymerase [133], melanin in hair or skin: binds to DNA and inhibits polymerase activity [134], calcium ions, urea, and degraded tissue components (e.g., from bones, feces) [135]) and exogenous inhibitors (from collection/storage materials or environments which are indigo dye from denim and clothing fibers [136], humic substances in soil (e.g., humic acid from peat), textile dyes, detergents, preservatives, fixatives(e.g., doctors and medical professionals are requested to not use formalin to keep fetus or tissue rather use normal saline)). PCR inhibitors can affect reactions in multiple ways as Mg^{2+} ions are essential cofactors for DNA polymerase activity and disruption in its level can causes a huge impact on PCR, direct binding or denaturation of Taq polymerase render its inactive, blocking primer-template annealing reduce amplification efficiency and DNA co-precipitation during extraction causes poor elution and loss of target DNA. Because different inhibitors act *via* different mechanisms, a single mitigation strategy (e.g., dilution) may not be universally effective [136, 137].

The most critical consequence of PCR inhibition is the production of false-negative results especially in low-template or degraded DNA cases where samples with sufficient DNA may yield no signal due to complete inhibition, stochastic amplification in partially inhibited reactions can result in allelic dropout, misinterpretation, or loss of critical loci and Internal Positive Controls (IPCs), if not included, may fail to indicate that inhibition occurred, leading to misinterpretation as sample failure [137, 138].

This is problematic in high-stakes applications like forensic evidence or clinical diagnostics. Inhibitory effects may mimic low DNA yield - both result in weak or absent PCR products, without internal PCR controls (amplifying a known target alongside the test sample), inhibition can remain undetected and even qPCR assays that include IPCs may not accurately reflect partial inhibition affecting only certain loci or target regions. Hence, many labs may report “no DNA detected” when in fact PCR was inhibited [138].

Enzyme selection becomes a bottleneck in assay standardization

and validation as not all DNA polymerases respond equally to inhibitors. Taq polymerase is highly sensitive to hemoglobin, humic acid, and melanin however some engineered enzymes (e.g., HotStarTaq, Phusion, Platinum Taq) show better inhibitor tolerance, but are not universally adopted due to cost or validation limitations [136-139].

Data interpretation errors

The use of Short Tandem Repeats (STRs) has revolutionized forensic DNA analysis due to their high polymorphism, reproducibility, and statistical power in individual identification. However, one of the most persistent challenges in forensic genetics is the interpretation of STR mixtures, which are DNA profiles derived from two or more individuals. Such mixtures are common in forensic casework - especially in sexual assault investigations, touch DNA samples, or mixed biological evidence - and they present complex interpretational challenges. Misinterpretation of these mixtures can result in false inclusions, false exclusions, or ambiguous reporting, thereby impacting judicial outcomes. One of the primary sources of error in STR mixture interpretation is allelic dropout and drop-in. Allelic dropout refers to the failure of one or more alleles to amplify during PCR, typically due to low DNA quantity or degradation. In a mixture, dropout from a minor contributor can cause the mixture to resemble a single-source profile or obscure critical allelic diversity. Allelic drop-in, on the other hand, is the appearance of a spurious allele, often due to contamination or stochastic effects. Both phenomena can mislead analysts into over- or underestimating the number of contributors, especially in low-template DNA [140, 141].

Another major challenge is the interpretation of stutter peaks, which are minor byproducts of the PCR process. These peaks are typically one repeat unit shorter than the actual allele and can be misinterpreted as real alleles from a secondary contributor in a mixture. This is particularly problematic when the stutter peaks coincide with alleles expected from a minor contributor or in highly imbalanced mixtures. Proper stutter threshold calibration is essential to reduce the risk of misinterpreting these artifacts [142]. Peak height imbalance also contributes to interpretational difficulties. In a two-person mixture, one individual may contribute significantly more DNA than the other, resulting in a major/minor profile imbalance. When minor contributor alleles fall below analytical or stochastic thresholds, they may be entirely missed or partially detected, leading to incomplete or misleading interpretations. Analysts must carefully consider the possibility of peak height variation due to differential amplification, degradation, or inhibition, all of which are amplified in complex mixtures [143].

Determining the number of contributors to a DNA mixture is itself a source of uncertainty. Overestimating contributors can inflate the probability of inclusion, while underestimating can mask the presence of legitimate contributors. Studies have shown that when the number of contributors exceeds three, distinguishing individual genotypes becomes increasingly unreliable without probabilistic modeling tools [144]. Even experienced analysts may reach different conclusions when interpreting complex mixtures, especially when constrained by binary (included/excluded) interpretations. Traditional manual interpretation methods are increasingly being supplemented or replaced by Probabilistic Genotyping (PG) systems, such as STRmix, TrueAllele, and Lab Retriever. These systems model DNA mixture data using likelihood ratios and Bayesian inference to quantify the probability of inclusion or exclusion. While PG tools reduce subjectivity, they introduce new complexities related to transparency, validation, and error propagation, especially when the defense must understand or challenge statistical models in court [145].

Overall, the risk of human error, bias, and analytical ambiguity in STR mixture interpretation underscores the need for rigorous standards, transparent reporting, and continual validation of both manual and computational methods. Analysts must also account for laboratory limitations, environmental factors affecting DNA integrity, and biological variability across contributors. Training, proficiency testing, and the use of blind samples are essential to maintaining reliability in forensic interpretation.

Proposed Workflow Optimization

For DNA fingerprinting to be reliable and used ethically, findings interpretation must be improved. There are a number of ways to deal with the difficulties in interpretation [9]. Variability and bias in DNA analysis can be greatly decreased by creating and following standardized interpretation processes. International forensic science organizations created these procedures, which offer instructions on how to handle complicated DNA profiles and guarantee accurate and consistent interpretations in various labs and jurisdictions [3, 9]. Complex DNA profiles can be easier to interpret with the use of more sophisticated statistical methods. These technologies can help better informed decision-making by offering more precise insights into the likelihood of matches, particularly when partial or mixed profiles are involved [9].

Extended-STRs, SNPs, MtDNA, enhanced extraction buffers

In recent years, scientific advancements have introduced a range of alternative and complementary tools including extended STRs, Single Nucleotide Polymorphisms (SNPs), Mitochondrial DNA (MtDNA) analysis, and enhanced extraction buffers, to improve sensitivity, discrimination, and reliability in forensic casework [146].

Traditional STR kits typically target 15-20 loci, but extended STR kits now include 24 or more loci to enhance discrimination power and mixture resolution. Extended STRs improve the probability of obtaining informative profiles from complex mixtures and partially degraded DNA. This is particularly beneficial in cases involving low-template DNA, kinship testing, or database comparisons where increased allele diversity strengthens statistical confidence [147]. The inclusion of global loci like SE33 and D22S1045 has further broadened the utility of extended STR panels in international casework. SNPs are single base changes in the genome and are highly stable markers with low mutation rates. They are especially valuable in challenging forensic samples, such as highly degraded remains or minute DNA quantities. Unlike STRs, SNP markers require shorter amplicons (as little as 50-80 bp), making them ideal for ancient or environmentally exposed samples [148]. Moreover, panels of ancestry-informative SNPs (aiSNPs) and phenotype-informative SNPs (piSNPs) are increasingly being used to infer bio-geographic ancestry and externally visible traits, aiding investigative leads when no suspect is available [149].

Unlike nuclear DNA, mitochondrial DNA is maternally inherited and present in hundreds to thousands of copies per cell, making it especially valuable when nuclear DNA is limited or degraded. MtDNA analysis is widely used in forensic anthropology, disaster victim identification, and historical remains. The hypervariable

regions (HVR I and II) within the control region of MtDNA allow for maternal lineage tracing, and full mitogenome sequencing now provides even greater discriminatory power [150]. However, MtDNA has limitations in individualization due to its lower variability and maternal inheritance, but it remains an essential tool when STRs fail.

The development of enhanced DNA extraction buffers has significantly improved the recovery of DNA from challenging substrates, including bone, hair shafts, and environmental swabs. These buffers often contain detergents, proteinases, chelating agents, and other components that help to lyse cells, release DNA, and remove inhibitors. For instance, buffers enriched with Dithiothreitol (DTT) are used to break down keratin-rich tissues like hair, while EDTA-containing buffers are effective in bone demineralization [151]. Additionally, commercial kits such as QIAamp DNA Investigator and PrepFiler BTA are optimized for low-template and degraded forensic samples, enhancing both yield and purity.

As forensic science continues to evolve, alternative and complementary DNA analysis strategies are essential for overcoming limitations in conventional STR-based profiling. Extended STRs, SNPs, MtDNA analysis, and enhanced extraction techniques collectively provide robust solutions for handling degraded, low-template, and complex forensic samples. Together, these advancements expand the capability of forensic laboratories to extract meaningful genetic evidence even from the most challenging casework.

Combining automated extraction + qPCR with extended -STR

Human subjectivity and mistake can be reduced in DNA analysis by incorporating automation and machine learning. Accuracy can be increased by using automated systems to consistently apply predetermined criteria for interpreting DNA profiles. Large databases of DNA profiles can be used to train machine learning algorithms, which can help find trends and make probabilistic decisions that would be difficult for human analysts [9].

The reliability and sensitivity of forensic DNA analysis depend heavily on the quality and quantity of DNA extracted from evidentiary samples. In modern forensic laboratories, automated DNA extraction systems, quantitative PCR (qPCR), and extended Short Tandem Repeat (STR) typing are increasingly used in combination to optimize forensic workflows. These integrated methodologies significantly enhance the ability to process complex or compromised samples, improve reproducibility, and ensure quality control.

Automated extraction platforms from Qiagen (e.g., QIAcube, QIAamp DNA Investigator Kits) and HiMedia (e.g., InstaGene™ Forensic Kit) are widely used for consistent and contamination-free DNA isolation. These systems reduce human error, improve sample throughput, and standardize protocols across various sample types such as bone, blood stains, touch DNA, and buccal swabs. Qiagen kits, for instance, use silica-membrane technology optimized for trace and degraded DNA, while HiMedia offers buffers compatible with a wide range of downstream applications, including PCR and STR analysis [152, 153].

Following extraction, qPCR quantification ensures that the DNA is amplifiable and free of inhibitors. Commonly used qPCR kits include VeriFiler™ Express, VeriFiler™ Plus, and Y-Filer™, which not only quantify total and male DNA but also evaluate degradation indices and inhibition levels. InGenoMix qPCR kits, available in some regions, provide additional flexibility for quantifying low-copy DNA from complex or environmental samples. Internal Positive Controls (IPC) within these kits serve as built-in quality assurance, indicating whether substances like hemoglobin, humic acid, or dyes are interfering with amplification [154].

Once DNA quantity and quality are confirmed, extended STR typing is performed using kits such as GlobalFiler™, PowerPlex® Fusion 6C, or VeriFiler™ Plus, which target 21-27 autosomal loci along with sex markers and Y-STRs. Extended STR kits are critical for resolving complex DNA mixtures and kinship testing, offering greater statistical power and discrimination than standard 13- or 15-locus STR panels. For example, VeriFiler™ Plus includes additional loci like D10S1248 and SE33, improving performance on degraded samples and aiding in cross-border or database comparisons [147].

This integrated workflow is automated extraction (Qiagen/ HiMedia) →qPCR (VeriFiler, InGenoMix) →extended STR profiling, which offers multiple advantages such as reduced manual steps through automation, qPCR quantification guides the DNA input volume for STR amplification, extended STR kits provide enhanced mixture deconvolution and increased match probabilities, suitable for casework, missing persons, mass disasters, and databasing efforts. Together, these systems form a seamless pipeline that ensures high sensitivity and reliability, even for samples with minimal or compromised DNA. Forensic laboratories aiming to meet both judicial and scientific standards benefit greatly from combining Qiagen or HiMedia automated DNA extraction, VeriFiler family qPCR quantification, and extended STR typing kits. This end-to-end approach ensures that DNA profiles are both accurate and legally defensible, even in the face of degraded, inhibited, or low-copy-number samples [152-154].

Probabilistic genotyping tools, software training

Developing and implementing more sophisticated statistical models for DNA match probability calculations enhances the accuracy of analysis, particularly in complex cases. These models can more accurately assess the likelihood of a match, especially in situations involving mixed or partial DNA profiles [9].

Probabilistic genotyping uses mathematical algorithms and Bayesian or likelihood-based models to evaluate possible genotype combinations and assign statistical weight to hypotheses (e.g., inclusion *vs*. exclusion of a suspect). The output is typically a Likelihood Ratio (LR), which compares the probability of observing the DNA evidence under two competing scenarios (e.g., suspect contributed *vs*. suspect did not contribute). Unlike threshold-based methods, PG considers allelic dropout, drop-in, stutter, and noise in a structured way [155]. As DNA analysis in forensic science becomes increasingly complex - especially with the interpretation of low-template, mixed, or degraded DNA profiles - traditional manual methods of interpretation have shown limitations. To address these complexities and enhance objectivity, forensic laboratories are increasingly adopting Probabilistic Genotyping (PG) software. These tools apply statistical modeling to interpret DNA mixtures and provide Likelihood Ratios (LRs), helping scientists quantify the strength of the DNA evidence. However, the effectiveness of such tools depends heavily on proper software validation, analyst training, and transparent interpretation protocols [145].

Several PG software tools are in routine forensic use worldwide:

- STRmix™ – Used widely in government and accredited forensic labs. It uses a fully continuous Bayesian model and considers

peak height information.

- TrueAllele® – Developed by Cybergenetics, this system uses a continuous probabilistic model and is
- Known for automation.
- Lab Retriever – Developed by the University of Washington, focuses on a likelihood-based approach.
- EuroForMix - An open-source continuous PG software platform developed in Europe.

Each tool has unique features and modeling assumptions, and requires rigorous validation and documentation before casework application [156]. Implementing probabilistic genotyping requires more than just software installation. Analysts must undergo extensive training in both the operational use of the software and the underlying statistical theory. Investing in ongoing training and education for personnel involved in DNA collection and analysis is vital. Keeping up-to-date with the latest techniques and understanding the nuances of DNA handling can significantly improve sample quality. Training includes understanding the model's assumptions (e.g., allele frequencies, number of contributors), correct input of parameters (e.g., stutter rates, degradation models) and interpretation and explanation of likelihood ratios in court.

Improper use without understanding can lead to misinterpretation, especially during expert testimony.

Establishing and adhering to standardized procedures for DNA collection, storage, and analysis across different labs and agencies ensures consistency and reliability of results. Furthermore, cross-validation, sensitivity testing, and inter-laboratory studies are critical to ensure reproducibility and compliance with SWGDAM or ISO 17025 standards [157]. Probabilistic tools are gaining increasing legal acceptance, but courts have also emphasized the need for transparent methodologies, accessible documentation, and defensible statistics. Defense attorneys often request discovery of source code, validation studies, and training records. This highlights the need for rigorous documentation, analyst certification, and ongoing proficiency testing in labs that use PG tools [158].

Probabilistic genotyping tools have transformed forensic DNA interpretation by introducing statistical rigor and reducing human bias in complex mixture analysis. However, their success depends not just on software capability but on proper analyst training, transparent validation, and informed interpretation. As forensic science moves further into the era of complex DNA profiling, investing in both technology and human expertise remains critical.

Quality control with real-time DNA fingerprinting

In forensic DNA analysis, Quality Control (QC) is essential to maintain accuracy, prevent contamination, and ensure the validity of results - especially in high-stakes environments like criminal investigations and court trials. Traditional QC measures include reagent blank checks, duplicate testing, and manual verification of results. However, with the growing complexity and volume of forensic casework, laboratories are increasingly adopting real-time DNA fingerprinting as a proactive QC strategy, enabling the rapid detection of sample contamination, sample switches, and handling errors during the workflow. Implementing strict quality control protocols at every step of the DNA handling process is crucial. This includes regular checks for equipment and environmental contamination, as well as meticulous record-keeping and sample tracking. Automation in sample processing can also reduce human error and improve consistency [9].

Real-time DNA fingerprinting involves performing intermediate STR profiling at key steps in the laboratory pipeline to verify sample identity and detect contamination or mix-ups as they occur. Unlike full forensic STR typing, which may take hours, this QC strategy uses fast PCR kits and pre-set panels to rapidly compare profiles often focusing on key loci (e.g., TH01, D5S818, D13S317) sufficient for identity matching. Some commercial solutions and laboratory-developed protocols integrate real-time QC into automated workflows. Laboratories may use compact thermocyclers with rapid STR kits, followed by real-time capillary electrophoresis or microfluidics-based genotyping. The NIST standard operating procedures recommend integrating DNA QC checkpoints in high-throughput environments [159]. Moreover, software platforms like GeneMapper® ID-X and LIMS systems can automatically flag mismatches between expected and observed profiles, providing alerts for reanalysis [160].

In a recent study (PMC9689305), researchers demonstrated that using intermediate STR fingerprinting during DNA processing significantly reduced sample switch errors and helped promptly detect contamination events, ultimately improving downstream profile quality [161]. Real time DNA QC fingerprinting has a lot pros such as the following:

- Early Error Detection: Identifies issues before full STR typing is done.
- Contamination Control: Detects low-level cross-contamination early in workflow.
- Sample Tracking: Prevents mislabeling or switch during batch processing.
- Improved Credibility: Adds an objective layer of verification to forensic findings.
- Legal Defense: Strengthens the defensibility of DNA evidence in court by showing documented quality checks.

Incorporating real-time DNA fingerprinting as a quality control tool in forensic workflows enhances sample integrity, reduces rework, and safeguards the credibility of DNA evidence. As forensic labs move toward automation and high-throughput processing, embedding such proactive QC measures is critical for ensuring both scientific rigor and judicial reliability.

Centralized case tracking for chain of custody

Sustaining public confidence in DNA fingerprinting requires the establishment of stringent ethical standards and strong privacy protection measures. Interpreting legal and cross-jurisdictional concerns can also be difficult. Standards and procedures for DNA analysis and interpretation may differ throughout jurisdictions, which could result in discrepancies and possible legal issues. This is made worse by the fact that DNA technology is developing at a rate that frequently surpasses the creation of legal frameworks and standards, resulting in a disconnect between legal requirements and scientific capability.

A more comprehensive knowledge of the consequences of DNA evidence can result from fostering cooperation between forensic scientists, statisticians, ethicists, and legal experts. This entails creating more effective means of conveying intricate DNA results to

non-scientific audiences, such jurors and judges, and making sure that interpretations are correctly interpreted in legal settings [9].

The chronological records demonstrating the seizure, custody, control, transfer, analysis, and disposal of tangible or digital evidence are referred to as the chain of custody [162]. In the context of DNA forensics, it ensures that the identity and integrity of biological samples are maintained throughout their lifecycle. A broken or ambiguous chain of custody can lead to the inadmissibility of evidence in court, regardless of the scientific reliability of the DNA results [163].

DNA analysis can be made more successful by encouraging cooperation and setting up procedures for data exchange across various jurisdictions. To make cross-jurisdictional comparisons simpler and more trustworthy, this entails standardizing formats for DNA data and guaranteeing interoperability across various database systems. These technologies can greatly increase the accuracy and dependability of DNA data processing, resulting in more assured interpretations and conclusions in a variety of DNA fingerprinting applications. The difficulties in interpreting DNA fingerprinting results and the solutions being explored to overcome these difficulties will be covered in the article's next part [164, 165].

A centralized case tracking system digitally unifies the entire chain of custody, including evidence intake, sample processing, analyst assignments, quality control checkpoints, and report generation. These systems are typically integrated into a Laboratory Information Management System (LIMS) or a dedicated forensic evidence management platform. All evidence movements are timestamped and visible to authorized personnel. Digital audit trails reduce the need for manual logging and minimize clerical errors. Ensures that only trained and authorized personnel handle specific samples. Automated reminders for pending analysis, overdue transfers, or discrepancies. Facilitates compliance with accreditation standards such as ISO 17025, SWGDAM, and FBI QAS. Some commercial systems like JusticeTrax LIMS-plus, STaCS® DNA, and Thermo Fisher's HID LIMS provide customized solutions for forensiclaboratories, offering modules specifically for DNA casework [166].

In addition to operational efficiency, centralized tracking systems enhance transparency and accountability, especially during legal scrutiny. Courts often request detailed records of who handled each sample, when and where it was transferred, and whether proper storage and documentation were followed. A centralized digital system can produce instant audit reports, dramatically reducing the risk of evidence exclusion on procedural grounds [167]. Moreover, such systems support broader inter-agency collaboration, allowing authorized access by law enforcement, prosecutors, and forensic experts while maintaining data security. In high-profile cases or mass disaster scenarios, the ability to centrally manage thousands of samples becomes not only efficient but essential [162].

As forensic DNA analysis becomes more complex and legally scrutinized, the role of centralized case tracking systems becomes indispensable. By digitally reinforcing the chain of custody, these systems uphold the scientific and legal integrity of forensic evidence, reduce the risk of human error, and streamline case processing. Forensic laboratories seeking accreditation and excellence must prioritize the implementation of such systems to meet modern demands in justice and accountability.

In modern forensic DNA laboratories, it is crucial to maintain the integrity and quality of evidence. Laboratories use advanced techniques to tackle issues such as low-quality DNA, contamination, and complicated profile analysis [146-150]. Methods like extended Short Tandem Repeats (STRs), Single Nucleotide Polymorphisms (SNPs), Mitochondrial DNA (MtDNA), and improved extraction buffers help when working with old or damaged samples. Mini-STRs and SNPs are particularly good for degraded DNA, while MtDNA is useful in challenging cases due to its high copy number. To minimize human error, many labs automate DNA extraction and use quantitative PCR (qPCR) alongside extended STR analysis [152-156]. Automated systems and DNA extraction kits ensure consistent workflows. After extraction, qPCR assesses DNA quality before STR amplification. For complex DNA profiles, labs utilize software like STRmix™, TrueAllele®, and EuroForMix, which help analyze mixed samples using statistical methods. However, proper training is necessary, and the results must be transparently validated to be legally acceptable. Real-time DNA fingerprinting detects issues early in the process, while secure chain-of-custody systems ensure proper handling of samples. Overall, these innovations create a strong framework for ensuring the reliability and legal validity of forensic DNA evidence [157-163, 166].

Conclusion

Forensic DNA laboratories today face an evolving landscape of technical and operational challenges that require integrated, science-driven solutions. Among the most persistent obstacles are degraded and low-template DNA, sample contamination, PCR inhibition, interpretation errors in STR mixtures, and lapses in chain of custody. These challenges can significantly compromise both the scientific validity and legal admissibility of DNA evidence if not proactively addressed. Degraded DNA, often the result of environmental exposure or sample aging, leads to fragmentation and loss of amplifiable regions. This complicates conventional STR profiling and increases the risk of allele drop-out. Similarly, low-template samples suffer from stochastic effects, including imbalance and drop-in events, which can obscure contributor identity and reduce match confidence. Inhibitors such as hemoglobin and indigo dye further compromise PCR amplification, increasing the likelihood of false negatives or partial profiles. Interpretation challenges also arise in complex STR mixtures, where traditional manual methods are insufficient to resolve overlapping alleles or determine contributor numbers with certainty.

The evolution of forensic DNA analysis has led to the integration of advanced tools and standardized protocols that collectively improve the reliability, sensitivity, and legal defensibility of genetic evidence. This paper explored how various innovations from extended STR panels to centralized digital tracking have addressed long-standing technical challenges in the forensic workflow. Extended STR kits, along with SNP and MtDNA analysis, offer critical alternatives for analyzing compromised, degraded, or trace DNA samples that previously would have yielded incomplete or unusable profiles. These tools significantly enhance discriminatory power and expand the forensic toolkit, especially in cases involving unidentified remains, aged samples, or mass disaster investigations. Complementing these advancements are enhanced DNA extraction protocols and buffers, which increase yield from difficult biological materials such as bone, hair, or environmentally exposed samples.

Integrating automated DNA extraction systems with quantitative PCR (qPCR) and extended STR amplification creates a streamlined and reproducible workflow that minimizes operator error and sample loss. qPCR not only provides critical quantification and inhibition

assessment but also serves as a decision point for determining sample suitability before STR typing. These enhancements significantly reduce bottlenecks caused by manual handling, contamination, or downstream reaction failure. Interpretation of complex or mixed DNA profiles remains a critical bottleneck in forensic casework. Here, probabilistic genotyping software tools such as STRmix™, TrueAllele®, and EuroForMix provide a scientifically rigorous approach by applying statistical models to evaluate the likelihood of various contributor combinations. These tools minimize subjective interpretation, reduce bias, and produce quantifiable likelihood ratios, making them highly suitable for complex DNA evidence. However, proper implementation requires not just software acquisition, but also structured analyst training, validation, and court-readiness, highlighting the importance of capacity building and continuous education in forensic laboratories.

Additionally, real-time DNA fingerprinting for quality control offers an essential layer of error detection and contamination monitoring at multiple stages of the laboratory pipeline. This proactive approach allows early correction of sample switches or cross-contamination, improving the reliability of results before full STR typing. Combined with centralized case tracking systems and digital chain-of-custody protocols, these QC enhancements ensure traceability, accountability, and compliance with accreditation standards such as ISO 17025 and SWGDAM guidelines. In conclusion, the forensic DNA field benefits substantially from the convergence of automation, statistical interpretation, and robust quality control mechanisms. These advancements not only streamline laboratory workflows and reduce case processing delays but also reinforce the credibility and scientific rigor of forensic outcomes. Laboratories are encouraged to adopt these technologies and practices as standard, ensuring that forensic DNA evidence continues to meet the highest standards of accuracy, reproducibility, and legal admissibility in both routine casework and complex investigative contexts.

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